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ROLE OF PROTEIN KINASE C ISOFORMS IN CARDIOPROTECTIVE MECHANISM OF CHRONIC HYPOXIA

Ph.D. THESIS

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Declaration

I hereby declare that I completed this Ph.D. thesis independently, except where explicitly indicated otherwise. It documents my own work, carried out under the supervision of Doc. RNDr. František Novák, CSc. Throughout, I have properly acknowledged and cited all sources used. Neither this thesis nor its substantial part has been submitted to obtain this or other academic degree.

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Declaration of co-authorship

On behalf of all co-authors, I hereby declare that Mgr. Markéta Hlaváčková has substantially contributed (30-90 %) to all 4 articles which represent an integral part of this Ph.D. thesis. She performed most of the experiments, especially in the papers where she is the first author and she actively participated in the set-up of the experiments, in the interpretation of the results and in the preparation of the manuscripts.

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ABSTRACT

Cardiovascular diseases, particularly acute myocardial infarction, are one of the leading causes of death in developed countries. It is well known that adaptation to chronic intermittent hypobaric hypoxia (IHH) confers long-lasting cardiac protection against acute ischemia/reperfusion injury. Protein kinase C (PKC) appears to play a role in its cardioprotective mechanism since the administration of general PKC inhibitor completely abolished the improvement of ischemic tolerance in IHH hearts. However, the involvement of individual PKC isoforms remains unclear. Therefore, the primary aim of this study was to investigate the potential involvement of PKCδ and PKCε, the most prevalent PKC isoforms in rat heart, in the mechanism of IHH-induced cardioprotection. We showed that IHH up-regulated PKCδ protein in left ventricle, enhanced its phosphorylation on Ser643 and increased its co-localization with markers of mitochondrial and sarcolemmal membranes. PKCδ subcellular redistribution induced by IHH as well as the infarct size-limiting effect of IHH was reversed by acute treatment with PKCδ inhibitor rottlerin. These data support the view that PKCδ plays a significant role in IHH-induced cardioprotection. On the other hand, adaptation to IHH decreased the PKCε total protein level without affecting its subcellular distribution and the level of phosphorylated PKCε (Ser729). Moreover, we demonstrated that the PKCε inhibitor peptide KP-1633 was not able to block the protective effects of IHH in isolated left ventricular myocytes exposed to metabolic inhibition/reenergization, simulating acute ischemia/reperfusion injury. These findings support the idea that PKCε is not a key player in cardioprotection induced by IHH.

Furthermore, we assumed that the increased accumulation of n-3 polyunsaturated fatty acids (n-3 PUFAs) in myocardial phospholipids induced by IHH may contribute to improved ischemic tolerance. PKC activity and function are influenced by lipid membrane composition and by the quality of lipid signaling molecules such as diacylglycerols (DAGs). The second goal of this study was to find out whether an altered phospholipid FA profile by diets enriched with saturated FAs (SFAs), n-3 PUFAs and n-6 PUFAs, plays a role in the cardioprotective mechanism of IHH in a PKC-dependent manner. Whereas the (SFA+monounsaturated FA)/PUFA ratio in heart DAGs corresponded to the ratio in the respective diet (in the sequence SFA>n-3 PUFA>n-6 PUFA diet), heart phospholipids maintained constant content of SFAs, monounsaturated FAs and total PUFAs, independent of diet and IHH. On the other hand, the n-6/n-3 PUFA ratio was influenced to various extents by either the dietary PUFA supply or IHH in both heart phospholipids and DAGs. Whereas the groups fed on SFA and n-3 PUFA enriched diets were protected by IHH, the protective effect of the n-6 PUFA-enriched diet on myocardial infarct size was not further enhanced by adaptation to IHH. As the IHH-induced decrease in n-6/n-3 PUFA ratio in membranes was proportional in all groups, it seems unlikely that this response is directly involved in the mechanism of infarct size-limiting effect in IHH hearts. The infarct size decreased with increasing relative PKCε protein content within the normoxic or hypoxic groups, which is in line with a generally accepted view that this PKC isoform is cardioprotective. However, all hypoxic groups had smaller infarction and lower PKCε content, suggesting that IHH protects the myocardium by a mechanism independent of PKCε. Unlike in other diet groups, IHH did not increase myocardial ischemic tolerance and did not cause up-regulation of PKCδ protein in rats supplemented with n-6 PUFA-enriched diet. The relative content of PKCδ in myocardial particulate fraction exhibited a close negative correlation with myocardial infarct size. These results suggest that PKCδ plays an important role in the infarct size-limiting mechanism of IHH in adult rat hearts.

ABSTRAKT

Kardiovaskulární onemocnění, především akutní infarkt myokardu, jsou jednou z hlavních příčin úmrtí ve vyspělých zemích. Je známo, že dlouhodobá adaptace na chronickou intermitentní hypobarickou hypoxii (IHH) snižuje rozsah ischemicko-reperfúzního poškození. Zdá se, že proteinkinasa C (PKC) se uplatňuje v mechanismu kardioprotektivního účinku IHH, neboť obecný inhibitor PKC zcela zablokoval kardioprotekci vyvolanou adaptací na IHH. Nicméně zapojení jednotlivých isoforem PKC v tomto kardioprotektivním fenoménu zůstává nejasné. Cílem této práce bylo studovat úlohu isoforem PKCδ a PKCε, které jsou nejvíce zastoupenými isoformami PKC v myokardu potkana, v mechanismu kardioprotektivního působení IHH. Ukázali jsme, že adaptace na IHH zvyšuje v myokardu levé komory potkana celkové množství proteinu PKCδ společně s množstvím její fosforylované/aktivní formy a současně zvyšuje přítomnost této isoformy PKC na sarkolemální membráně a v mitochondriích. Redistribuce PKCδ na sarkolemální membránu a do mitochondrií stejně jako snížení velikosti infarktového ložiska v srdcích adaptovaných na IHH byly potlačeny podáním inhibitoru PKCδ delta, rottlerinu. Adaptace na IHH naopak snížila množství celkového proteinu PKCε a neovlivnila buněčnou distribuci a fosforylaci této isoformy PKC. Podání inhibitoru PKCε, KP-1633, neovlivnilo protektivní efekt adaptace na IHH v izolovaných kardiomyocytech vystavených metabolické inhibici/reenergizaci simulující ischemii/reperfúzi. Druhý cíl této práce vychází z předpokladu, že pozorovaný nárůst n-3 polynenasycených mastných kyselin (n-3 PUFA) v srdečních fosfolipidech vyvolaný adaptací na IHH by mohl přispívat k zvýšené odolnosti vůči ischemicko/reperfúznímu poškození. Aktivita a funkce PKC může být ovlivněna lipidovým složením membrán a kvalitou lipidových signálních molekul. Druhým cílem bylo zjistit, zda změněný profil mastných kyselin v srdečních fosfolipidech a diacylglycerolech vyvolaný dietou obohacenou saturovanými mastnými kyselinami (SFA), n-3 nebo n-6 PUFA hraje roli v kardioprotektivním působení adaptace na IHH a zda se v tomto mechanismu podílí PKC. Zatímco poměr (SFA+mononenasycené FA)/PUFA v srdečních diacylglycerolech kopíruje tento poměr v jednotlivých dietách (v pořadí SFA>n-3 PUFA>n-6 PUFA dieta), srdeční fosfolipidy si drží konstantní podíl SFA, mononenasycených FA a celkových PUFA nezávisle na dietě a adaptaci na IHH. Naopak, jednotlivé diety i IHH významně ovlivnily poměr n-6/n-3 PUFA jak v srdečních diacylglycerolech, tak fosfolipidech. Zatímco u zvířat krmených dietou obohacenou SFA a n-3 PUFA snížila adaptace na IHH velikost infarktového ložiska, u skupiny krmené n-6 PUFA, kde byla velikost infarktového ložiska signifikantně nižší ve srovnání se skupinou krmenou n-3 PUFA, se protektivní efekt IHH na velikosti infarktu neprojevil. Jelikož byl pokles poměru n-6/n-3 PUFA v srdečních membránových fosfolipidech po adaptaci na IHH proporcionálně stejný u všech dietních skupin, zdá se nepravděpodobné, že je tato změna přímo zapojená v protektivním efektu IHH na velikost infarktu. Velikost infarktu klesala se zvyšujícím se relativním množstvím PKCε v partikulární/membránové frakci u normoxických či hypoxických zvířat, což je v souladu s obecně uznávanou představou, že je tato isoforma kardioprotektivní. Nicméně, u všech hypoxických skupin jsme pozorovali menší velikost infarktu společně se snížením relativním množstvím PKCε v partikulární frakci, což naznačuje, že IHH chrání srdce mechanismem nezávislým na PKCε. Na rozdíl od ostatních dietních skupin, IHH nezvýšila ischemickou odolnost myokardu u potkanů krmených dietou obohacenou n-6 PUFA a zároveň nedošlo u této dietní skupiny k nárůstu PKCδ v partikulární frakci. Množství PKCδ v partikulární frakci negativně korelovalo s velikostí infarktu. Tyto výsledky naznačují, že PKCδ, ale ne PKCε, hraje významnou roli v ischemické odolnosti myokardu potkana vyvolané adaptací na IHH.

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1 INTRODUCTION

1.1 Cardiac protection

In the mammalian myocardium, adenosine triphosphate (ATP) is utilized at a high rate with respect to its steady-state intracellular concentration (Hochachka et al., 1992). Therefore, myocardial performance depends critically on energy-yielding metabolic processes and a continuous supply of oxygen and substrates. Myocardial ischemia occurs when blood flow to the heart is impaired, such as during acute myocardial infarction. Whenever the oxygen supply becomes limited with respect to need, a potentially lethal condition ensues leading to rapid ATP depletion, intracellular Ca^{2+} overload, cell failure and necrosis. There are two distinct components of damage: i) ischemic injury, which results from the initial loss of blood flow and ii) reperfusion injury, which occurs upon restoration of oxygenated blood flow. Although timely reperfusion remains the only efficient strategy for myocardial salvage, it may paradoxically further exacerbate injury caused by ischemia (Katz, 2010).

The degree of ischemia/reperfusion (I/R) injury depends not only on the intensity and duration of the ischemic insult but also on the intrinsic heart tolerance to oxygen deficit. It is well known that the heart is capable of protecting itself from I/R injury if exposed to an appropriate stimulus. Cardiac tolerance to oxygen deprivation can be increased by exposure to:

- pharmaceuticals (e. g. beta-blockers, opioids, adenosine, atrial natriuretic peptide, and cyclosporine) (Gerczuk and Kloner, 2012),
- acute adaptive protective mechanisms such as several forms of ischemic and pharmacological pre- and postconditioning which induce short-lasting cardioprotection persisting for hours or days,
- chronic adaptive protective mechanisms which induce long-lasting cardioprotection in the range of weeks or months, to which adaptation to chronic hypoxia belongs, but also include adaptation to other forms of stress such as exercise or dietary intervention (Peart and Headrick, 2008).

Ischemic preconditioning, which occurs after short episodes of ischemia, increases the resistance of the myocardium to subsequent more prolonged reductions of coronary flow. This phenomenon was discovered by Murry and colleagues in 1986 and represents the most powerful way to increase cardiac ischemic tolerance. However, the cardiac

protection induced by ischemic preconditioning persists only for hours or days (Kuzuya et al., 1993) which could be considered as one of the serious practical limitations for the introduction of preconditioning-based therapy in clinics for patients at risk of infarction. Therefore several attempts have been made to overcome this limitation by maintaining a protected phenotype of the heart for a prolonged period of time. One of these approaches, sometimes referred to as chronic preconditioning, applies knowledge derived from preconditioning studies and induces longer-lasting myocardial protection by repeated administration of a protective mimetic (e. g. an activator of mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channels, opioids) to maintain rat hearts in a constant preconditioned state (Peart and Gross, 2004; 2006; Wang et al., 2007). Another way is prophylactic gene therapy based on transferring to the myocardium the gene(s) responsible for the protective effect of delayed preconditioning, such as inducible nitric oxide (NO) synthase (iNOS) (Li et al., 2006). Similarly, exposure to chronic hypoxia results in a long-term adaptation inducing protected cardiac phenotype which persists for weeks or months (Neckar et al., 2004). Moreover it is a relatively non-invasive (as compared with ischemic preconditioning or gene therapy) and cost-effective (as compared with pharmacological preconditioning) approach. However, adaptation to chronic hypoxia has been studied much less than preconditioning and the understanding of its protective mechanisms is still far behind that of preconditioning.

1.2 Chronic hypoxia

The incidence of myocardial infarction is lower in people living at high altitude which was first discovered already in the late 1950s (Hurtado, 1960). The first experimental study showing the cardioprotective effect of adaptation to chronic hypoxia was carried out by Kopecky and Daum (1958). Subsequent experiments on animals kept in hypoxia chambers for prolonged periods of time confirmed that chronically hypoxic myocardium is more tolerant to I/R injury than normoxic myocardium (reviewed by Ostadal and Kolar, 2007).

1.2.1 Experimental models of chronic hypoxia

Hypoxic states of the heart may be induced by several mechanisms:

- ischemic hypoxia (often described as "cardiac ischemia"), induced by a reduction or interruption of coronary blood flow,
- systemic hypoxia caused by environmental factors (e.g. high altitude) or by various cardiopulmonary disorders (e. g. chronic obstructive pulmonary disease, sleep apnea) is characterized by a drop in PO_2 in the arterial blood but normal perfusion,
- anemic hypoxia, in which the arterial PO_2 is normal but the oxygen transport capacity of the blood is decreased (Ostadal and Kolar, 2007).

Organisms can adapt to chronic systemic hypoxia either in the natural high altitude environment or under laboratory conditions in a normobaric chamber (by reducing the O_2 fraction in the gas mixture; normobaric hypoxia) or in a hypobaric chamber (which simulates high altitude conditions by using a vacuum pump to reduce the air pressure in the airtight chamber; hypobaric hypoxia) (Table 1). Hypobaric hypoxia is also called high altitude hypoxia since the PO_2 of the chamber corresponds to a certain altitude (e. g. 7,000 m; P_B = 308 mm Hg, 41 kPa; PO_2 = 65 mm Hg, 8.6 kPa). Adaptation to chronic hypoxia could be continuous (exposure to a prolonged sustained imbalance between oxygen supply and oxygen demand without interruption) or intermittent (repetitive short-term or long-term exposures to hypoxia).

Table 1 Experimental models of chronic hypoxia.

Model	Human relevance
Hypobaric hypoxia	
Continuous (CHH)	Life at high altitude
Intermittent (IHH)	Repeated ascents of mountains (mountaineering, tourism, pilgrimages), high-altitude training
Normobaric hypoxia	
Continuous (CNH)	Hypoxemic congenital heart disease, severe chronic obstructive lung disease, severe chronic ischemic heart disease
Intermittent (INH)	Exacerbations of chronic obstructive lung disease, ischemic heart disease (acute coronary syndrome, exercise), sleep apnea

Adopted from Ostadal and Kolar (2007) and modified.

The vast variety of experimental protocols used to study the mechanisms of chronic hypoxia induced cardioprotection complicates the comparison and summarization of data obtained from different research groups. Hypoxic protocols vary in the intensity of hypoxia, the type of hypoxic chamber (hypobaric or normobaric), the length and frequency of reoxygenation periods and also in the total duration of adaptation. The results of this thesis were obtained from experiments using chronic intermittent hypobaric hypoxia (PO_2 8.6 kPa corresponding to altitude of 7,000m, 8 h/day, 5 days a week, 25-30 exposures) model. The following chapters concentrate primarily on data from *in vivo* cardioprotective models of chronic intermittent normobaric and hypobaric hypoxia (INH/IHH). Some of the previously reported continuous hypoxia models did in fact include brief periods of normoxia (reoxygenation) when hypoxic chambers were opened to allow operations such as cleaning and animal feeding. The results of these studies were also taken into consideration in the discussion of data concerning cardioprotective effects induced by chronic intermittent hypoxia.

1.2.2 Cardioprotective effects of chronic hypoxia

Adaptation to chronic hypoxia is characterized by a variety of functional changes to maintain homeostasis with a minimum expenditure of energy. Such adjustments may protect the heart under conditions that require enhanced work and consequently increased metabolism. Adaptation to chronic hypoxia increases cardiac tolerance to all major deleterious consequences of acute oxygen deprivation including reduction of infarct size (Neckar et al., 2002a,b), improved recovery of contractile function during reperfusion

(Tajima et al., 1994), reduction in the incidence of ischemic/reperfusion arrhythmias (Asemu et al., 2000) and slowing in the rate of ATP depletion (Opie, 1978). The degree of protection afforded by intermittent hypoxia depends on many factors including cycle length, severity and number of hypoxic episodes per day and number of exposure days (Beguin et al., 2005; Belaidi et al., 2009; Samaja, 2009). Significant differences exist between males and females in cardiac adaptive responses to hypoxia (Netuka et al., 2006; Bohuslavova et al., 2010) and are in line with the generally accepted observation that female hearts tolerate stress much better than male ones (Ostadal et al., 2009). The cardioprotective effect of adaptation to chronic hypoxia is also markedly influenced by the age of experimental animals (La Paluda and Costa, 2005). The following chapters concentrate primarily on the effects of chronic hypoxia on adult male hearts. In addition to the protective effects, adaptation to chronic hypoxia may also induce adverse influences on the cardiopulmonary system such as polycythemia, pulmonary hypertension and right ventricular hypertrophy (Ostadal and Kolar, 1999). Nevertheless, the protective signs of adaptation to chronic hypoxia persist long after the other chronic hypoxia-induced changes have already normalized (Ostadal and Kolar, 2007).

1.2.3 Molecular mechanisms of chronic hypoxia-induced cardioprotection

Although the cardioprotective effect of adaptation to chronic hypoxia has been known for half a century, its molecular mechanism has rarely been studied until recently and thus it remains far from being understood. It was shown that a combination of adaptation to chronic hypoxia with ischemic preconditioning fails to produce an additive cardioprotective effect (Neckar et al., 2002a,b), suggesting activation of common pathways, although with different efficiency and duration. Unlike the early phase of preconditioning leading to activation of these protective signaling pathways, chronic hypoxia affects also the expression of their components and of other proteins involved in maintaining oxygen homeostasis via transcription factors such as hypoxia-inducible factor 1 (HIF-1α) (Semenza, 2004).

HIF-1 functions as a global regulator of oxygen homeostasis and plays critical roles in the responses of the cardiovascular and respiratory systems to hypoxia (Semenza, 2012). HIF-1 is a heterodimeric transcription factor that is composed of an oxygen-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit (Wang and Semenza, 1995). HIF-1 activates the transcription of genes that are essential for adaptive responses to

hypoxia such as glycolysis, erythropoiesis, angiogenesis, and vascular remodeling (Semenza, 2012). Cai et al. (2003) showed that in INH mouse hearts, the HIF-1α-dependent production of erythropoietin is connected not only with the stimulation of hematopoiesis but also with the increased ischemic tolerance. Erythropoietin was recognized as a protective agent against ischemic injury since its binding to cardiac erythropoietin receptors can activate various protective signaling pathways in cardiomyocytes (Joyeux-Faure et al., 2005). Downstream of receptor interactions, the activation of phosphatidylinositol-3 kinase (PI3K) and Akt appears to mediate many of erythropoietin cardioprotective effects (Burger et al., 2009). In line with these findings, the PI3K/Akt signaling pathway appears to be involved in cardioprotection induced by adaptation to IHH since the specific PI3K inhibitors (such as LY294002 or wortmannin) attenuated the reduction of infarct size in chronic IHH rats (Ravingerova et al., 2007) and abrogated the IHH-improved postischemic LV performance (Wang et al., 2011).

It is well known that exposure to IHH is associated with increased oxidative stress (Kolar et al., 2007). The increase of reactive oxygen species (ROS) was traditionally considered as injurious but now it appears that ROS signaling is involved in chronic hypoxia-induced cardioprotection. The administration of the antioxidant N-acetylcysteine during the adaptation of rats to IHH significantly attenuated the protective effect of IHH on infarct size reduction (Kolar et al., 2007). Wang et al. (2011) have demonstrated that IHH confers cardioprotection against I/R injury by enhancing the production of ROS during early reperfusion, which subsequently activates downstream protective signaling pathways. The authors suggest that the endogenous ROS generated during early reperfusion in non-adapted I/R hearts do not seem to reach the threshold which would trigger efficient cardioprotection.

The exposure of rats to IHH is associated with transiently increased adrenergic activity and elevated plasma levels of catecholamines (Ostadal et al., 1984). According to a recent study by Mallet and colleagues (2006) it appears that episodic β_1-adrenergic activation during adaptation of dogs to INH evokes progressive development of powerful resistance to myocardial ischemia. Metoprolol (β_1-adrenoceptor antagonist) administered throughout the INH program blunted the INH-evoked cardioprotection.

Among the potential mediators of IHH-induced cardioprotection, NO is a likely candidate since chronic IHH up-regulated myocardial iNOS mRNA and protein levels in rats, and the selective iNOS-inhibitor aminoguanidine abolished the enhanced ischemic

tolerance (Ding et al., 2005). The NO/cyclic guanosine 3′,5′-monophosphate (cGMP) pathway was shown to play a role in cardioprotection (Ferdinandy and Schulz, 2003). The intracellular level of cGMP is controlled by the activity of phosphodiesterase-5. Milano et al. (2011) have found that administration of phosphodiesterase-5 inhibitor sildenafil has the potential to mimic the cardioprotective effects provoked by INH. NO can also originate from constitutive/endothelial NOS (eNOS); the increased expression of eNOS was detected in CNH hearts of neonatal rabbits and the NOS inhibitors L-NAME and L-NMA abolished the cardioprotective effect of hypoxia (Baker et al., 1999). Nevertheless, the involvement of eNOS in chronic hypoxia-induced cardioprotection is not clear since in some studies its expression was down-regulated (Ferreiro et al., 2001). In line with these data, INH suppressed myocardial NOS activity, eNOS content and excessive NO formation upon reperfusion in canine hearts (Ryou et al., 2008). According to these studies, the role of NO in adaptive protective responses of chronically hypoxic hearts is far from clear, the different experimental models and selected species used could play a role in these controversial results. It appears that the observed beneficial influence of NO is critically dependent on its concentration: too little or too much may be detrimental (Manukhina et al., 2006; Zaobornyj et al., 2007).

Both adrenergic stimulation and increased production of ROS and NO can change the activity and/or expression of numerous signaling molecules. Besides the already mentioned PI3K, several reports demonstrated the involvement of the protein kinase C (PKC), the cAMP-dependent protein kinase (PKA) and the Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) in the protective signaling pathways of chronically hypoxic hearts (Table 2). The cardioprotective effects of IHH, an improved postischemic recovery of cardiac contractile function and reduced myocardial infarct size, were inhibited by the general PKC inhibitor chelerythrine or attenuated by the PKCδ-selective inhibitor rottlerin (Ding et al., 2004; Neckar et al., 2005). Moreover, a PKC-related prevention of Ca^{2+} and Na^{+} overload in cardiomyocytes isolated from IHH rats (Ding et al., 2004) and PKC-related improved Ca^{2+} handling in INH rat hearts (Yeung et al., 2007) have been reported. Stable PKC activation also contributed to the preservation of the intracellular pH which may be beneficial in maintaining cardiac function during I/R in IHH rats (Li et al., 2007).

Table 2 The involvement of various protein kinases in cardioprotection by chronic hypoxia.

Model of chronic hypoxia	Species	Model of acute O_2 deprivation	Protective effect	Protein kinase	Effect of protein kinase inhibitor on protection	Reference
IHH, 5000 m, 6 h/day, 6 wk	Adult rats	Perfused heart, global I/R Ventricular myocytes, A/R	↑ Recovery of function Prevention of Na^+ and Ca^{2+} overload	PKC α, δ, ε	Abolished (chelerythrine) Abolished (chelerythrine)	Ding et al., 2004
IHH, 7000 m, 8 h/day, 5-6 wk	Adult rats	In vivo, regional I/R	↓ Infarct size	PKCδ PKCε	Abolished (chelerythrine) Attenuated (rottlerin)	Neckar et al., 2005
IHH, 5000 m, 6 h/day, 6 wk	Adult rats	Ventricular myocytes, A/R	↓ Intracellular acidification	PKC	Abolished (chelerythrine)	Li et al., 2007
INH, 10% O_2, 6 h/day, 1 wk	Adult rats	Perfused heart, regional I/R	Improved Ca^{2+} handling	PKC PKA CAMKII	Abolished (chelerythrine, calphostin C) Abolished (KT5720) Abolished (KN-93)	Yeung et al., 2007
IHH, 5000 m, 6 h/day, 6 wk	Adult rats	Perfused heart, global I/R	↑ Recovery of function	PKA CAMKII	Abolished (H-89) Abolished (KN-93)	Xie et al., 2005
IHH, 7000 m, 8 h/day, 5 wk	Adult rat	In vivo, regional I/R	↓ Infarct size	PI3K	Attenuated (LY294002)	Ravingerova et al., 2007
IHH, 5000 m, 4 h/day, 4 wk	Adult rat	Perfused heart, global I/R	↑ Recovery of function	PKCε PI3K	Abolished (εV1-2) Abolished (wortmannin)	Wang et al., 2011
CNH, 10% O_2, 10 days	Neonatal rabbits	Perfused heart, global I/R	↑ Recovery of function	PKCε p38 MAPK JNK ERK	Abolished (chelerythrine) Abolished (SB203580) Abolished (curcumin) No effect (PD98059)	Rafiee et al., 2002

IHH, intermittent hypobaric hypoxia; INH, intermittent normobaric hypoxia; CNH, continuous normobaric hypoxia; I/R, ischemia/reperfusion; A/R, anoxia/reoxygenation; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; CAMKII, Ca^{2+}/calmodulin-dependent protein kinase II; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; ERK (p42/p44 MAPK), extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase. Adopted from Kolar et al. (2009: Supplement 5) and modified.

Wang et al. (2011) have found that the IHH-improved postischemic LV performance in rats was abrogated by PKCε-selective inhibitor peptide εV1-2, but the precise εV1-2 peptide form used in this study was not specified. The inhibitory peptide ability to cross biological membranes is rendered through linking with cell-permeable peptides such as TAT (Chen et al., 2001). Wang et al. (2011) did not mention whether the εV1-2 peptide used included this sequence, which renders the interpretation of their findings rather difficult. Nevertheless, the involvement of PKCε in cardioprotection induced by IHH in this study supports the notion that IHH promoted the PKCε translocation to membranes and increased the phosphorylation level of PKCε after I/R which, together with IHH-induced cardioprotective effects, were abolished by the specific PI3K inhibitor wortmannin and the ROS scavenger *N*-2-mercaptopropionylglycine, suggesting the activation of the protective ROS/PI3K/PKCε-dependent pathway in IHH hearts. Taken together, these studies clearly indicate that the activity of PKC during the acute ischemic insult is required for the manifestation of protected phenotype of chronically hypoxic hearts. There is also a limited evidence in favor of the involvement of other protein kinases, such as PKA, CAMKII, p38 MAPK and JNK in IHH-induced cardioprotection; for the details see Kolar et al. (2009: Supplement 5).

Activated protein kinases may exert their protective effects by phosphorylation of numerous target proteins. One of the potential targets is the K_{ATP} channel which is localized either on the sarcolemma ($sarcK_{ATP}$) or in the mitochondrial inner membrane ($mitoK_{ATP}$). Whereas the molecular structure of cardiac $sarcK_{ATP}$ has already been determined, the molecular identity of $mitoK_{ATP}$ has not yet been established. Therefore, the exact function/involvement of $mitoK_{ATP}$ in cardioprotection is still matter of debate (Garlid and Halestrap, 2012). The studies based on the use of selective $mitoK_{ATP}$ blockers (such as 5-hydroxydecanoate or MCC-134) and openers (such as diazoxide or BMS-191095) suggest that the $mitoK_{ATP}$ activation is involved in IHH-induced cardioprotective effects (Asemu et al., 1999; Neckar et al., 2002a; Zhu et al., 2003; Kolar et al., 2005). It can be assumed that activation of these channels during adaptation enhances mitochondrial tolerance to the pathogenic action of I/R stress. This hypothesis is supported by studies, which showed that pharmacological inhibition of $mitoK_{ATP}$ channels attenuated ATP resynthesis and led to irreversible damage of cardiomyocytes during reperfusion (Fryer et al., 2000). Also, it can be hypothesized that activation of $mitoK_{ATP}$ channels before I/R exposure intensifies the production of ROS that act as intracellular messengers and activate protective pathways

involved in improvement of heart tolerance to I/R (Gross et al., 2003). However, these results and hypotheses can be verified only when the molecular identity of mitoK_{ATP} is determined. Then it will become possible to use genetic ablation and overexpression techniques rather than the less specific pharmacological interventions to establish the role of mitoK_{ATP} channel in IHH-induced protection.

Recent studies have revealed that, besides K_{ATP} channels, the inner mitochondrial membrane contains large-conductance Ca^{2+}-activated K^+ channels that are opened by hypoxia (Cheng et al., 2008) and contribute to myocardial protection induced by CNH (Borchert et al., 2011).

It was shown that IHH can protect cardiac myocytes against I/R-induced cytosolic Ca^{2+} overload by preserving the functions of regulatory proteins that are involved in maintaining intracellular Ca^{2+} homeostasis, such as the Na^+/Ca^{2+} exchangers, the sarcoplasmic reticulum Ca^{2+} pumps and ryanodine receptors (Chen et al., 2006). Also, an increase in Na^+/K^+ pump activity following IHH was observed (Guo et al., 2011), which can play an essential role in maintaining the equilibrium of intracellular Na^+ and Ca^{2+} during I/R.

Chronic IHH also appears to inhibit the opening of mitochondrial permeability transition pores (MPTP), the non-specific large pores in the inner mitochondrial membrane, which open under the conditions of Ca^{2+} overload, especially when it is accompanied by adenine nucleotide depletion, elevated inorganic phosphate and oxidative stress, which are all features of I/R injury. MPTP opening allows water and solutes to enter the mitochondria, leading to matrix swelling, inner membrane potential collapse, uncoupling of the respiratory chain, efflux of Ca^{2+}, and the release of small proteins such as cytochrome *c* (Halestrap and Pasdois, 2009). Zhu et al. (2006) observed that the opening of the MPTP with atractyloside at the immediate start of reperfusion abolished the protective effect of IHH on functional recovery and infarct size in rat hearts. In this study, the authors have shown that IHH protected mitochondria against Ca^{2+} overload and cytochrom *c* release upon reperfusion. The MPTP opening is regulated by glycogen synthase kinase-3β (GSK-3β) activity (Juhaszova et al., 2004; 2009). Activation of cardioprotective signaling pathways is associated with phosphorylation and inhibition of a discrete pool of GSK-3β relevant to mitochondrial signaling which was also observed in IHH rat hearts (Wang et al., 2011). The protective effects of both mitoK_{ATP} and large-conductance Ca^{2+}-activated K^+

channels activation are most probably based on the K^+ influx to the mitochondrial matrix and thus prevention of Ca^{2+} overload and MPTP opening.

The participation of heat shock proteins (Xu et al., 2011), opioid peptides (Lishmanov et al., 1998) and antioxidant enzymes (Balkova et al., 2010: Supplement 4; Guo HC et al., 2009) were also reported in connection with IHH cardioprotective effects, and many others factors such as thyroid hormones and prostanoids, etc. can be supposed to play a role in this complex cardioprotective mechanism.

The fact that a combination of adaptation to IHH with ischemic preconditioning fails to produce an additive cardioprotective effect suggests that these phenomena utilize basically the same endogenous pool of protective pathways, albeit with different efficiency. Three major pathways, which can potentially be activated during IHH, have been proposed based on studies investigating the signal transduction of cardioprotection induced by different forms of preconditioning: i) the cGMP/protein kinase G (PKG)-pathway (Burley et al., 2007), ii) the reperfusion injury salvage kinase (RISK)-pathway (Hausenloy and Yellon, 2004) and iii) the survivor activating factor enhancement (SAFE)-pathway (Lecour, 2009) (Fig. 1). These three major pathways are potentially cooperative and they are recruited by the cardioprotective phenomena to differing extents. The cardioprotective signaling pathways are thought to converge on the mitochondria, where various mitochondrial proteins with or without channel structure (mitoK_{ATP}, MPTP, GSK-3β etc.) have been identified as central elements in cardioprotection. In the first program (Fig. 1 A), NO is a central step with the upstream activation of PI3K/Akt and downstream activation of mitoK_{ATP} channels and inhibition of MPTP. The second program (Fig. 1 B) involves the activation of PI3K/Akt and the ERK system with the downstream inhibition of GSK-3β. The participation of elements of cGMP/PKG-pathway and RISK-pathway in IHH-induced cardioprotection was mentioned in text above. Concerning the third program (Fig. 1 C), SAFE-pathway, the increase in myocardial interleukin 6 (IL-6)/Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) expression in infants with cyanotic cardiac defects suggests that tissue levels of IL-6/JAK/STAT3 could be induced by chronic hypoxia (Gu et al., 2011). Experimental studies have provided ample evidence that activated STAT3 protects cardiomyocytes from apoptosis and promotes expression of cardioprotective genes during the processes of hypoxia/reoxygenation and ischemia/reperfusion (Lecour, 2009). The involvement of these pathways in IHH-induced cardioprotection, their regulation and mutual interactions remain to be elucidated.

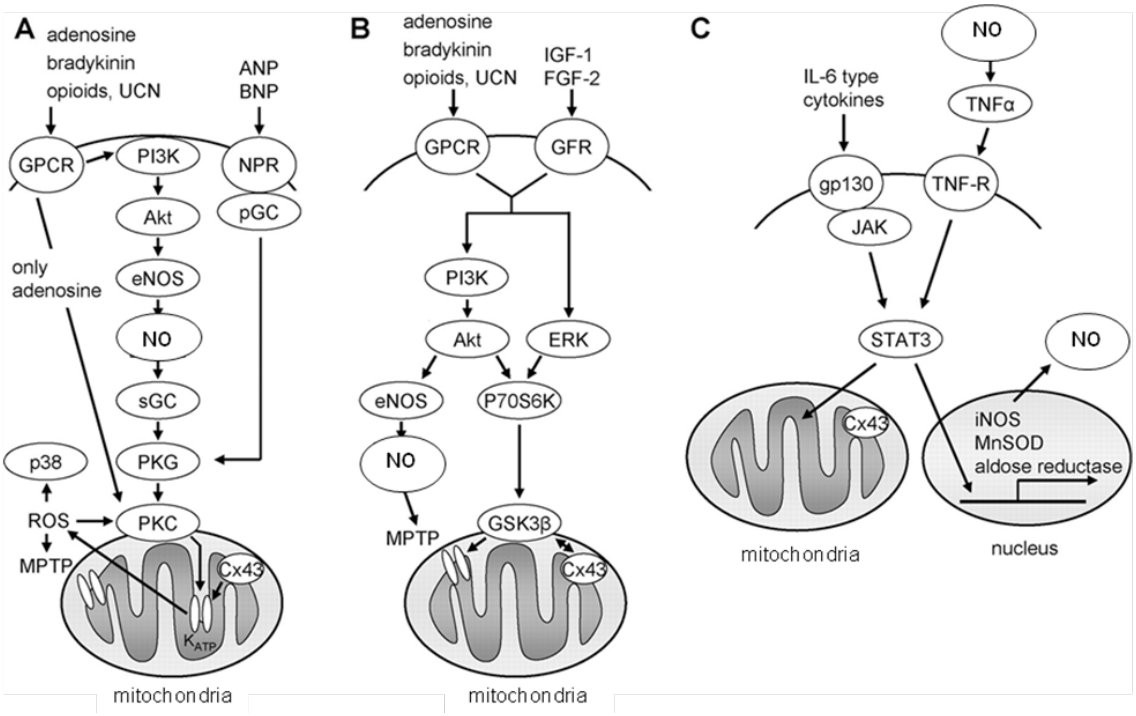

Fig. 1 *Protein kinase activation in cardioprotection. (A) GPCR/NPR-AKT-eNOS-PKG pathway. (B) RISK pathway. (C) gp130-JAK-STAT pathway. ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; Cx43, connexin 43; eNOS, endothelial nitric oxide synthase; ERK, extracellular regulated kinase; FGF-2, fibroblast growth factor 2; GFR, growth factor receptor; gp130, glycoprotein 130; GPCR, G-protein–coupled receptor; GSK3β, glycogen synthase kinase 3β; IGF-1, insulin-like growth factor 1; IL-6, interleukin 6; iNOS, inducible nitric oxide synthase; JAK, Janus kinase; K_{ATP}, ATP-sensitive potassium channel; MnSOD, manganese superoxide dismutase; MPTP, mitochondrial permeability transition pore; NO, nitric oxide; NPR, natriuretic peptide receptor; p38, p38 mitogen-activated protein kinase; P70S6K, p70 ribsosomal S6 protein kinase; pGC, particulate guanylyl cyclase; PI3K, phosphatidylinositol 3 kinase; PKC, protein kinase C; PKG, protein kinase G; ROS, reactive oxygen species; sGC, soluble guanylyl cyclase; STAT3, signal transducer and activator of transcription 3; TNFα, tumor necrosis factor α; TNF-R, tumor necrosis factor receptor; and UCN, urocortins. Adopted from Heusch et al. (2008) and modified.*

1.2.4 Further candidate mechanisms of cardioprotective adaptation to chronic hypoxia

There are many additional effects induced by adaptation to chronic hypoxia that might potentially contribute to the protection of the heart against acute oxygen deprivation.

Changes in energy metabolism, such as increased capacity of cardiac anaerobic metabolism, increased energy utilization capacity and selection of metabolic pathways or substrates with higher energy efficiency, were proposed to decrease the oxygen requirements in chronically hypoxic hearts (Moret, 1980). This view is supported by a study which showed that acclimatization to IHH increased capacity for oxidation of glucose and lactate at the expense of fatty acids in both ventricles (Bass et al., 1989). Similarly, decreased oxidation of palmitoyl carnitine was observed in both ventricles

following CHH (Nouette-Gaulain et al., 2005). The production of 1 mol of ATP from either fatty acid or glucose requires 0.177 or 0.156 moles of oxygen, respectively (Grynberg, 2005), thus the decrease of fatty acid/glucose oxidation ratio induced by IHH can contribute to the protection of the heart against acute oxygen deprivation.

One of the initial adaptive mechanisms to chronic hypoxia is a balanced suppression of ATP demand and supply pathways (Hochachka et al., 1996). This regulation allows the ATP level to remain constant, even when the ATP turnover rate greatly declines (Nouette-Gaulain et al., 2005). Thus, many ATP-dependent processes, like ion pumping or protein synthesis, are down-regulated during exposure to chronic hypoxia (Hochachka and Lutz, 2001).

Mitochondrial metabolism is involved in adaptation to chronic hypoxia via energy regulation, generation of ROS and apoptosis (Chandel et al., 2000). It was reported that one critical cell-autonomous adaptive response to chronic hypoxia controlled by HIF-1 is reduced mitochondrial mass and/or metabolism (Semenza, 2011). HIF-1 induces the transition from oxidative to glycolytic metabolism and reduces ROS production under hypoxic conditions by multiple mechanisms including: i) induction of pyruvate dehydrogenase kinase 1, which shunts pyruvate away from the mitochondria (Kim et al., 2006), ii) a subunit switch in cytochrome *c* oxidase (COX) from the COX4-1 to the COX4-2 regulatory subunit that increases the efficiency of complex IV (Fukuda et al., 2007), iii) induction of BNIP3 (Bcl-2/adenovirus E1B 19-kDa protein-interacting protein 3, a member of proapoptotic Bcl-2 proteins), which triggers mitochondrial selective autophagy (mitophagy) (Zhang et al., 2008), and iv) induction of microRNA-210, which blocks assembly of iron-sulfur clusters that are required for oxidative phosphorylation (Chan et al., 2009). It was observed that adaptation to CHH increased the number of cardiac mitochondria and decreased their size (Nouette-Gaulain et al., 2005). Hypoxia accelerates mitochondrial protein turnover, reflecting increased destruction and biogenesis (Gottlieb and Gustafsson, 2011). Mitochondrial fission, fusion and autophagy have been shown to be essential mechanisms of mitochondrial quality control (Twig et al., 2008). Mitophagy is required to eliminate damaged mitochondria; removal of mitochondria that are producing ROS can prevent dissemination of injury to neighboring mitochondria and thereby preclude widespread MPTP opening (Gottlieb and Gustafsson, 2011).

Adaptation to IHH influences the properties of cardiac cell membranes by altering their phospholipid fatty acids (FAs) profile (Jezkova et al., 2002). These changes, in particular the greater incorporation of n-3 polyunsaturated FAs (PUFAs) into

phospholipids and increased unsaturation index, can affect a number of cellular functions, including ion transport and transmembrane signal transduction, and may lead to a better preservation of membrane integrity and thereby contribute to the improved ischemic tolerance of IHH hearts.

1.3 The effects of dietary polyunsaturated fatty acids (PUFAs) in cardiovascular disease

A low incidence of cardiovascular death among Alaskan and Greenland Eskimos was reported in pivotal papers (Bang et al., 1980; Middaugh, 1990). These observations led to a hypothesis that a high dietary intake of n-3 PUFAs from oily fishes (e.g. wild salmon, sardines and mackerel) might decrease cardiovascular mortality. Since these observations, many experimental and clinical studies have shown that dietary lipids can influence the incidence and severity of coronary heart disease. The heart is very sensitive to the dietary FA supply and responds to dietary modifications by alterations in membrane phospholipid FA composition (Jude et al., 2007; Hlavackova et al., 2007: Supplement 2).

Fatty acids can be divided into three general categories: saturated (SFAs), monounsaturated (MUFAs) and PUFAs with two or more double bonds. The family of PUFAs with a double bond between the third and fourth carbon from the methyl end of the chain are called n-3 PUFAs, and those with a double bond between the sixth and seventh carbon atoms are n-6 PUFAs (Fig. 2).

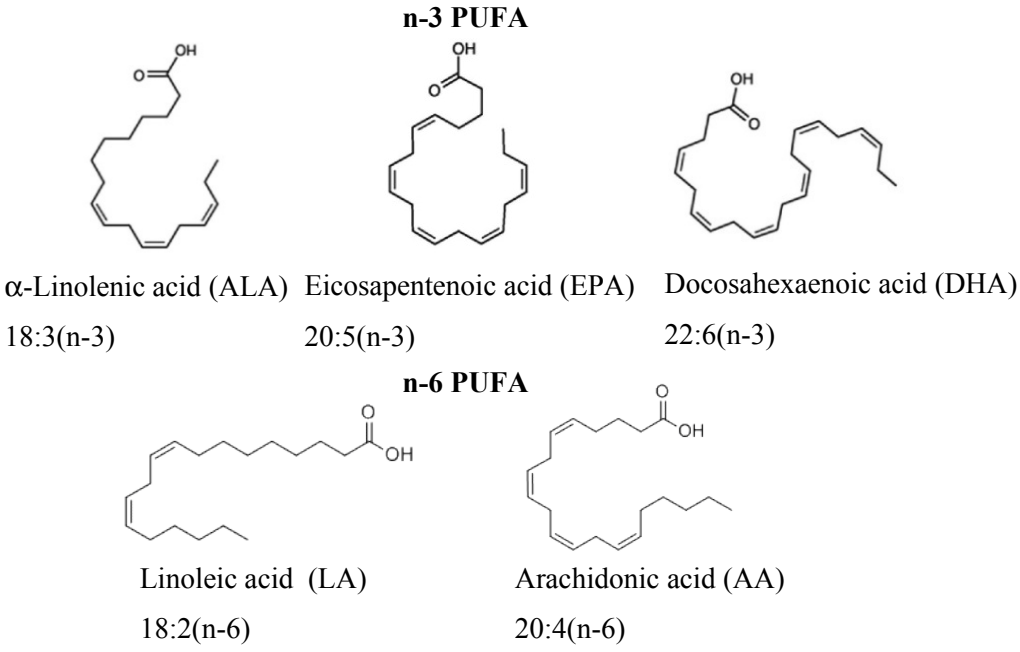

Fig. 2 *The structure of n-3 and n-6 PUFA.*

The essential fatty acids, linoleic acid (LA; 18:2n-6) and α-linolenic acid (ALA; 18:3n-3), are a necessary part of the human diet because the body has no biochemical pathway to produce these molecules on its own. LA and ALA are transformed to their higher

unsaturated derivates (arachidonic acid (AA; 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3)) by the activities of consecutive desaturation and elongation reactions. Unsaturated FAs also include the n-9 series, derived from oleic acid (18:1n-9) and the n-7 series, derived from palmitoleic acid (16:1n-7), which are not essential. Dietary sources of n-6 PUFAs are abundantly present in liquid vegetable oils, including soybean, corn, sunflower, safflower oil, cotton seed oils, while linseed and canola oils are rich in n-3 PUFAs. EPA and DHA belong to the n-3 series of PUFAs and are abundantly present in fish and shellfish tissue (Russo, 2009). The long hydrocarbon backbones, multiple double bonds and location of the first double bond in the n-3 or n-6 position result in complex and unique 3-dimensional configurations that contribute to the biological properties of these FAs.

MUFAs and PUFAs (n-3 and n-6 classes) usually display beneficial effects, while SFAs may have an adverse impact on the heart (White, 2009; Russo, 2009). Dietary replacement of SFAs with n-3 and n-6 PUFAs leads to a significantly decreased risk of coronary heart disease (Harris, 2010). Both classes of PUFAs collectively protect the heart against I/R injury but n-3 PUFAs are considered more potent in their cardioprotective activity. Consumption of n-3 PUFA improves vascular and cardiac hemodynamics, lowers plasma triglycerides, and possibly improves endothelial function and reduces inflammation, thrombosis and arrhythmia (Mozaffarian and Wu, 2011). More controversial is the role of n-6 PUFAs in cardiovascular disease prevention. While some studies have shown that LA improves lipid profile by lowering total cholesterol and low-density lipoprotein-cholesterol (reviewed by Hu et al., 2001), others have not confirmed these observations (Benatti et al., 2004). Animal studies have suggested an anti-arrhythmic effect of sunflower oil (rich in LA), although the effect was less prominent compared with that of fish oil rich diet (McLennan, 2001; Hlavackova et al., 2007: Supplement 2). Several authors tended to explain the PUFA effects in terms of the ratio between the respective dietary content of n-6 and n-3 PUFAs. A very high n-6/n-3 ratio is considered detrimental for human health, while a value as close as possible to 1 is considered protective against degenerative pathologies (Russo, 2009). During the last 150 years human population experienced a drastic increase in consumption of vegetable seed oils rich in n-6 PUFAs and a parallel decrease of n-3 PUFA intake leading to actual n-6/n-3 PUFA ratio ranges between 15:1 and 20:1 in the Western diet (Simopoulos, 2006) which could be one of the reasons of increased frequency of heart disease nowadays. Besides the n-6/n-3 PUFA ratio in diet, the n-3 PUFA index measured in red blood cells and expressed as a percentage of

EPA + DHA of total FAs was proposed as a new biomarker for cardiovascular risk (von Schacky and Harris, 2007).

Interestingly, adaptation to IHH also influences the properties of cardiac cell membranes by altering their phospholipid FA profile (Jezkova et al., 2002). Table 3 shows the main FAs present in the rat myocardial phospholipids. The SFAs account in total phospholipids in control rat left ventricle for about 35% and PUFAs for about 56% of total FAs. The proportion of SFAs and MUFAs in total phospholipids is not influenced by adaptation to IHH. However, adaptation to IHH causes a decrease of phospholipid n-6 PUFA content mainly due to a decrease of LA which is opposed by increased n-3 PUFA mainly due to DHA.

Table 3 Fatty acid composition (mol %) of total phospholipids in left ventricle of rats adapted to IHH (7,000 m) and of normoxic controls.

Fatty acids	Names of fatty acids	Total phospholipids / Left ventricle	
		Normoxia	Hypoxia
16:0	Palmitic acid	10.95 ± 0.29	13.34 ± 0.38*
18:0	Stearic acid	23.24 ± 0.56	22.30 ± 1.01
18:1n-9	Oleic acid	4.13 ± 0.11	3.95 ± 0.25
18:1n-7	Vaccenic acid	4.26 ± 0.13	4.28 ± 0.18
18:2n-6	Linoleic acid (LA)	28.40 ± 1.06	20.82 ± 1.28*
20:4n-6	Arachidonic acid (AA)	15.21 ± 0.41	15.56 ± 0.21
22:5n-3	Docosapentaenoic acid	1.60 ± 0.07	2.15 ± 0.09*
22:6n-3	Docosahexaenoic acid (DHA)	9.94 ± 0.50	15.35 ± 0.82*
Σ SFA		34.37 ± 0.78	35.85 ± 0.99
Σ MUFA		8.86 ± 0.26	8.68 ± 0.44
Σ n-6 PUFA		44.71 ± 0.87	37.53 ± 1.21*
Σ n-3 PUFA		12.07 ± 0.56	17.94 ± 0.89*
n-6/n-3 PUFA		3.77 ± 0.22	2.13 ± 0.17*

*Values are mean ± SEM of 7 hearts in each group. SFA, saturated fatty acids (FA); MUFA, monounsaturated FA; PUFA, polyunsaturated FA; *p < 0.05 vs. normoxic ventricle; FA reaching at least 1% of the total are shown only. Adopted from Jezkova et al. (2002).*

1.3.1 Molecular mechanisms of PUFAs action

PUFAs play important and diverse roles in structure and function of biological membranes, tissue metabolism and genetic regulation.

PUFAs contribute to membrane fluidity that is an important determinant for the correct hormone-receptor binding. Lipid microdomains, such as cholesterol and sphingolipid enriched rafts and caveolae, function as operational platforms to modulate numerous cellular functions, including signal transduction, protein and membrane

trafficking and ion channel kinetics (Dart, 2010). The incorporation of n-3 and n-6 PUFAs into membrane phospholipids alters the physico-chemical properties of membrane rafts and caveolae, thereby influencing membrane-associated protein localization and function. These changes may contribute to potential anti-inflammatory and anti-arrhythmic effects (Mozaffarian and Wu, 2011). Among the proteins influenced by changes of phospholipid FA composition rank PKC isoforms, which are activated by lipid second messengers such as 1,2-diacylglyceroles (DAGs). PUFA composition of DAGs can significantly affect the pattern of PKC activation/translocation to membranes and thus the signaling pathways transduced by PKCs (Madani et al., 2001; Nair et al., 2001).

N-3 and n-6 PUFAs are natural ligands of several nuclear receptors and transcription factors, such as peroxisome proliferator-activated receptors (PPARs), liver X receptors and sterol regulatory element-binding protein 1c, that regulate gene expression in multiple tissues. The finding that PUFAs are potent PPAR activators parallels the metabolic findings that n-6 and n-3 PUFAs are potent inducers of fatty acid oxidation and potent suppressors of fatty acid and triacylglycerol synthesis in animal models (Benatti et al., 2004). For regulating gene expression, cell membrane fluidity and raft composition, the effects of n-6 and n-3 PUFAs are often interchangeable with n-3 PUFAs acting as more potent ligands to nuclear receptors (Russo, 2009).

Dietary n-3 PUFAs have profound effects on mitochondrial phospholipid composition and mitochondrial resistance to stress (Stanley et al., 2012). Dietary supplementation with DHA, but not with EPA, increased DHA and total n-3 PUFAs in cardiac mitochondrial phospholipids and the tolerance of isolated mitochondria to Ca^{2+}-induced MPTP opening (Khairallah et al., 2010). This dietary intervention was also associated with an increase of the LA-rich form of cardiolipin, a mitochondrial phospholipid that fundamentally contributes to the function of many proteins in the inner mitochondrial membrane; the loss of cardiolipin LA content is associated with cardiac disorders (Sparagna and Lesnefsky, 2009).

PUFAs and their metabolites exert a second messenger action when intercalated in the cell membrane. Following the binding of a ligand such as growth factors and hormones to membrane receptors, phospholipase A2 is activated and releases AA, EPA and DHA from the sn-2 position of phospholipids. These molecules become substrates for eicosanoid biosynthesis mediated by cyclooxygenases, lipoxygenases, cytochrome P450s and non-enzymatic pathways (Bucszynski et al., 2009). Earlier conception has considered the lowering of AA-derived eicosanoids, which are regarded to be pro-inflammatory or pre-

thrombotic, and replacing by n-3 PUFAs as important for health benefits. Nowadays, this hypothesis seems to be overly simplistic, since it was reported that the anti-inflammatory effects of n-3 PUFAs may be independent of AA (e.g. via direct interactions with G-protein-coupled receptors (Oh et al., 2010)) and several AA-derived eicosanoids, such as epoxyeicosatrienoic acid (EETs) and lipoxins, exhibit anti-inflammatory activities (Serhan, 2010). The ability of higher EETs levels to improve cardiac function has been established in several animal models (Imig and Hammock, 2009). Both AA-derived lipoxins and recently identified classes of n-3 PUFA-derived eicosanoids called specialized pro-resolving mediators (such as resolvins, protectins and maresins) are key players of cellular specific resolution programs, which appear to be essential in the control of the duration and magnitude of acute inflammation (Serhan, 2010). Moreover, cytochrome P450-generated mono-epoxides from EPA and DHA were recently shown to be highly active anti-arrhythmic agents which possessed nearly 1,000-fold greater potency than EPA and DHA in suppressing the Ca^{2+}-induced increased rate of spontaneous beating of neonatal rat cardiomyocytes (Arnold et al., 2010).

The anti-arrhythmic effects of n-3 PUFAs are among their most intriguing potential physiological impacts. Both *in vitro* and *in vivo* experiments suggest that n-3 PUFAs directly influence atrial and ventricular myocyte electrophysiology, potentially mediated by effects on membrane ion channels or the cell-cell connexins/gap junctions (McLennan, 2001; Radosinska et al., 2011). The decreased conductance of gap junction is closely related to the occurrence of arrhythmias (De Groot and Coronel, 2004; Severs et al., 2008). Zhang et al. (1999) found that endothelial cell incubation with EPA was able to prevent hypoxia/reoxygenation-induced gap junction dysfunction. N-3 PUFA might contribute to reduced myocyte excitability and cytosolic Ca^{2+} fluctuations, particularly in ischemic or damaged cells, by altering the function of membrane Na^{+} channels, L-type Ca^{2+} channels and Na^{+}/Ca^{2+} exchangers (Xiao et al., 2006; Ander et al., 2007; Ferrier et al., 2002) and thus prevent arrhythmia triggering. It was also reported that ion channels are sensitive to some peroxidized products of n-3 PUFAs (Jude et al., 2003) which can be released under the particular pathophysiological conditions, such as myocardial infarction. The authors suggested that a high content of n-3 PUFAs in membrane phospholipids will permit the production of bioactive peroxidized molecules inhibiting ion channels involved in the occurrence of arrhythmias (Le Guennec et al., 2010). At the same time, n-3 PUFAs can modify the FA composition of cardiac phospholipids, including phosphoinositides. The changes in FA composition of phosphoinositide can lead to a reduction in the ratio of

activated PKCδ/PKCε (Jude et al., 2007) and thus subsequently to a different regulation of PKC-modulated ion channels involved in arrhythmias (Wang et al., 2009; Yang et al., 2009).

1.4 Protein kinase C (PKC)

Since the discovery of the protein kinase C (PKC) in 1977 by Nishizuka and co-workers (Takai et al., 1977), PKC has been demonstrated to be central to many signal transduction processes, including chronic cardiac disease, acute cardiac injuries (Churchill et al., 2008) and preconditioning-induced cardioprotection (Budas et al., 2007). PKC is a member of the AGC (PKA, PKG, PKC) family of serin/threonine kinases and is part of the inositol phospholipid signaling cascade, which is ubiquitous in tissues and organs. In the rat heart, PKC α, δ, ε and ζ are the most abundant isoforms (Rybin and Steinberg, 1994).

1.4.1 PKC structure

The PKC family is encoded by nine genes and is classified into three groups based on differences in structure and activation by second messengers (Fig. 3): i) classical or conventional PKCs (cPKCs; PKC α, βI, and the alternatively spliced βII containing an additional 43 residues at the NH_2 terminus and γ), which are calcium-dependent and activated by both DAG and phosphatidylserine (PS), ii) novel PKCs (nPKCs; PKC δ, ε, η and θ), which are calcium independent and regulated by DAG and PS, and iii) atypical PKCs (aPKCs; PKC ζ and ι/λ), which are calcium-independent and do not require DAG for activation, although PS can regulate their activity (Steinberg, 2008).

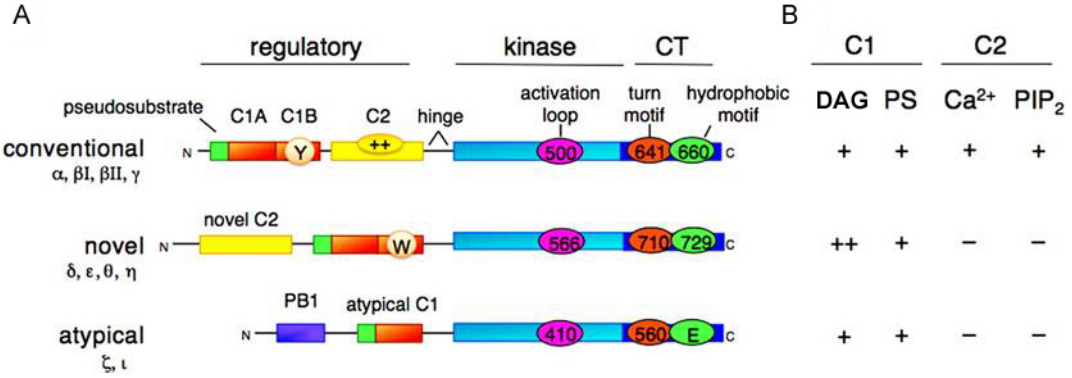

Fig. 3 *(A) Domain composition of protein kinase C (PKC) family members, showing pseudosubstrate (green), C1 domain [orange; tyrosine/tryptophan (Y/W) switch that dictates affinity to 1,2-diacylglycerol (DAG)-containing membranes indicated by circle in C1B domain], C2 domain [yellow; basic patch that drives binding to PIP_2 (phosphatidylinositol-4,5-bisphosphate), indicated by oval with ++], connecting hinge segment, kinase domain (cyan), and carboxyl-terminal tail (CT; dark blue). Also shown are the 3 priming phosphorylations in the kinase domain and CT with numbering indicated for PKCβII, PKCε, and PKCζ. (B) Table showing dependence of PKC family members on C1 domain cofactors, DAG and phosphatidylserine (PS) and C2 domain cofactors Ca^{2+} and PIP_2. PB1, Phox and Bem1 protein-protein interaction module domain. Adopted from Newton (2010).*

All family members share the same architecture: an amino-terminal region (20–40 kDa), containing regulatory modules, linked by a flexible hinge segment to a carboxylterminal catalytic domain (~ 45 kDa) (Fig. 3). The regulatory domain serves several functions: i) it maintains the enzyme in an autoinhibited state in the absence of appropriate second messengers, ii) it targets the enzyme to specific cellular locations, and iii) it mediates protein-protein interactions (Gould and Newton, 2008). When PKC is inactive, the pseudosubstrate sequence, resembling that of a substrate except for an alanine at the phospho-acceptor position, occupies the substrate-binding cavity of PKC (House and Kemp, 1987). Binding of the C1 and C2 domains to membranes provides the energy to release the pseudosubstrate from the substrate-binding cavity, thus allowing substrate phosphorylation. Both cPKCs and nPKCs have a tandem C1 domain (C1A and C1B) that bind their ligands, DAGs (Hurley et al., 1997). In addition to binding DAGs, the C1 domain also specifically binds the anionic phospholipid, PS (Johnson et al., 2000). In the case of cPKCs, the other module, the C2 domain also binds anionic phospholipids but in a Ca^{2+}-dependent manner. Novel PKCs also contain the C2 domain but it lacks the key residues required to bind Ca^{2+}; the C2 domain in nPKCs play a role in protein-protein interactions (Cho and Stahelin, 2006). Atypical PKCs lack a C2 domain entirely but have an additional N-terminal domain PB1 (Phox and Bem 1), which has been shown to serve an important role in mediating protein-protein interactions (Moscat et al., 2006). The hinge regions of certain PKCs have been identified as targets for caspase-dependent cleavage, protein-protein interactions, and tyrosine phosphorylations. The C-terminal catalytic domain (that contains motifs required for ATP/substrate binding and catalytic activity) of PKCs is highly conserved, primarily differing in the C-terminal tail that serves as a phosphorylation-dependent docking site for key regulatory molecules (Newton, 2010).

1.4.2 PKC regulation

PKCs are processed by phosphorylation and regulated by cofactor binding and subcellular localization. PKC translocation to the plasma membrane has generally been considered the hallmark of its activation. However, this model of PKC activation is not sufficient to explain the complex spatiotemporal controls of PKC localization in cells since PKCs achieve specificity besides translocation events through other mechanisms such as i) PKCs phosphorylations on both serine/threonine and tyrosine residues that influence the stability, protease/phosphatase resistance, protein-protein interactions, subcellular

targeting, and activity of the enzyme, ii) PKCs cleavage by caspases, generating a catalytically active kinase domain and a freed regulatory domain fragment that can act both as an inhibitor of the full-length enzyme and as an activator of certain signaling responses, and iii) PKCs activation by less traditional lipid cofactors (such as ceramide or AA) or through lipid-independent mechanisms (such as oxidative modifications or tyrosine nitration) that allow for PKC signaling throughout the cell, not just at DAG-containing membranes (Steinberg, 2008; Newton, 2009; Newton, 2010).

Regulation of PKC by phosphorylation

Before PKC can respond to lipid second messengers, the enzyme must first be properly processed by three ordered phosphorylations: activation loop phosphorylation (localized in the catalytic domain), turn motif phosphorylation and hydrophobic motif phosphorylation (localized in the C-terminal tail) (Fig. 3 and 4). These phosphorylations on the highly conserved serine/threonine phosphorylation motifs in all PKC isoforms lock the enzyme in a conformation which is closed, stabilized, catalytically competent and protease/phosphatase-resistant. In the PKC, maturation occurs due to the indispensable role played by i) the heat shock protein-90 (HSP90), whose interaction with a specific motif on PKC is essential to allow phosphorylations to occur (Gould et al., 2009), and ii) the mammalian target of rapamycin (mTOR) complex 2 (mTORC2) whose integrity is required to allow the priming phosphorylations (Ikenoue et al., 2008). The first step in the maturation process of cPKC/aPKC isoforms is phosphorylation of the activation loop by the upstream kinase, phosphoinositide-dependent kinase-1 (PDK1) (Le Good et al., 1998). PKCδ is unusual among PKCs in that it does not require activation loop phosphorylation (Thr505) for catalytic competence. This has been attributed to an acidic Glu at position 500 in PKCδ that assumes the role of the phosphorylated activation-loop Thr in cPKCs (Stempka et al., 1999). Nevertheless, Thr505 phosphorylation has been implicated as a mechanism that regulates PKCδ substrate specificity (rather than absolute PKCδ activity) (Steinberg, 2008). Rybin et al. (2007) identified a novel PKCδ-Thr505 autophosphorylation mechanism in cardiomyocytes that is triggered by PKCε overexpression and involves Src-dependent PKCδ-Tyr311/Tyr332 phosphorylation in the hinge domain. Unlike phosphorylation of the activation loop site which is dispensable for activity, phosphorylation of the turn motif is absolutely essential to maintain the catalytic competence of the enzyme and depends on mTORC2 complex involvement (Ikenoue et al.,

2008). Phosphate on the turn motif locks PKC in a thermally stable conformation by anchoring the carboxyl-terminal tail on the upper lobe of the kinase (Hauge et al., 2007). The final step in the maturation and processing of PKC is HSP90 and mTORC2-controlled phosphorylation at the C-terminal hydrophobic motif which affects the subcellular localization and stability of PKC.

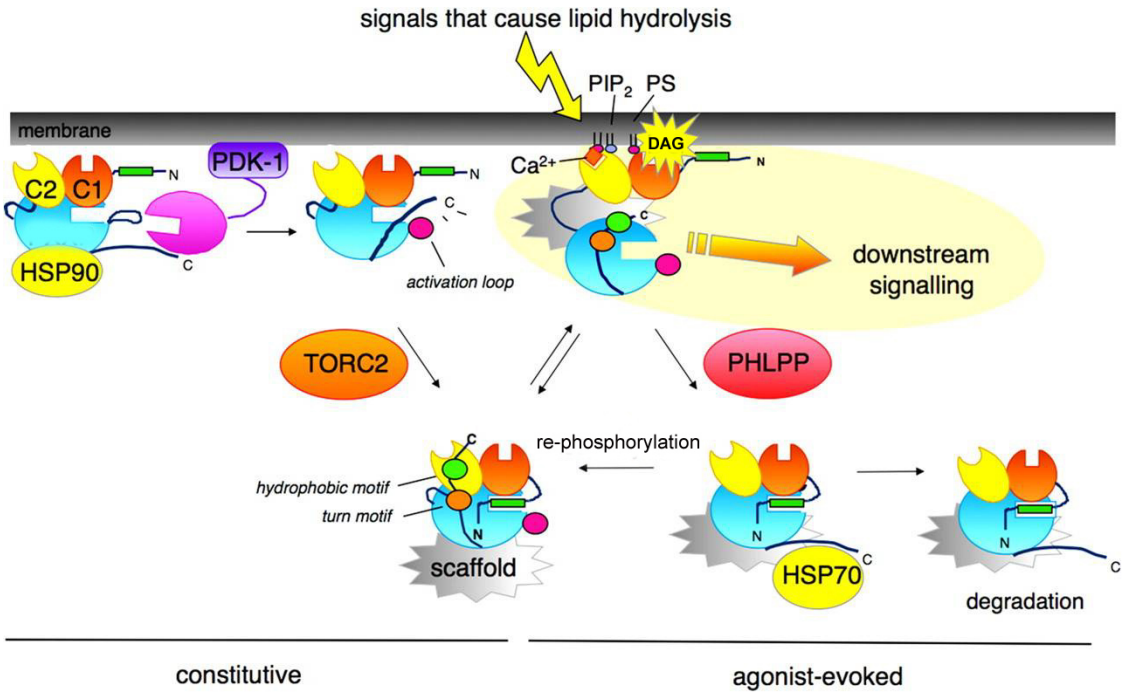

Fig. 4 *Model for the life cycle of PKC, from biosynthesis to degradation. Newly synthesized PKC associates with a membrane fraction, where it is processed by a series of ordered and tightly coupled phosphorylations. The heat shock protein-90 (HSP90) binds to the kinase domain, an event required for priming phosphorylations. Two upstream kinases control priming phosphorylations: i) phosphoinositide-dependent kinase-1 (PDK-1), bound to the exposed carboxyl terminus of newly synthesized PKC, phosphorylates the activation loop, ii) the mammalian target of rapamycin (mTOR) complex 2 (mTORC2) promotes the phosphorylation of the turn and hydrophobic motifs. The fully phosphorylated "mature" PKC localizes to the cytosol with the pseudosubstrate (green rectangle) occupying the substrate-binding cavity. Signals that cause lipid hydrolysis recruit PKC to membranes. Membrane-bound PKC adopts an open conformation, in which the pseudosubstrate is released from the kinase domain, allowing downstream signaling. This open conformation is sensitive to dephosphorylation: the phosphatase PHLPP (PH domain leucine-rich repeat protein phosphatase) dephosphorylates the hydrophobic motif. The molecular chaperone HSP70 binds to the dephosphorylated turn motif, an event that promotes rephosphorylation of PKC and reentry into the pool of signaling-competent enzymes. Specific protein scaffolds bind to themselves specific isoforms and specific species [e.g., Receptors for Activated C Kinase (RACKs) bind active PKC, but other scaffolds bind inactive PKC] in order to position PKC in specific cellular microdomains. The amplitude of the PKC signal and similarly the responsiveness of the cell to low concentrations of agonist are ultimately controlled by how much PKC is in the cell. Adopted from Newton (2010).*

Conventional, novel, and atypical PKC isoforms are also phosphorylated on tyrosine residues (Konishi et al. 1997). This phosphorylation emerges as an additional mechanism to fine-tune PKC activity and has been most studied with the nPKC isoform, PKCδ. In general, tyrosine phosphorylation of the catalytic domain increases the kinase activity of PKCδ, whereas phosphotyrosines in the PKCδ regulatory domain influence the cellular actions of PKCδ without influencing its kinase activity (Steinberg, 2004).

Regulation of PKC by lipid second messengers

Lipids acutely control the amplitude, duration, and subcellular location of signaling by lipid second messenger responsive PKCs. Despite the enormity of the kinome, few kinases (approximately 10%) directly bind and transduce lipid second messenger signals. Conventional and novel PKC isoforms contain DAG-binding C1 domains, and transduce signals that trigger DAG production. Agonist-evoked hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) generates two key second messengers for cPKC isoforms: Ca^{2+} and DAG. The binding of Ca^{2+} to the C2 domain pretargets cPKC to the plasma membrane, where it binds to anionic phospholipids, with preference for PIP_2 (Fig. 4). Once attached to the membrane, the C1 domain binds to DAG, an interaction that is enhanced by stereospecific binding to PS (Newton, 2009). nPKC isoforms, which do not have a Ca^{2+}-binding C2 domain and thus lack the membrane-pretargeting mechanism, are able to respond to agonist-evoked changes in DAG because their C1B domain has a twice as high affinity for DAG-containing membranes than the C1B domain of cPKCs (Giorgione et al., 2006). The structural basis for isoform-specific differences in the C1B domain affinity for DAG is determined by a single residue on the C1B domain; an invariant tryptophan residue that is conserved in the lipid-binding surface of nPKCs is replaced by a tyrosine in cPKC C1B domains (Fig. 3) (Dries et al., 2007). cPKC isoforms respond rapidly at the plasma membrane with the same kinetics as Ca^{2+} formation, driven by rapid recruitment via the C2 domain to the PIP_2-rich plasma membrane, which in turn allows efficient binding of DAG via the C1 domain. nPKC isoforms respond more slowly at Golgi apparatus, driven by direct recruitment via their C1 domain to Golgi-bound DAGs. Whereas cPKC activity terminates rapidly because of the faster turnover of DAGs at the plasma membrane, nPKC activity is sustained because of prolonged DAG accumulation at the Golgi (Gallegos and Newton, 2008). DAGs are produced upon appropriate agonist cell stimulation primarily by hydrolysis of PIP_2 catalyzed by phosphoinositide-specific phospholipase C (PI-specific

PLC) or by phospholipase D (PLD)-catalyzed phosphatidylcholine (PC) (but also PI and phosphatidylethanolamin) hydrolysis (Fig. 5). It is generally considered that the FA composition of DAGs generated by the action of PLD differs from that resulting from hydrolysis by PI-specific PLC (Hodgkin et al., 1998). PI usually has stearic acid in position 1 of the glycerol molecule and AA in position 2, whereas PC, which is much more abundant than PI (comprising 40% of total cell lipids versus 5% for PI in cardiac cells (Jezkova et al., 2002)), has mainly SFAs or MUFAs in position 2. The FA composition of DAGs can significantly influence the activation of different isoforms of cPKCs and nPKCs which differ in their responses to DAGs containing AA, EPA, or DHA (Madani et al., 2001). DAG signals are terminated by the action of DAG kinases (DGKs) which convert DAGs into phosphatidic acid (Merida et al., 2008). It was shown that PKCs phosphorylate DGK and thus can regulate its activity (Luo et al., 2003). Moreover, activation of PKC can also cause translocation of DGK to where PKC is localized and thus possibly initiate a negative feedback mechanism designed to regulate its own enzyme activity (van Baal et al., 2005).

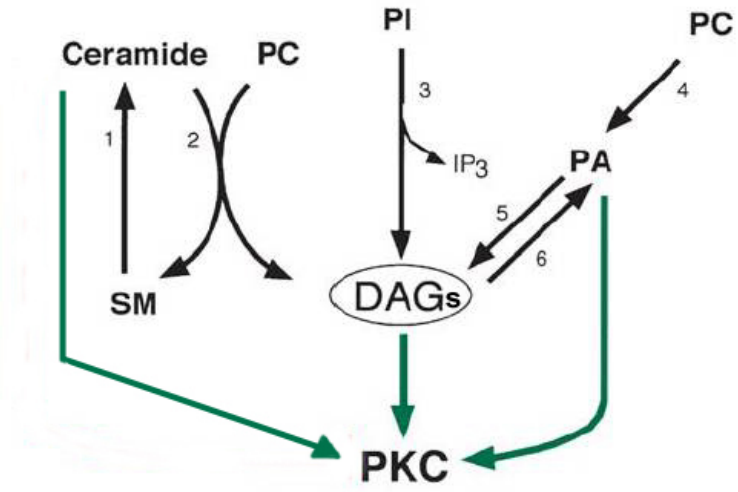

Fig. 5 *Multiple sources of 1,2-diacylglycerols (DAGs) in the cell and the enzymes that are involved. 1) sphingomyelinase; 2) sphingomyelin (SM) synthase; 3) phosphatidylinositol (PI)-specific phospholipase C; 4) phosphatidylcholine (PC)-specific phospholipase D; 5) phosphatidic acid (PA)-phosphohydrolyase; (6) DAG kinase. PKC, protein kinase C. Adopted from Becker and Hannun (2005) and modified.*

The different effects of free FAs on individual PKC isoenzyme translocation and activation were also reported (Shirai et al., 1998; Huang et al., 1997; Mackay and Mochly-Rosen, 2001a). AA selectively activates PKCε and PKCδ in neonatal rat cardiomyocytes, leading to protection from ischemia (Mackay and Mochly-Rosen, 2001a). On the other hand, n-3 PUFA (DHA and EPA) supplementation of adult porcine cardiac myocytes decreased the activity of PKC in comparison with lack of the decrease in AA supplemented cells (Nair et al., 2001).

PKC isoforms also translocate to specialized membrane compartments such as lipid rafts or caveolae (Parton and Simons, 2007). Ceramide (formed in lipid rafts) provides the driving force for raft fusion into platforms. The ceramide-enriched raft platforms have been shown as important redox signaling membrane domains that amplify activation of ROS generating enzymes such as NADPH oxidase family enzymes (Li et al., 2010). The interaction of PKCs with ceramide has been observed (Kashiwagi et al., 2002). PKCδ was shown to regulate ceramide formation by the activation of acid sphingomyelinase, an enzyme that catalyzes the hydrolysis of sphingomyelin to form ceramide at the plasma membrane (Zeidan and Hannun, 2007). This effect of PKCδ to promote local ceramide accumulation at the plasma membrane drives raft fusion and may provide a nonspecific mechanism to localize signaling proteins such as PKCs to membrane rafts.

Regulation of PKC by scaffolding proteins

Targeted kinase activity reporters have shown that PKCs operate in all regions of the cell: plasma membrane, nucleus, Golgi apparatus, mitochondria, and cytosol (Gallegos et al., 2006). Each PKC isoform localizes to unique sub-cellular sites upon stimulation of cardiac myocytes which suggests that the various PKC isoform mediate unique functions (Disatnik et al., 1994). This process is mediated by binding of each isoform to its selective receptor for activated C kinase, RACK (Mochly-Rosen, 1995), a family of membrane-associated PKC anchoring proteins. RACKs act as molecular scaffolds to localize individual PKCs to distinct membrane microdomains in close proximity with their activators and unique intracellular substrates. It was proposed that cells express a unique RACK for each PKC isoform and that PKC-RACK interactions are essential for isoform-specific cellular responses. To date, proteins with characteristics of RACKs have been identified for PKCβ (RACK1) and PKCε (RACK2 or β-COP) (Mackay and Mochly-Rosen, 2001b). These RACK proteins share a seven-WD40-motif repeat structure, similar to the protein-protein binding motifs found in heterotrimeric G protein β-subunits (Ron et al., 1994). Concerning PKCδ, p32/gC1qBP has been identified as a PKCδ–binding partner which exhibits properties of a RACK (Robles-Flores et al., 2002), and annexin V was shown as a shuttle protein on microtubules to transport PKCδ to its site of action in the particulate fraction; this interaction between PKCδ and annexin V in the cytosol precedes and is required for the subsequent PKCδ translocation to membranes (Kheifets et al., 2006). PKC mediates protein-protein interactions through its regulatory C1 and C2

domains as well as through the C-terminal tail (Kheifets and Mochly-Rosen, 2007). Short peptides have been derived from the C2 domain and other PKC domains to serve as isoform-specific activators and inhibitors by regulating PKC interaction with its RACK (Fig. 6) (Souroujon and Mochly-Rosen, 1998; Dorn et al., 1999). Peptide inhibitors bind to the specific RACKs and disrupt the anchoring and function of the corresponding PKC isoforms; peptides derived from a sequence from the C2 region of PKCδ and PKCε, δV1-1 and εV1–2, have been shown to selectively inhibit PKCδ and PKCε translocation and function in cardiomycytes and hearts (Johnson et al, 1996; Inagaki et al., 2003).

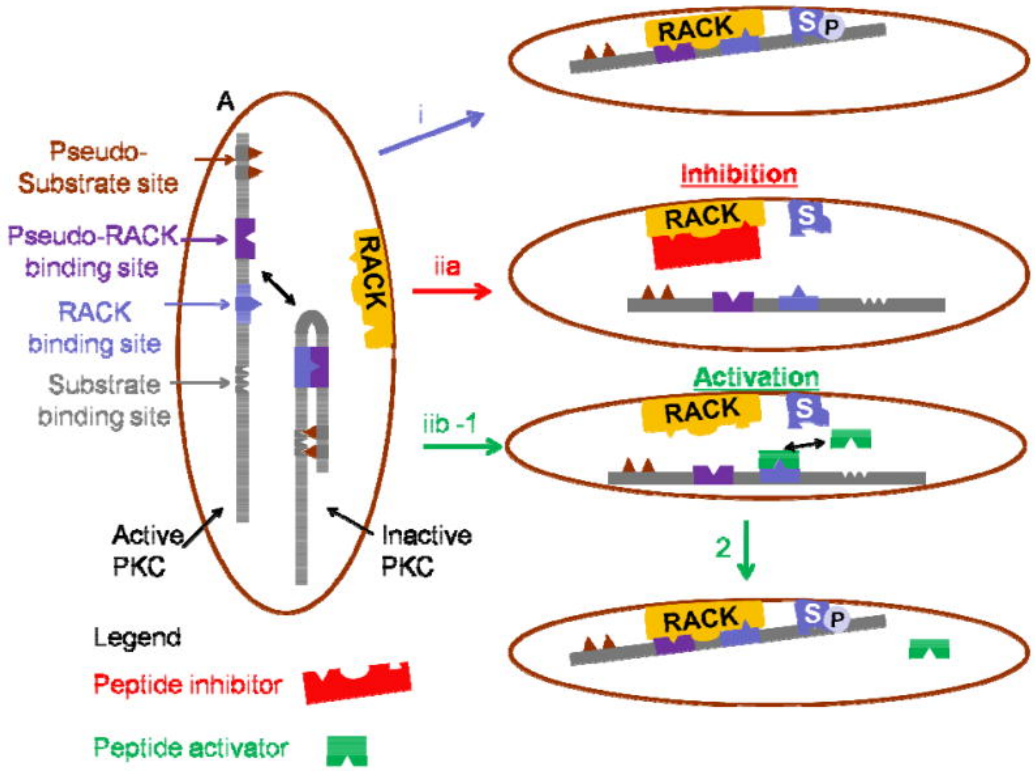

Fig. 6 *Model of peptide regulators of protein-protein interactions between PKC isoforms and their anchoring proteins, Receptors for Activated C Kinase (RACKs). A) The PKC isoform is in equilibrium between active and inactive conformations. Four intra-molecular interactions sites are depicted: pseudo substrate site, pseudo RACK site, the RACK-binding site and the substrate-binding site. i) Upon activation, PKC isoforms bind to their RACKs, enabling the phosphorylation of their respective substrates (S) that are localized near the RACK. iia) Competitive antagonist - peptide inhibitor, corresponding to a sequence in PKC, binds to the PKC-binding site in the RACK, competing with the protein-protein interaction between PKC and RACK and thereby inhibiting phosphorylation of the substrate. iib) Allosteric agonist - 1) peptide activator is derived from the PKC pseudo-RACK site. It binds to a PKC isoform at the RACK-binding site, tilting the equilibrium between the active and inactive conformations towards the active conformation. 2) The affinity of the pseudo-RACK peptide to the PKC is lower than the affinity of RACK, allowing PKC to bind to its RACK and phosphorylate the substrate. Adopted from Qvit and Mochly-Rosen (2010).*

Direct redox regulation of PKC

All PKCs contain redox-sensitive cysteine residues in both regulatory and catalytic domains, which are required for autoinhibition and catalytic activity, respectively (Gopalakrishna and Jaken, 2000). Mild oxidative stress promotes the oxidation of regulatory C1 domain cysteine residues. Oxidation of these residues leads to the release of Zn ions, reduced autoinhibitory function, and cofactor-independent PKC activation (Gopalakrishna et al., 2008; Knock and Ward, 2011). In contrast, PKC may be inhibited by high concentrations of oxidants through further oxidation of catalytic domain cysteine residues (Knock and Ward, 2011). Under physiological conditions, the catalytically essential cysteine residues are protected from oxidation by preferential reduction by thioredoxin (Gopalakrishna et al., 2008). The existence of a thioredoxin-PKC complex has been demonstrated and this close proximity was shown to be critical for restoring the catalytic activity of PKC (Watson et al., 1999). As well as being activated (or inhibited) by ROS, it is well established that PKC is an important activator of most isoforms of NADPH oxidase (Bedard and Krause, 2007). This suggest that the activation of PKC directly or indirectly by ROS can lead to amplification of the ROS signal which could regulate the PKC activity.

NO and NO-derived reactive nitrogen species, such as peroxynitrite, which is formed when NO reacts with superoxide, react with reduced cysteines or tyrosines, forming cysteine *S*-nitrosothiols or 3-nitrotyrosines, respectively. PKC nitration has been reported to increase PKC signaling by enhancing PKC-protein complex formation. It was shown that NO donors increase PKCε tyrosine nitration and PKCε binding to its anchoring protein RACK2, leading to increased PKCε protein and activity in the particulate fraction of rabbit cardiomyocytes (Balafanova et al. 2002). Indeed, exposure to high levels of NO results in loss of the catalytic activity of PKCs (Knapp et al., 2001).

Caspase cleavage of PKC at the hinge region

PKCs can be cleaved by caspases, generating a catalytically active kinase domain and a freed regulatory domain fragment that can act both as an inhibitor of the full-length enzyme and as an activator of certain signaling responses. PKCδ, PKCθ, PKCε, and PKCζ undergo caspase-dependent cleavage in response to a range of apoptogenic stimuli (Steinberg, 2008). This model of PKC activation is valid only if kinase domains are per se

catalytically competent. This condition is fulfilled for PKCδ, which is active without activation loop phosphorylation (Stempka et al., 1999). Nevertheless, the differences in the enzymology of full-length PKCδ, which is inhibited by sphingosine, and the freed PKCδ catalytic domain, which acts as a sphingosine-dependent kinase, have been detected (Hamaguchi et al., 2003).

Mechanisms of PKC inactivation and down-regulation

In the absence of chronic stimulation, PKC isoforms have a relatively long half-life, in the order of days for cPKC in cells grown in culture. However, the membrane bound conformation is sensitive to dephosphorylation and, upon prolonged activation, becomes dephosphorylated, ubiquitinated, and degraded (Newton, 2010). PKC reversibly translocates on and off membranes in response to second messenger levels. A mechanism by which PKCs can be down-regulated is the decrease in concentration of cofactors. PKC activity is correlated to cofactor concentrations and DGK activity (Violin et al., 2003; Miele et al. 2007). Dephosphorylation of the hydrophobic motif residue is suggested to be another mechanism of PKC down-regulation. The PH domain leucine-rich repeat protein phosphatase was shown to be responsible for regulation of phosphorylation status and protein levels of PKCs (Gao et al., 2008). The molecular chaperone heat shock protein 70 binds to the dephosphorylated species of PKC, which allows the enzyme to become rephosphorylated and re-enter the pool of signaling-competent enzymes (Fig. 4) (Gao and Newton, 2006). The signal that triggers PKC ubiquitination and proteosomal degradation has not been elucidated yet. The trigger appears to be dependent on the type of PKC isoform, cell-type, type and duration of stimuli and localization of PKC in the cell. Debate currently exists as to whether or not the phosphorylation state of PKC dictates whether it will be ubiquitinated. Several studies have suggested that dephosphorylation of the three priming sites of PKC and inactivation precedes the degradation of the enzyme (Lee et al., 1997), whereas some studies show that PKC requires phosphorylation and catalytic activity for proteosomal degradation (Lu et al., 1998). The most common mechanism that initiates ubiquitination of proteins is phosphorylation of the PEST (proline, glutamate, serine and threonine) sequence; PKCs also contain this sequence and it may contribute to degradation (Lee et al., 1997). However, the precise mechanisms for degradation remain to be elucidated, and it is likely that different PKC isoforms will be controlled by unique "turn-off" mechanisms.

1.4.3 Specific roles of PKC in the heart

PKC isoforms signaling in the heart has been the subject of intensive investigation since the discovery that PKCs play a crucial role in cardioprotection induced by ischemic preconditioning (Ytrehus et al., 1994). Whereas numerous studies have implicated individual PKC isoforms, primarily PKCε, in cardioprotection induced by preconditioning, data concerning their potential role in the cardioprotective mechanism of chronic hypoxia are rather limited and controversial probably due to different animal and hypoxia models (Kolar et al., 2009: Supplement 5). Table 4 summarizes the available experimental data that describe changes in the left ventricular myocardium expression, phosphorylation, subcellular distribution and activity of PKC isoforms induced by chronic hypoxia. Right ventricle data could be influenced by PKCs involvement in hypertrophic growth of chronically hypoxic animals and therefore were not included. In adult rats, 4 and 6 weeks of IHH corresponding to an altitude of 5,000 m led to an increased phosphorylation of PKCε (Wang et al., 2011) and an abundance of PKC α, δ and ε in the myocardial particulate fraction (Ding et al., 2004), respectively. After 12 days of INH (10% O_2) increased phosphorylation of PKCα was found (Cataldi et al., 2004). In contrast, two other reports did not show any significant effect of intermittent hypoxia at comparable intensity either on the PKC activity and content in total tissue homogenate (Rouet-Benzineb et al., 1999) or on the content and distribution of PKC isoforms α, δ, ε and ζ in cellular fractions (Morel et al., 2003). In our studies, using a more severe model of IHH (7,000 m), an increased abundance of PKCδ in the myocardial particulate fraction compared to normoxic controls was detected. This effect was dependent on oxidative stress associated with intermittent hypoxia as it was completely prevented by chronic antioxidant treatment of animals during the hypoxic adaptation period (Kolar et al., 2007). In addition, the content of PKCε, which is a key player in various forms of preconditioning, was reduced in the particulate fraction after exposure to chronic IHH (Kolar et al., 2007). In line with these results, PKCε content was significantly reduced in the cytosolic fraction and tended to decrease in the membrane fraction after adaptation to CHH (5,500 m) for 21 days, while the content of PKC α, βII, γ, and δ was not changed (Uenoyama et al., 2010). On the other hand, the exposure of neonatal rabbits to CNH (10% O_2) for 10 days resulted in increased phosphorylation of PKCε and its redistribution to the particulate fraction (Rafiee et al., 2002). Last but not least, the exposure of pregnant rats to CNH for 7 days led to a decreased level of PKCε in the myocardium of their offspring at adulthood while levels of PKC α, βI, βII, δ, and ζ were not affected (Li et

al., 2004). According to these studies, it appears that chronic hypoxia results, in at least some cases, in the up-regulation of specific PKC isoforms and/or their subcellular redistribution and activation. Together with the results of studies where PKC involvement in cardioprotection induced by chronic hypoxia was explored using pharmacological tools (mentioned in chapter 1.2.3, Table 3), it seems that the activity of PKC during the acute ischemic insult is required for the manifestation of protected phenotype of chronically hypoxic hearts. However, the identity of PKC isoforms involved and their potential downstream molecular targets responsible for cardioprotective effects of chronic hypoxia remains to be elucidated.

Table 4 Effects of chronic hypoxia on protein kinase C in the left ventricular myocardium.

Model of chronic hypoxia	Species	Protein kinase C isoform	Effect	Reference
IHH, 5,000 m, 6 h/day, 6 wk	Adult rats	PKC α, δ, ε	↑ Level in particulate fraction	Ding et al., 2004
IHH, 5,500 m, 23 h/day, 2 wk	Adult rats	PKC α, δ, ε, ζ	No effects on levels, subcellular distribution and activity	Morel et al., 2003
IHH, 7,000 m, 8 h/day, 5-6 wk	Adult rats	PKCδ PKCε	↑ Levels in all cellular fractions No effect	Neckar et al., 2005
IHH, 7,000 m, 8 h/day, 5-6 wk	Adult rats	PKCδ PKCε	↑ Level in particulate fraction ↓ Level in particulate fraction	Kolar et al., 2007 Hlavackova et al., 2007
IHH, 7,000 m, 8 h/day, 5-6 wk	Adult rats	PKCδ PKCε	↑ Level in homogenate and phosphorylation, translocation to mitochondria and sarcolemmal membranes ↓ Level in homogenate, no effect on phosphorylation and translocation	Hlavackova et al., 2010
IHH, 5,000 m, 4 h/day, 4 wk	Adult rats	PKCε	↑ Phosphorylation in homogenate	Wang et al., 2011
CHH, 5,500m, 21 days	Adult rats	PKCε PKC α, βII, γ, δ	↓ Level in cytosolic fraction No effect on level in cytosolic and particulate fraction	Uenoyama et al., 2010
INH, 10% O_2, 23 h/day, 1-3 wk	Adult rats	PKC	No effect on activity or level in homogenate	Rouet-Benzineb et al., 1999
INH, 10 % O_2, 12 h/day, 12 days	Neonatal to senescent rats	PKCα	↑ Phosphorylation in homogenate	Cataldi et al., 2004
CNH, 10.5% O_2, 7 days	Prenatal rats	PKCε PKC α, βI, βII, δ, ζ	↓ Level in homogenate (late effect examined at adulthood) No effect	Li et al., 2004
CNH, 10% O_2, 10 days	Neonatal rabbits	PKCε	↑ Phosphorylation and translocation to particulate fraction	Rafiee et al., 2002

CHH, continuous hypobaric hypoxia; CNH, continuous normobaric hypoxia; IHH, intermittent hypobaric hypoxia; INH, intermittent normobaric hypoxia. Adopted from Kolar et al. (2009: Supplement 5) and modified.

Based on changes in their expression and subcellular distribution, mainly the two novel PKC isoforms, δ and ε, can be considered as potential mediators of protection in chronically hypoxic hearts. It was shown that ischemic and pharmacological preconditioning activates both PKCδ and PKCε in rat and mouse cardiomyocytes (Hirotani and Sadoshima. 2005; Budas et al., 2007) whereas preconditioning was not observed in either PKCδ or PKCε null mice (Mayr et al., 2004a; Gray et al., 2004). The cooperation of these two PKC isoforms in cardiomyocytes was noted. Transient activation of PKCδ before ischemia has been shown to induce PKCε activation via stimulation of adenosine A_1 receptors leading to cardioprotection (Inagaki and Mochly-Rosen, 2005). PKCε and PKCδ influence key steps in the glucose metabolism of murine hearts; inhibition of PKCε resulted in compensatory phosphorylation and mitochondrial translocation of PKCδ providing a possible explanation for the synergistic effect of PKCδ and PKCε in cardioprotection (Mayr et al., 2009). However, the function of PKCδ in the heart could also be detrimental since it was shown that its activation in the middle of reperfusion increases the reperfusion-induced cell damage (Inagaki et al., 2003). This detrimental effect of PKCδ seems to be due to stimulation of excess ROS production and activation of proapototic pathways (Murriel et al., 2004). These findings clearly show the importance of the timing of PKCδ activation in relation to its protective or detrimental function in the heart. Although PKCδ and PKCε are members of the same subgroup of PKCs, they have multiple differences in their structure (a 41% amino acid homology with 9% gaps), which could be one of reasons for their different and sometimes opposite effects (Duquesnes et al., 2011). PKCδ seems to have the greatest flexibility among all PKC isoforms to affect various cellular functions because its activation and subcellular localization is finely modulated by phosphorylation of multiple serine/threonine and tyrosine residues (Steinberg, 2004). Moreover, PKCδ and PKCε mutual phosphorylation of residues influencing their activity, localization and stability in cardiomyocytes was observed (Mayr et al., 2009; Rybin et al., 2003; Rybin et al., 2007).

PKCε appears to play cytoprotective roles in three intracellular compartments, the cytosol, sarcolemma and mitochondria. As cytosolic targets of PKCε in cardioprotection Akt and eNOS were identified (Zhang et al., 2005). Concerning the sarcolemmal targets, it was shown that activation of PKC significantly sensitized the A_{2b} receptors to adenosine and that increased activation of PI3/Akt- and ERK-mediated signaling upon reperfusion in the preconditioned myocardium was abrogated either by a PKC inhibitor or by an A_{2b}

receptor blocker (Kuno et al., 2007; 2008). PKCε was demonstrated to phosphorylate connexin 43 in rat cardiomyocytes (Doble et al., 2000). Connexins are components of gap junctions which mediate electrical coupling between cardiomyocytes (Garcia-Dorado et al., 2004). Connexin 43 progressive dephosphorylation occurs during ischemia and leads to high conductance (Beardslee et al., 2000), thus PKCε phosphorylation of connexin 43 may prevent spreading of injury by reducing gap junction permeability. Proposed mitochondrial targets of PKC ε include mitoK_{ATP} (Jaburek et al., 2006), components of MPTP (Baines et al., 2003) and components of the electron transport chain (Guo et al., 2007). In mitochondria, PKCε was shown to form a complex with mitogen-activated protein kinases (i.e. ERKs, JNKs, and p38MAPK) and components of MPTP (i.e. the voltage-dependent anion channel, the adenine nucleotide translocase, and hexokinase II) (Baines et al., 2002; 2003). ERK phosphorylation was shown to inhibit MPTP opening via GSK-3β phosphorylation in non-cardiac cells (Rasola et al., 2010). GSK-3β phosphorylation (and inhibition) is a necessary step for preconditioning-mediated attenuation of MPTP opening (Juhaszova et al., 2004; 2009). Moreover, an increased level of phoshorylated ERK and GSK-3β were observed in constitutively active PKCε transgenic mouse hearts (Baines et al., 2002; McCarthy et al., 2011). According to these studies, it can be suggested the connection of PKCε activation in the intermembrane space of mitochondria and inhibition of the MPTP opening by ERK-GSK-3β signaling. Mitochondrial aldehyde dehydrogenase-2, which oxidized cytotoxic aldehydes to carboxylic acids, is another target of PKCε playing an important role in cardioprotection (Chen CH et al., 2008). Other study has identified cytochrome *c* oxidase subunit IV as a substrate for PKCε, an interaction of PKCε with cytochrome *c* oxidase subunit IV and improved cytochrome *c* oxidase activity was observed in preconditioned rat myocardium (Guo et al., 2007). Lastly, some studies have suggested that PKCε may also regulate the levels of pro- and anti-apoptotic Bcl-2 proteins and thus mediates its anti-apoptotic effects (Baines et al., 2002).

Concerning PKCδ, it seems that its protective effects are manifested when PKCδ is stimulated well before the detrimental ischemic insult. In the ethanol-induced cardioprotection, the initial PKCδ activation leads to PKCε activation through release of adenosine, suggesting that PKCδ could play a role as initial trigger/sensor of preconditioning. In rat cardiomyocytes, PKCδ and PKCε cooperate to regulate phosphorylation of p66Shc (Guo J et al., 2009), which has recently emerged as a master regulator of intracellular ROS production and cardiovascular oxidative stress responses (Pinton et al., 2007; Nemoto et al.,

2006; Rota et al., 2006). PKCδ was shown as a novel regulatory molecule of oxidative phosphorylation by targeting the pyruvate dehydrogenase (PDH) complex (Actin-Perez et al., 2010a). Activation of PKCδ leads to the dephosphorylation of pyruvate dehydrogenase kinase 2 (PDK2), thereby decreasing PDK2 activity and increasing PDH activity, accelerating oxygen consumption, and augmenting ATP synthesis. PKCδ activation results from interaction with oxidized form of cytochrome *c,* which is dependent on vitamin A (retinol) bound to the zinc-finger domain of PKCδ enabling the site-specific oxidation of PKCδ by cytochrome *c*, and requires the presence of the adaptor protein p66Shc (Acin-Perez et al., 2010b). This study indicates the existence of a PKCδ signalosome in mitochondria that gauges the redox state of cytochrome *c*, responds by enzymatic activation, and transmits this signal to the PDH complex for increased flux of fuel into the Krebs cycle. In contrast, PDH declines in activity during cardiac ischemia and prevention of PKCδ translocation to the mitochondria during reperfusion was shown to result in recovery of PDH activity (Churchil et al., 2005). Translocation of PKCδ to mitochondria during reperfusion also enhanced the release of cytochrome *c* and led to propagation of further downstream apoptotic effects such as the accumulation and dephosphorylation of the pro-apoptotic BAD (Bcl-2-associated death promoter), dephosphorylation of Akt, and DNA laddering (Murriel et al., 2004). Besides PKCδ apoptotic effects, a link between PKCδ and autophagy was reported (Huang et al., 2010; Chen JL et al., 2008). Autophagy and cardioprotection were abolished in rat hearts perfused with recombinant inhibitor of autophagy Tat-Atg5 (K130R) (Huang et al., 2010). Autophagy, a lysosome-dependent degradation pathway, and apoptosis are two fundamental cellular pathways that respond to stress conditions, such as hypoxia and oxidative stress. In the early stage of hypoxic response, PKCδ activates autophagy in an attempt to protect cells from cell death by promoting JNK1-mediated Bcl-2 phosphorylation and dissociation of the Bcl-2/Beclin 1 complex (Chen JL et al., 2008). In contrast, prolonged hypoxic stress leads to the proteolytic activation of PKCδ and caspase-3, which stops the protection from autophagy and causes cells to irreversibly commit to apoptosis (Clavijo et al., 2007). Thus, PKCδ-dependent signaling not only represents a mechanism for inducing autophagy for protecting cells from stress conditions and a mechanism for promoting apoptosis to eliminate irreversibly damaged cells, but also seems to provide a mechanism for switching or regulating cells between survival and death.

2 AIMS OF THE THESIS

Aim I

This work follows on the pivotal findings of our laboratory which suggested that PKC can play a role in cardioprotection induced by adaptation of rats to IHH: administration of general PKC inhibitor completely abolished, and PKCδ inhibitor attenuated, the improvement of ischemic tolerance in IHH hearts (Neckar et al., 2005). Therefore, the first aim of this study was to investigate the potential involvement of PKCδ and PKCε, the most prevalent PKC isoforms in rat heart, in the cardiac adaptation to IHH.

Our specific aims were:

- To examine the effects of IHH on the myocardial mRNA level (Hlavackova, unpublished data), protein level of total and phosphorylated forms of PKCδ and PKCε and their detailed subcellular distribution (results published in Hlavackova et al., 2010: Supplement 1).
- To study the effects of the PKCδ inhibitor rottlerin on myocardial PKCδ detailed subcellular redistribution after the adaptation of rats to IHH using the same experimental protocol under which the inhibitory effect of rottlerin on myocardial ischemic tolerance was observed (results published in Hlavackova et al., 2010: Supplement 1).
- To assess the involvement of PKCε in cardioprotection induced by adaptation to IHH was explored using the PKCε peptide inhibitor in the left ventricle myocytes subjected to simulated acute anoxia followed by reoxygenation (Hlavackova, unpublished data).

Aim II

It was assumed that the increased accumulation of n-3 PUFA in myocardial phospholipids induced by IHH may contribute to improved ischemic tolerance (Jezkova et al., 2002). PKC transduces lipid second messenger signals and its activity and function are influenced by lipid membrane composition and by the quality of lipid signaling molecules (Giorgione et al., 1998; Madani et al., 2001). Thus, the second aim of this study was to find out whether an altered phospholipid FA profile plays a role in the cardioprotective mechanism of IHH in a PKC-dependent manner.

Our specific aims were:

- To compare the effects of diets enriched with SFAs, n-3 PUFAs or n-6 PUFAs on FA composition of myocardial phospholipids, PKC activators DAGs and products of lipid peroxidation (conjugated dienes) in normoxic and to IHH-adapted hearts.
- To assess the protein level and distribution of PKCδ and PKCε between cytosolic and particulate fractions, and the susceptibility of normoxic and chronically hypoxic rat hearts to acute I/R injury induced by coronary artery occlusion (results published in Hlavackova et al., 2007: Supplement 2; Balkova et al., 2009: Supplement 3).

3 METHODS

All the chemical compounds were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

3.1 Animals and model of intermittent hypobaric hypoxia (IHH)

Adult male Wistar rats (250–280 g body wt) were exposed to IHH corresponding to an altitude of 7,000 m for 8 h/day, 5 days/wk. The barometric pressure (P_B) was lowered in steps so that the level equivalent to an altitude of 7,000 m (P_B = 308 mm Hg, 41 kPa; PO_2 = 65 mm Hg, 8.6 kPa) was reached after 13 exposures; the total number of exposures was 24–30. The animals were used the day after the last hypoxic exposure. The control normoxic subgroups of animals were kept for the same period of time at P_B and PO_2 equivalent to an altitude of 200 m (P_B = 742 mm Hg, 99 kPa; PO_2 = 155 mm Hg, 20.7 kPa). All animals had free access to water. The study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

3.2 Aim I

Within this aim we studied the effect of IHH on the subcellular distribution, protein and mRNA levels of PKCδ and PKCε and their role in IHH-induced cardioprotection.

The animals chosen for determination of the effect of IHH on PKC mRNA levels were killed by decapitation, and their hearts were rapidly excised, washed in cold (0°C) saline, and dissected into the left and right free ventricular walls and the septum on the ice dish. The left ventricles were immediately frozen and stored in liquid nitrogen until use. The animals designed for determination of the effect of IHH on PKCε protein level and subcellular distribution (Western blot and immunofluorescence microscopy analysis) were killed by decapitation, and their hearts were rapidly excised and perfused with Krebs-Henseleit solution containing (in mmol/l): 120 NaCl, 5.0 KCl, 2.0 $MgSO_4$, 1.2 NaH_2PO_4, 20 $NaHCO_3$, 10 glucose, 0.25 $CaCl_2$, and 20 2,3-butanedione monoxime. The apex and the remaining part of the left ventricular free wall were dissected, weighed, instantly frozen and stored in liquid nitrogen until use. Concerning the effect of PKCδ inhibitor rottlerin, animals were anesthetised with sodium pentobarbital (60 mg/kg body weight, ip; Sanofi, Montpellier, France). Rottlerin (Biomol, Plymouth Meeting, PA) was dissolved in dimethyl sulfoxide (DMSO) and then diluted with saline and administered into the jugular

vein in a dose of 0.3 mg/kg as a single bolus (1 ml/kg) 15 min before the excision of the heart; the final dose of DMSO was 6 µl/kg (Neckar et al., 2005). Controls were given saline (1 ml/kg) with DMSO. The animals were decapitated, and their hearts were rapidly excised and perfused with Krebs-Henseleit solution containing 20 mmol/l 2,3-butanedione monoxime. The apex and the remaining part of the left ventricular free wall were dissected, weighed, frozen and stored in liquid nitrogen until use.

3.2.1 Tissue fractionation and Western blot analysis

Myocardial left ventricular samples were pulverized to a fine powder, followed by Potter-Elvehjem homogenisation in 8 volumes of ice-cold buffer composed of (in mmol/l): 12.5 Tris-HCl (pH 7.4), 250 sucrose, 2.5 EGTA, 1 EDTA, 100 NaF, 5 DTT, 0.3 phenylmethylsulfonyl fluoride, 0.2 leupeptin and 0.02 aprotinin. The homogenization buffer for analyses of phosphorylated PKC isoforms also contained the phosphatases inhibitor sodium orthovanadate (0.1 mM) and glycerol-3-phosphate (10 mM). The homogenate was centrifuged to obtain the particulate (all membranes) fraction (100,000×g for 60 min) or nuclear-cytoskeletal-enriched (Nucl) (1,000×*g* for 10 min), mitochondria-enriched (Mito) (8,000×*g* for 10 min), microsomal (Micro) (100,000×*g* for 60 min) and cytosolic (Cyto) fractions. The homogenate and pellets of all fractions were re-suspended in a homogenization buffer containing 1% Triton X-100, held on ice for 60 min and then centrifuged at 100,000×*g* for a further 60 min. Triton X-100 was also added to the cytosolic fraction to reach the final concentration of 1%. Triton X-100-extraction represents a partial purification step, which improves sensitivity of PKC detection.

The protein content was determined according to Lowry's assay modified by Peterson (1977). Detergent-treated extracts of subcellular fractions were subjected to SDS-PAGE electrophoresis on 10% bis-acrylamide polyacrylamide gel at 20 mA/gel for 90 min on a Mini-Protean III apparatus (Bio-Rad, Hercules, CA), the resolved proteins were transferred to a nitro-cellulose membrane (Amersham Int., Freiburg, Germany). Equal protein transfer efficiency was verified by staining of membranes with Ponceau S. After blocking with 5% dry low-fat milk in Tris-buffered saline with Tween 20 (TTBS) for 60 min at room temperature, membranes were washed and incubated (90 min at room temperature) with polyclonal antibodies against PKCδ (amino acids 662-673), PKCε (amino acids 728-737) (Research & Diagnostic Antibodies, Benicia, CA), PKCε (amino acids 726-737) (Sigma-Aldrich; used in previous studies) or (overnight at 4°C)

phosphorylated form of PKCδ (p-PKCδ) (Ser643) (Cell Signaling Technology, Beverly, MA) and p-PKCε (Ser729) (Upstate, Billerica, MA). After the membranes were washed with TTBS, they were incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase for 60 min at room temperature. Before enhanced chemiluminescence, the membranes were washed and stored in TTBS for at least 2 h. For enhanced chemiluminescence, substrates A (Luminol solution) and B (H_2O_2 solution) were prepared, mixed 1:1 just before use, and poured on the membranes. Bands were visualized by enhanced chemiluminescence on the autoradiographic film (Amersham Biosciences, Freiburg, Germany) and ImageQuant software was used for quantification of the relative abundance of PKC isoforms. To ensure the specificity of PKCδ and PKCε immunoreactive proteins, prestained molar-mass protein standards, recombinant human PKCδ and PKCε standards, rat brain extract, and the blocking immunizing peptides were used (Fig. 7).

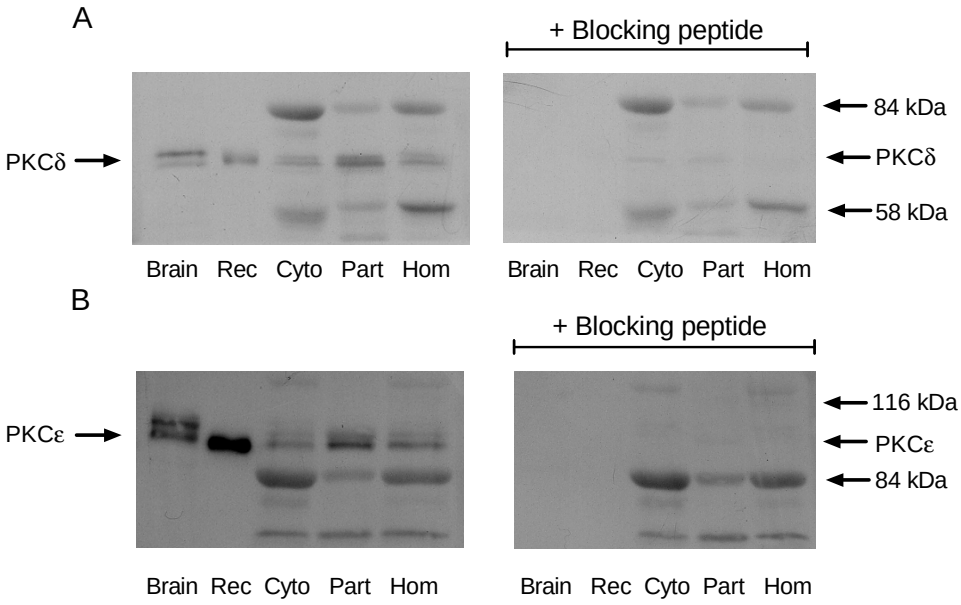

Fig. 7 *Representative Western blots of PKCδ (A) and PKCε (B) isoforms in positive controls from rat brain homogenate (Brain) and recombinant human PKCδ and PKCε standards (Rec), cytosolic (Cyto) and particular (Part) fractions and homogenate (Hom) from the left ventricular myocardium of the normoxic rats, in the absence and presence of the respective blocking peptides. Arrows mark the positions of 116, 84, and 58 kDa prestained protein molecular mass standards.*

For comparative quantification of the amount of monitored protein in Western blot analysis, an appropriate housekeeping protein is usually used as an internal control. We examined the effect of IHH on the expression of four common housekeeping proteins: actin (Sigma-Aldrich, St. Louis, USA), calsequestrin (Abcam, Cambridge, USA), glyceraldehyde-3-phosphate dehydrogenase (Abcam, Cambridge, USA), and β-tubulin (Abcam, Cambridge, USA) in the heart homogenates. The expression of all these proteins

increased significantly after the adaptation to IHH (Balkova et al., 2011: Supplement 4). On that ground, we used the total protein concentration as the most suitable referential value, because it was not altered under our experimental conditions. Neither IHH nor PKCδ inhibitor rottlerin influenced the protein yields in cellular fractions. From each group, one sample was run on the same gel and quantified on the same membrane. Obviously, the choice of a housekeeping protein as an internal standard in chronic experiments should always be carefully validated.

3.2.2 RNA isolation and real-time PCR analysis

Total cellular RNA was extracted from each left ventricle sample using the Trizol Reagent (Invitrogen-Molecular Probes, Eugene, OR). The purity and concentration of the RNA preparations was checked spectroscopically using NanoDrop. Prior to cDNA synthesis, the RNA was cleaned with a DNA-free kit (Ambion, Carlsbad, CA). One microgram of total RNA was converted to cDNA using the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas UAB, Vilnius, Lithuania) using oligo(dT) primers according to the manufacturer's instructions. Real-time PCR was performed on a Light Cycler 480 (Roche Applied Science, Mannheim, Germany) using the mono color hydrolysis probe method (Universal Probe – Roche Applied Sciences) with the appropriate Probe Master kit (Roche Applied Sciences) according to the manufacturer's protocol. Specific primers and probes for PKCδ mRNA, PKCε mRNA and the reference gene hypoxanthine-guanine phosphoribosyltransferase1 (HPRT1) mRNA were designed by Universal probe library software (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp) using the National Center for Biotechnology Information (NCBI) reference sequences (Table 5). PCR amplification was performed under the following conditions: initial denaturation at 95°C for 10 min, followed by 50 cycles consisting of denaturation at 95°C for 10 s, annealing at 60°C for 10 s where fluorescence was acquired, and elongation at 72°C for 5 s. The data used for calculation are the means of C_P values of triplicate samples. We verified that the variation of triplicates did not exceed 0.5 C_P. Standard curves were generated for each pair of primers using 3-fold serial dilution of cDNA. Melting curve analysis was performed to ascertain the presence of a single amplicon at the end of each standard curve run. The efficiency of the PCR amplification for each primer pair was then calculated from the standard curve to precisely state the relative expression. The level of analyzed transcripts was normalized to

the level of the reference Hprt1 gene transcripts (the high expression stability of HPRT1 mRNA under hypoxic condition was established by Bohuslavova et al., (2010)) with regard to the specific PCR efficiency (E) for each gene (Livak and Schmittgen, 2001) as: Normalized amount of mRNA = $(1+E_{reference})^{C_P \text{ reference transcript}} / (1+E_{target})^{C_P \text{ target transcript}}$. Non-template and non-RT reactions were performed as controls.

Table 5 Mono color hydrolysis probe numbers, primer and NCBI reference sequences

mRNA	Probe number	Primer	Primer length	Primer sequence	Amplicon length (nt)	NCBI reference sequence
PKCδ	20	Left	20	caagaagaacaacggcaagg	72	NM_133307.1
		Right	19	tgcacacacatcagcacct		
PKCε	38	Left	25	aaacacccttatctaacccaactct	73	NM_017171.1
		Right	23	catattccatgacgaagaagagc		
HPRT1	95	Left	19	gaccggttctgtcatgtcg	61	NM_012583.2
		Right	24	acctggttcatcatcactaatcac		

HPRT1, hypoxanthine-guanine phosphoribosyltransferase1; PKC, protein kinase C; nt, nucleotide

3.2.3 Quantitative immunofluorescence microscopy

The subcellular redistribution of PKC δ and ε induced by IHH and the effect of rottlerin were investigated by immunofluorescent staining followed by digital imaging fluorescence microscopy. Left ventricle apex cross cryo-sections (5 μm) were incubated with a primary antibody raised against rat PKCδ (662-673) and PKCε (728-737) (Research & Diagnostic Antibodies, Benicia, CA) and counterstained for nuclei with 4',6 diamidino-2-phenylindole (DAPI)-containing a mounting medium (Vectashield; Vector Laboratories, Burlingame, CA) and either for the sarcolemma with 10% (vol/vol) wheat-germ agglutinin (WGA; Molecular Probes) or for mitochondria with anti-oxidative phosphorylation (OXPHOS) complexes. They were then incubated with the appropriate Alexa Fluor secondary antibody conjugate (Molecular Probes) and visualized by immunofluorescence microscopy, as described by Bouwman et al. (2004). Sections were qualitatively and quantitatively analyzed with the use of imaging and analysis software (SlideBook™, version 4.1). Regions of interest from the digital images of the left ventricle cross cryo-sections were selected in a process termed masking, a mask being a binary overlay on a digital image. Masks of segments of cross cryo-sections were created by automatically

selecting standardized fluorescence thresholds of the counterstains (either sarcolemmal glycocalyx or mitochondrial OXPHOS complexes). Next, the mean intensities of the fluorescence of PKCδ and PKCε within these masks were calculated using SlideBook™ imaging software.

3.2.4 Isolation of cardiomyocytes

Cardiomyocytes were isolated as described previously (Klusonova et al., 2009; Borchert et al., 2011). The rats were heparinized (5,000 IU ip) and killed by cervical dislocation. The heart was quickly excised, mounted on a perfusion system, and perfused via the aorta with Ca^{2+}-Tyrode solution (mM: 140 NaCl, 5.4 KCl, 1 Na_2HPO_4, 1 $MgCl_2$, 10 D-glucose, 5 HEPES, 1 $CaCl_2$, pH 7.3-7.4) at 37°C under constant flow conditions (10 ml/min) for 5 min, followed by perfusion with nominally Ca^{2+}free-Tyrode for 8 min. Tissue digestion was initiated by adding 14,000 U collagenase (20 mg, 700 U/mg; Yakult, Tokyo, Japan) and 7 mg protease type XIV (type XIV, 0.2 mg/ml) into 30 ml of Ca^{2+}-free Tyrode containing 50 mg BSA. All solutions were gassed with 100% O_2 for 5 min before use. After 12-15 min, the collagenase-protease cocktail was washed out by 10-min perfusion with Ca^{2+}-free Tyrode. The right ventricle was cut off first and then the septum and then the left ventricle. Myocytes isolated from the left ventricle (LVM) were dispersed mechanically then filtered through a nylon mesh to remove non-dissociated tissue; LVM solutions were adjusted to the same cell density and transferred to a culture medium.

3.2.5 Cardiomyocytes treatment

The PKCε inhibitor peptide KP-1633 and control peptide (scrambled amino acid sequence) KP-1723 were obtained from KAI Pharmaceuticals, Inc. (South San Francisco, CA) on the basis of a Material transfer agreement. KP-1633 is a new analog of the PKCε peptide inhibitor εV1–2, a sequence from the C2 region of PKCε (amino acids 14–21; EAVSLKPT), that binds to the anchoring protein RACK2 (Mochly-Rosen, 1995; Souroujon and Mochly-Rosen, 1998). εV1–2 was shown to inhibit translocation and function of the activated PKCε in intact cells and hearts (Johnson et al., 1996; Dorn et al., 1999). The KP-1723 and KP-1633 peptides ability to cross biological membranes is rendered through linking with the cell-permeable “carrier” peptide TAT (amino acids 47-57; YGRKKRRQRRR) (Chen et al., 2001). KP-1633 contains the cargo peptide, PKCε

inhibitory peptide (EAVSLKPT), linked to the carrier peptide, TAT sequence (YGRKKRRQRRR), *via* a short cleavable dipeptid linker (G-G). The KP-1633 carboxy terminus is amidated which increases the peptide stability by protecting it from proteolytic degradation (MacLean, 2011).

Isolated LVM were cultured in a cell culture medium (50% Dulbecco's modified Eagle's medium and 50% Nutrient Mixture F12HAM, containing 0.2% BSA, 100 U/ml penicillin, and 100 μg/ml streptomycin) and kept in a CO_2 incubator (95% air, 5% CO_2, 28°C) for a 1-h stabilization period.

In the first series of experiments, we determined the dose response of the control peptide KP-1723 and PKCε peptide inhibitor KP-1633. Having considered the effective concentrations of the KP-1633 resembling peptide, εV1-2, used in previous studies (Chen et al., 1999), we chose concentrations of 0.1, 1, 5, 10 and 50 μM for the dose response study of KP-1633 and KP-1723. We determined the percentage of living cells compared to the untreated control cells at the beginning of the experiments (after stabilization) and after 2, 4 and 20 h using the SYTOX Green nucleic acid stain (S7020) (Invitrogen-Molecular Probes, Eugene, OR), a nucleic acid dye uptake assay. Sytox Green is a DNA-binding dye that only gets into dying cells where the plasma membranes are compromised. The dye becomes fluorescent on binding to nuclear DNA. The overall fluorescence of the cells is inversely related to the intactness of the cell membranes. The fluorescence of SYTOX Green was measured at 490 nm excitation and 520 nm emission wavelengths in 96-well Nunclon™Δ Surface plates (Nunc A/S, Roskilde, Denmark) using the Synergy™ HT Multi-Detection Microplate Reader (BioTek, Winooski, VT). We found that treatment with 50 μM concentration of KP-1723 or KP-1633 led to a decrease in the number of surviving LVM. Moreover we observed a decreasing viability of LVM by 10 μM KP-1633 after incubation for 4 and 20 h (data not shown), therefore we decided to use 5 μM concentration of KP-1723 and KP-1633 for the following experiments.

In the second set of experiments, KP-1633 activity as a PKCε inhibitor was studied. LVM isolated from normoxic rats were divided into four groups and pretreated either with 50 mM ethanol or 5 μM KP-1633, or a combination of both, for 15 min and subjected to metabolic inhibition (MI) and reenergization (MI/R).

In the third set of experiments, we studied the effect of 15-min pretreatment with 5 μM KP-1723 or 5 μM KP-1633 on LVM isolated from rats adapted to IHH and normoxic animals and subjected to MI/R.

LVM from each treatment group were split into two parts of equal volumes. Control cells were incubated in a normal Krebs solution and not exposed to MI/R. Experimental cells were subjected to 25 min of MI, followed by 30 min of reenergization. MI was induced by incubation of cells in the modified Krebs solution containing 1.5 mM NaCN and 20 mM 2-deoxyglucose instead of glucose. The reenergization was achieved by removing the metabolic inhibitors and replacing the MI solution with the normal cell culture medium (the same medium was applied to control cells).

3.2.6 Cell viability and lactate dehydrogenase release

Cell viability and lactate dehydrogenase (LDH) release were evaluated at the beginning of the experiments (after stabilization), after MI (LDH release only), and after reenergization. The number of viable and dead (stained) myocytes was determined by Trypan blue exclusion (Wu et al., 1999). Typically, 50–100 myocytes were counted in duplicates from 4–10 independent experiments. Viable myocytes were divided into two fractions: rod-shaped myocytes with the cell length-to-width ratio > 3:1 and non-rod-shaped myocytes with the ratio < 3:1. Viability after MI/R was expressed as a percentage of the rod-shaped cells that survived the MI/R insult, normalized to the appropriate control group not exposed to MI/R. LDH was determined spectrophotometrically (Buhl and Jackson, 1978) using the LDH Liqui-UV kit (Stanbio, Boerne, TX). LDH released during MI and during reenergization was normalized to total LDH content in the cells and expressed as a percentage of appropriate control values.

3.3 Aim II

Within this aim we studied the effect of SFA, n-3 PUFA and n-6 PUFA enriched diets and IHH on the fatty acid profile of myocardial phospholipids and diacylglycerols, ischemia/reperfusion injury and protein level of PKCδ and PKCε.

Adult male Wistar rats (250–280 g) were fed a nonfat diet based on standard ST1 (Velaz, Prague, Czech Republic) enriched with 10% lard (SFA), fish oil (n-3 PUFA; Lehmann & Voss, Hamburg, Germany) or corn oil (n-6 PUFA; Olmühle GmbH, Bruck, Austria) for 10 weeks. The FA composition of the dietary oils is shown in Table 6.

Table 6 Fatty acid composition (mol %) of the diet lipids.

	SFA diet	n-3 diet	n-6 diet
14:0	1.40	5.66	0.58
16:0	24.15	17.86	12.28
16:1n-7	2.48	7.71	0.93
18:0	10.87	2.13	1.81
18:1n-9	35.50	16.48	24.70
18:1n-7	2.49	3.44	0.95
18:2n-6	19.03	15.38	53.16
18:3n-3	1.62	2.61	2.31
20:1n-9	0.96	7.31	1.03
20:5n-3	0.18	9.94	N.D.
22:5n-3	0.07	1.78	N.D.
22:6n-3	0.21	8.04	N.D.
others	1.04	1.66	2.25
Σ SFA	36.42	25.65	14.67
Σ MUFA	41.43	34.94	27.61
Σ n-6 PUFA	19.30	15.38	53.16
Σ n-3 PUFA	2.08	22.7	2.31

Σ SFA, total saturated FA; Σ MUFA, total monounsaturated FA; Σ n-3 PUFA, total n-3 polyunsaturated FA; Σ n-6 PUFA, total n-6 polyunsaturated FA; N.D., not detected. Values are means of two separate analyses. Adopted from Hlavackova et al. (2007: Supplement 2).

The composition of the nonfat standard ST1 diet was as follows (per kg): 240 g crude protein; 37.2 g non-nutrient fibres; 65.1 g ash; 240 g N substances; antioxidants (butylhydroxytoluene, ethoxyquin, butylhydroxyanisol); 4500 mg (15 000 IU) vitamin A; 25 mg (1000 IU) vitamin D; 107.9 mg vitamin E; 0.34 mg Se; 22.6 mg Cu; 13.2 g Ca; 8.7 g P; 1.8 g Na; Myco ad (adsorbent of mycotoxins). After 4 weeks on the diet, each group was divided into two subgroups that were either exposed to IHH or kept at normoxia for an additional 5–6 weeks. All animals were used the day after the last hypoxic exposure. Rats designed for biochemical analyses were anesthetized with sodium pentobarbital (60 mg/kg body weight, ip; Sanofi, Montpellier, France). The right ventricle was catheterized *via* the

jugular vein and right ventricle pressure was measured with a Gould P23Gb transducer. Thereafter, the hearts were rapidly excised, washed in cold (0 °C) saline and dissected into the right ventricle and left ventricle free walls and the septum. All parts were weighed and the left ventricles were frozen and stored in liquid nitrogen until use.

Adaptation of rats to IHH led to a marked increase in hematocrit and a significant retardation of body growth compared with age-matched normoxic controls in all diet groups. Lipid diets had no effect on weight parameters and hematocrit in both normoxic and hypoxic rats (Hlavackova et al., 2007: Supplement 2).

3.3.1 Tissue fractionation and Western blot analysis

The homogenization of frozen left ventricular myocardium and preparation of 1% Triton X-100-treated cytosolic and particulate fractions followed the same protocol as was described in 3.2.1.

Proteins were separated by SDS-PAGE electrophoresis (8% bis-acrylamide polyacrylamide gel), transferred to nitro-cellulose membranes, treated with appropriate antibodies and quantified as described in 3.2.1. The PKCδ- or PKCε-specific polyclonal primary rabbit antisera from Sigma-Aldrich were used for detection of the specific signal of PKCδ or PKCε in this experiment. The amount of protein applied to the gel was 15 μg (cytosolic fraction) and 5 μg (particulate fraction).

3.3.2 Analysis of fatty acid profile of myocardial phospholipids and diacylglycerols

Frozen left ventricles were pulverized and homogenized. Lipids were extracted in three consecutive steps according to the modified method of Folch et al. (1957). The first extraction was performed in three portions of a chloroform-methanol mixture (1:3, 2:1 and 2:1) in a chilled mortar. Subsequent extractions were performed in the 2:1 mixture. Saline (20% volume of extract) was added and after vigorous shaking the lower lipid layer was dried at 40 °C under a stream of nitrogen. Total phospholipids and DAG were separated by one-dimensional thin-layer chromatography (0.5 mm Silica Gel H, Merck, Darmstadt, Germany) using the solvent mixture hexane-ether-acetic acid (80:20:3). Spots were observed under UV light after staining with 0.005% 2,7-dichlorofluorescein, scraped out and stored in nitrogen atmosphere at -20 °C until the next day when methyl esters were prepared. FA methyl esters were separated/assessed using with a gas chromatograph (CP 438 A, Chrompack, Middelburg, The Netherlands) (Tvrzicka et al., 2002).

The analysis of FA methyl esters by gas chromatography was performed by Mgr. Barbora Stankova, Fourth Department of Internal Medicine, First Faculty of Medicine, Charles University, Prague, Czech Republic.

3.3.3 Analysis of concentration of conjugated dienes

The extraction of lipids was carried out by the method of Folch et al. (1957) as described above. The dry lipid residue was redissolved in cyclohexane and the tissue content of conjugated dienes was measured spectrophotometrically (Ahotupa et al., 1996).

3.3.4 Infarct size determination and analysis of arrhythmias

The susceptibility of hearts to ventricular arrhythmias and myocardial infarction was evaluated in anesthetized open-chest animals subjected to 20-min coronary artery occlusion and 3-h reperfusion. The infarct size determination and analysis of arrhythmias were performed by RNDr. Jan Neckar, Ph.D., Institute of Physiology, Academy of Sciences of the Czech Republic. The details of the methods are described in Neckar et al. (2002b) and Hlavackova et al. (2007: Supplement 2).

3.4 Statistical analysis

The results are expressed as means ± SEM. A one-way (Aim I data) or a two-way ANOVA (Aim II data) and subsequent Student-Newman-Keuls test were used for comparison of differences in normally distributed variables between groups. Differences were considered as statistically significant when $P<0.05$.

4 RESULTS

4.1 Aim I

Within this aim we studied the effect of IHH on the subcellular distribution, protein and mRNA levels of PKCδ and PKCε and their role in IHH-induced cardioprotection.

4.1.1 IHH up-regulates PKCδ protein level but does not influence mRNA level

IHH increased the relative protein content of total PKCδ as well as the level of p-PKCδ (Ser643) in homogenate by 190% and 204%, respectively; similar changes were observed in cytosolic and particulate fractions (Fig. 8A,B). The ratio of p-PKCδ (Ser643) to total PKCδ was not influenced by IHH (Fig. 8C). IHH did not affect the PKCδ mRNA level in rat left ventricle (Fig. 9).

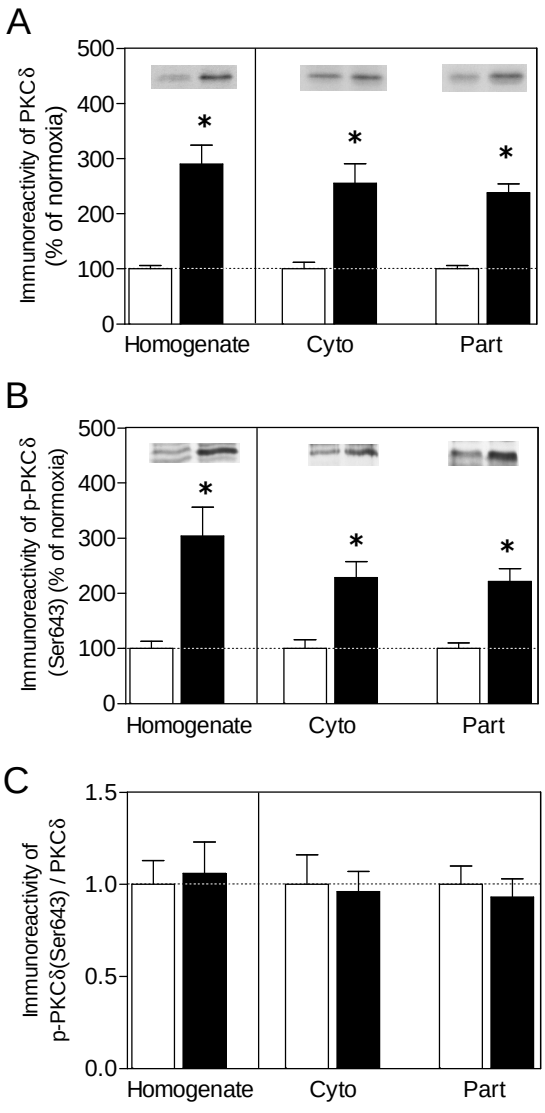

Fig. 8 *The effect of IHH on the level of total PKCδ (A), p-PKCδ (Ser643) (B) and p-PKCδ (Ser643) to total PKCδ ratio (C) in homogenate, cytosolic (Cyto), total particulate (Part) fractions from left ventricles of normoxic (open columns) and chronically hypoxic (black columns) rats, including representative Western blots. The amount of protein applied to the gel was 10 μg (homogenate), 15 μg (cytosolic fraction) or 5 μg (particulate fraction) for PKCδ and 40 μg (homogenate), 50 μg (cytosolic fraction) or 40 μg (particulate fraction) for p-PKCδ (Ser643). Values are means ± SEM from 6 hearts in each group. *P<0.05 vs. the corresponding normoxic group.*

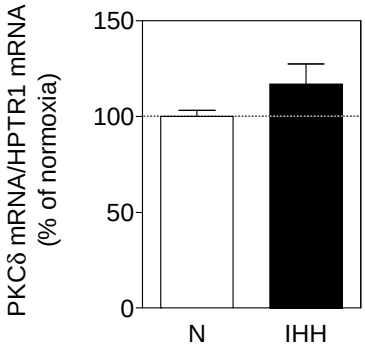

Fig. 9 *The effect of IHH on PKCδ mRNA level normalized to the level of the reference gene hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1) in left ventricles of normoxic (N; open columns) and chronically hypoxic (IHH; black columns) rats expressed as a percentage of normoxic values. Values are means ± SEM from 6 hearts in each group.*

4.1.2 IHH-induced effect on PKCδ subcellular redistribution is disrupted by PKCδ inhibitor rottlerin

PKCδ inhibitor rottlerin had no effect on PKCδ content in the homogenate from either normoxic or hypoxic hearts (Fig. 10A). Detailed analysis showed that IHH up-regulated PKCδ protein amount in all subcellular fractions with the highest increase in mitochondrial and microsomal fractions (Fig. 10B). Interestingly, acute rottlerin treatment significantly decreased the relative PKCδ protein content in the mitochondrial fraction in favor of cytosolic and nuclear-cytoskeletal fractions of IHH-adapted hearts (Fig. 10C).

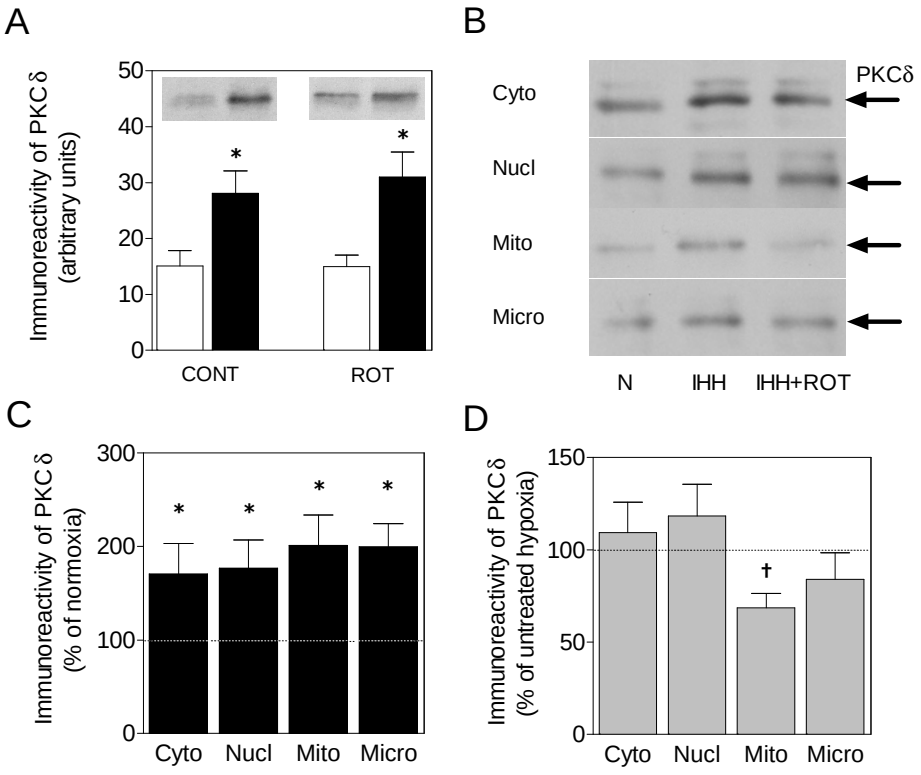

Fig. 10 *The effect of IHH and rottlerin on the level of PKCδ in homogenate from the left ventricles of normoxic (open columns) and chronically hypoxic (black columns) rats treated by vehicle (CONT) or rottlerin (ROT), including representative Western blots (A). Representative Western blots (B) showing the effects of IHH and rottlerin on PKCδ abundance in cytosolic (Cyto), nuclear-cytoskeletal (Nucl), mitochondrial (Mito), and microsomal (Micro) fractions. Quantified data (C) show the effect of IHH on PKCδ level in subcellular fractions expressed as percentage of normoxic values. (D) The effect of rottlerin on PKCδ level in subcellular fractions from chronically hypoxic rats expressed as a percentage of untreated hypoxic values. The amount of protein applied to the gel was 10 μg (homogenate), 15 μg (Cyto), 5 μg (Nucl), 10 μg (Mito) and 5 μg (Micro). Arbitrary units: PKCδ in each sample was quantified by densitometry measurements and expressed as the intensity of the stained band/μg of protein. Values are means ± SEM from 5 hearts in each group. *P<0.05 vs. the corresponding normoxic group; †P<0.05 vs. the untreated hypoxic group.*

Fig. 11 shows the PKCδ redistribution pattern in response to IHH and PKCδ inhibitor rottlerin in left ventricle cross cryo-sections. In the normoxic group, a diffuse staining of PKCδ can be observed. PKCδ was co-localized with nuclei in normoxic as well as in hypoxic hearts. IHH induced PKCδ redistribution and increased PKCδ co-localization with the sarcolemma (Fig. 12A, red WGA sarcolemmal glycocalyx counterstain) as well as with the mitochondria (Fig. 12B, red OXPHOS counterstain) as indicated by the increase in the yellow-orange color.

Figures 11A and 11C (left panel) show that the IHH-induced redistribution of PKCδ to the sarcolemma (the mean intensity of fluorescence of PKCδ in the sarcolemmal mask was increased by up to 25%) was inhibited by rottlerin (the mean intensity of fluorescence of PKCδ decreased to the normoxic value). In Fig. 11B, the PKCδ co-localization with mitochondria (the red OXPHOS counterstain) is shown. In sections of hearts adapted to IHH, PKCδ was present as a dotted-like distribution pattern with a yellow-orange color (arrows), which indicates the co-localization with the mitochondria. IHH increased the mean intensity of fluorescence of PKCδ in the mitochondrial OXPHOS complexes mask by 19% (Fig. 11C, right panel). Rottlerin reduced PKCδ localization in hypoxic mitochondria, decreasing the mean intensity of PKCδ fluorescence in the mitochondrial OXPHOS complexes mask to the normoxic control value. Rottlerin did not significantly affect the PKCδ distribution in normoxic tissue.

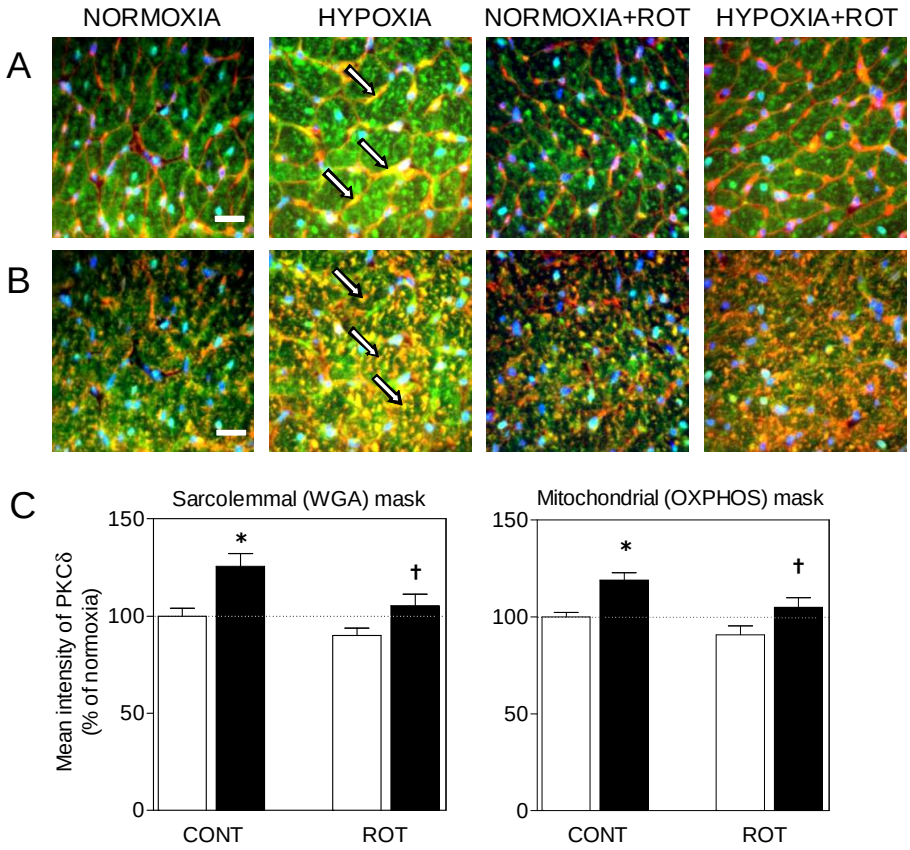

Fig. 11 *PKCδ distribution and co-localization with sarcolemma (A) and mitochondria (B) in normoxic and chronically hypoxic cross cryo-sections of left ventricles, and the effect of rottlerin (ROT). In all panels, green represents specific PKCδ staining and blue indicates the nuclear 4',6 diamidino-2-phenylindole staining. In panels (A) red represents the wheat-germ agglutinin (WGA) staining of the sarcolemmal glycocalyx, and in panels (B) red represents the OXPHOS complexes. Note the increase in the yellow-orange color in both panels, indicating increased IHH-induced co-localization of PKCδ with sarcolemma and mitochondria (arrows). Bar represents 20 μm. (C) Quantification of the mean intensity of fluorescence of PKCδ in sarcolemma (WGA staining, left panel) and mitochondria (OXPHOS complexes staining, right panel) in cryo-sections from normoxic (open columns) and chronically hypoxic (black columns) rats treated by vehicle (CONT) or rottlerin (ROT) expressed as percentage of normoxic values. Values are means ± SEM from 3 hearts in each group. *P<0.05 vs. the normoxic group; †P<0.05 vs. the untreated hypoxic group.*

4.1.3 IHH down-regulates PKCε protein level but does not affect its mRNA level and subcellular distribution

IHH decreased the total level of PKCε in left ventricular myocardium. Adaptation to IHH decreased the total level of PKCε in homogenate and particulate fraction (by 40% and 37%, respectively) without affecting the level of p-PKCε (Ser729) (Fig. 12A, B). The ratio of p-PKCε (Ser729) to total PKCε was increased by IHH in homogenate and cytosolic fraction; the increase in particulate fraction did not reach statistical significance due to greater variability (Fig. 12C).

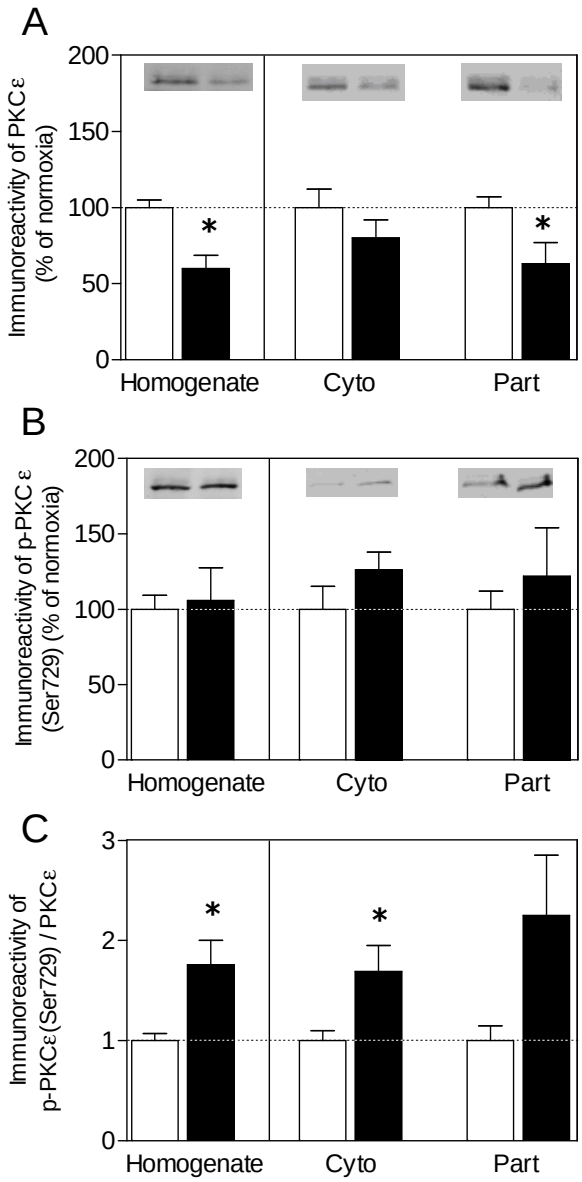

Fig. 12 *The effect of IHH on the level of total PKCε (A), p-PKCε (Ser729) (B) and p-PKCε (Ser729) to total PKCε ratio (C) in homogenate, cytosolic (Cyto), total particulate (Part) fractions from the left ventricles of normoxic (open columns) and chronically hypoxic (black columns) rats, including representative Western blots. The amount of protein applied to the gel was 10 μg (homogenate), 15 μg (cytosolic fraction) or 5 μg (particulate fraction) for PKCε and 40 μg (homogenate), 50 μg (cytosolic fraction) or 40 μg (particulate fraction) for p-PKCε (Ser729). Values are means ± SEM from 5 hearts in each group. *P<0.05 vs. the corresponding normoxic group.*

Although the protein content of PKCε in left ventricle myocardium was decreased by adaptation to IHH, the mRNA level of PKCε was not changed (Fig. 13).

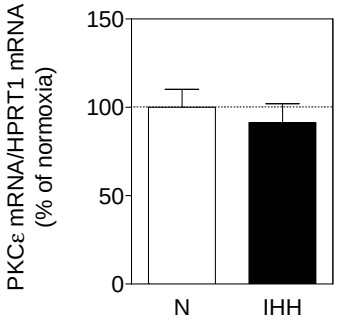

Fig. 13 *The effect of IHH on PKCε mRNA level normalized to the level of the reference gene hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1) in left ventricles of normoxic (N; open columns) and chronically hypoxic (IHH; black columns) rats, data are expressed as a percentage of normoxic values. Values are means ± SEM from 7 hearts in each group.*

The more detailed analysis of IHH effect on subcellular fractions revealed the decreased abundance of PKCε in the mitochondrial and microsomal fractions (Fig. 14). We verified our Western blot results with two different antibodies, the antibody from Sigma (used in our previous study (Neckar et al., 2005)) and the antibody from Research & Diagnostic (used also for immunofluorescence microscopy analysis) and obtained comparable results.

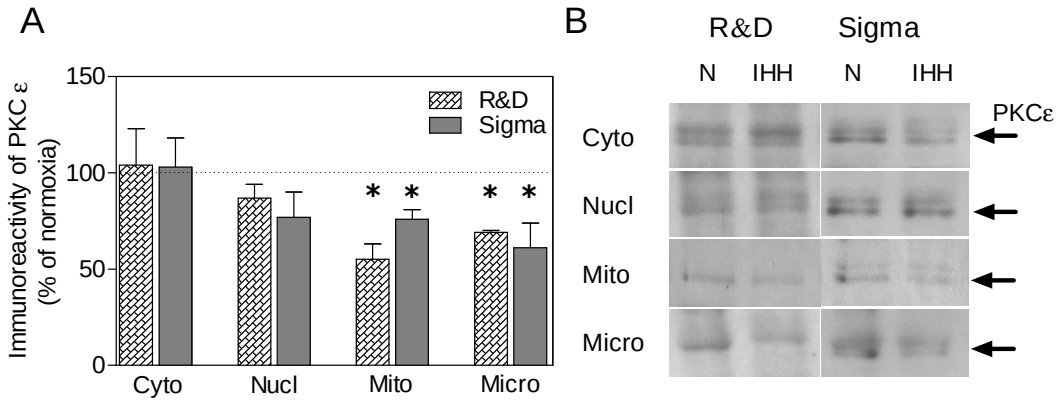

Fig. 14 *The effect of IHH on PKCε level (A) in cytosolic (Cyto), nuclear-cytoskeletal (Nucl), mitochondrial (Mito) and microsomal (Micro) myocardial fractions analysed by Research & Diagnostic (R&D) and Sigma antibodies, data are expressed as percentage of normoxic values; (B) representative Western blots. The amount of protein applied to the gel was 15 μg (Cyto), 5 μg (Nucl), 10 μg (Mito) and 5 μg (Micro). Values are means ± SEM from 4 hearts in each group. *P<0.05 vs. the corresponding normoxic group.*

PKCε staining in normoxic tissue displayed a dotted-like distribution pattern (Fig. 15A, B). Partial PKCε co-localization with OXPHOS complexes suggests its presence in the mitochondria (Fig. 15B). IHH did not affect the PKCε distribution quantified as the mean intensity of PKCε in sarcolemma (WGA staining) and mitochondria (OXPHOS complexes staining) (Fig. 15C).

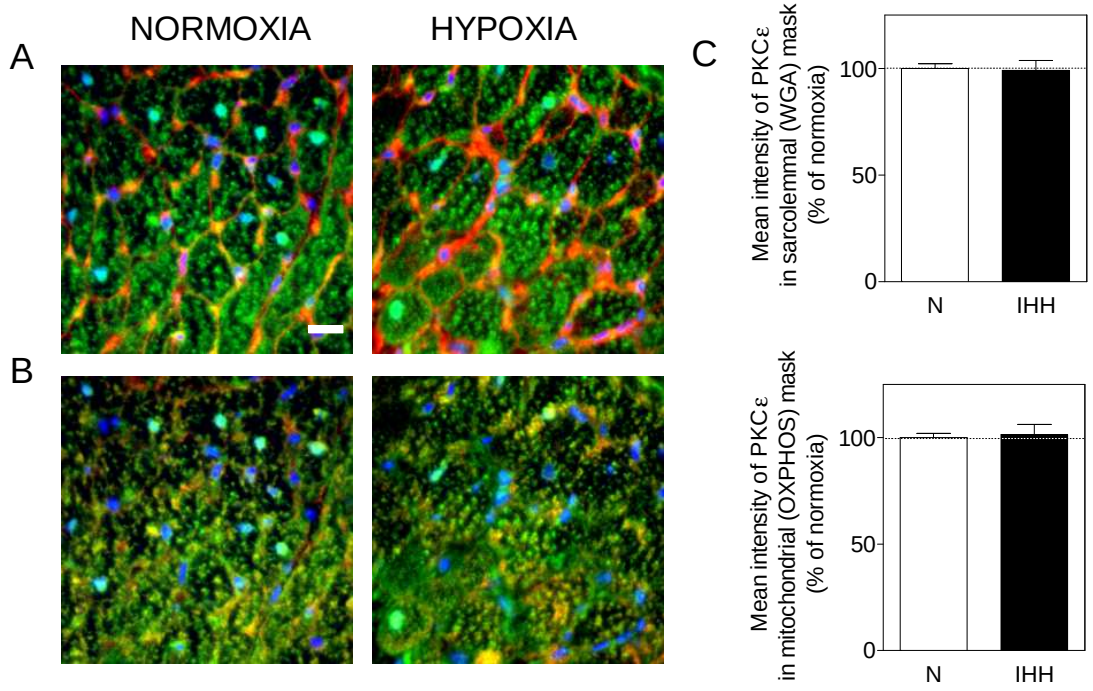

Fig. 15 *PKCε distribution and co-localization with sarcolemma (A) and mitochondria (B) in normoxic (N) and chronically hypoxic (IHH) left ventricle cross cryo-sections. In all panels, green represents specific PKCε staining, and blue indicates the nuclear 4',6 diamidino-2-phenylindole staining. In panels (A), red represents the the wheat-germ agglutinin (WGA) staining of the sarcolemmal glycocalyx, and in panels (B), red represents the OXPHOS complexes. The bar represents 20 μm. (C) Quantification of the mean intensity of fluorescence of PKCε in sarcolemma (WGA staining, upper panel) and mitochondria (OXPHOS complexes staining, lower panel) in cryo-sections from normoxic (open columns) and chronically hypoxic (black columns) rats expressed as percentage of normoxic values. Values are means ± SEM from 3 hearts in each group.*

4.1.4 Verification of KP-1633 as a PKCε inhibitor

It has been shown that exposure to physiologically attainable ethanol levels minutes before ischemia provides cardioprotection that is mediated by direct activation of PKCε in the cardiac myocytes. This cardioprotective effect of acute ethanol (50 mM EtOH) exposure was blocked by an isozyme-selective PKCε peptide inhibitor εV1-2 (Chen et al., 1999). We found that 15-min pretreatment with 50 mM EtOH decreased lactate dehydrogenase (LDH) release from LVM during metabolic inhibition (MI) and 5 μM PKCε inhibitor peptide KP-1633 blocked this effect (Fig. 16).

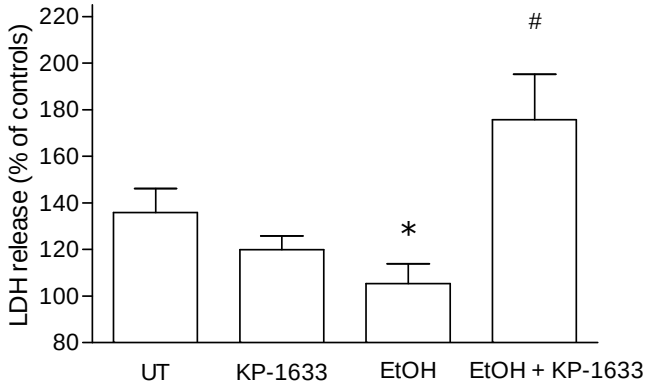

Fig. 16 *The effect of 5 μM KP-1633 and 50 mM EtOH on lactate dehydrogenase (LDH) release from cardiomyocytes during metabolic inhibition (MI) expressed as a percentage of LDH release from control cells not exposed to MI. Cells were isolated from the left ventricle of normoxic rats. UT, untreated cells. Values are means ± SEM from at least 4 hearts in each group. *P<0.05 vs. untreated group; #P<0.05 vs. other treated groups.*

4.1.5 PKCε peptide inhibitor KP-1633 does not affect IHH-induced protection in isolated cardiomyocytes

Figure 17, A–C, respectively, shows LDH release from cells during MI, during reenergization (R), and total release during MI/R, expressed as a percentage of appropriate control values. In LVM from normoxic rats, the LDH release during reenergization was higher than that caused by MI. The same is true for LVM isolated from rats adapted to IHH. Adaptation to IHH significantly reduced MI-induced, reenergization-induced and total LDH release during MI/R from LVM. Pre-treatment with KP-1723 and KP-1633 of LVM isolated from normoxic hearts tended to decrease LDH release during MI/R and had no effect on LDH release from LVM isolated from the hearts of rats adapted to IHH.

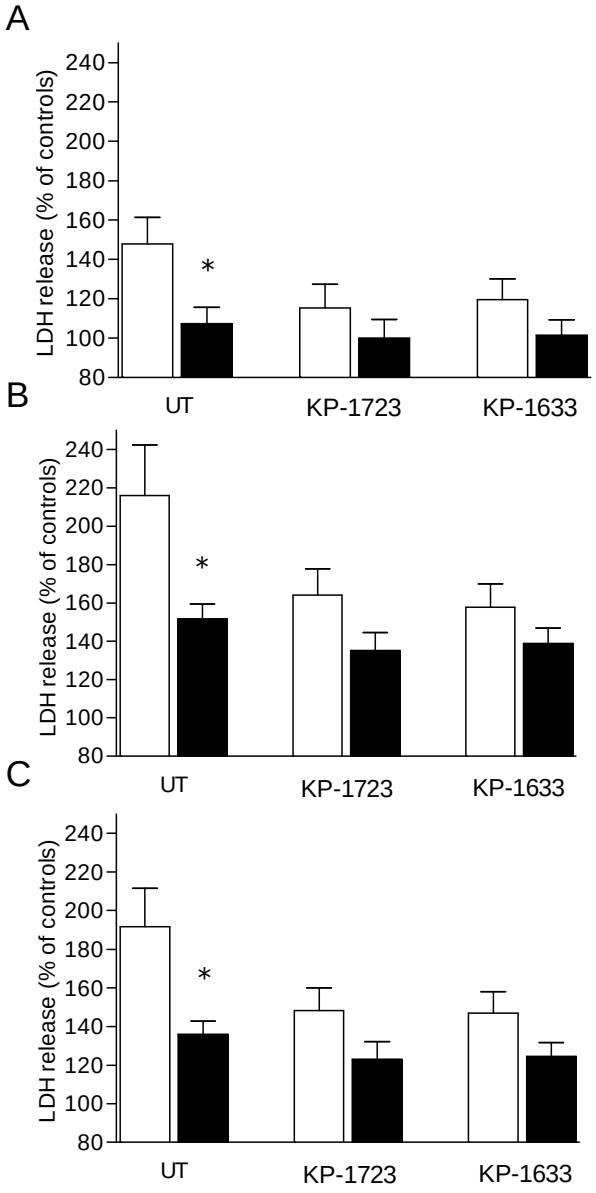

Fig. 17 *The effect of IHH, 5 μM control peptide KP-1723 or PKCε inhibitor peptide KP-1633 on lactate dehydrogenase (LDH) release from cardiomyocytes during metabolic inhibition (A) and during reenergization (B), and total release during MI/R (C), expressed as a percentage of LDH release from control cells not exposed to MI/R. Cells were isolated from the left ventricles of rats adapted to IHH (black columns) and of normoxic animals (open columns). UT, untreated cells. Values are means ± SEM from 10 hearts in each group. *P<0.05 vs. the corresponding normoxic groups.*

Exposure of LVM to MI/R insult decreased their survival in all groups in the same range (Fig. 18). Adaptation to IHH markedly improved the relative survival rate by lowering the reduction of LVM viability by about 50% after MI/R; KP-1723 and KP-1633 pre-treatment had no effect.

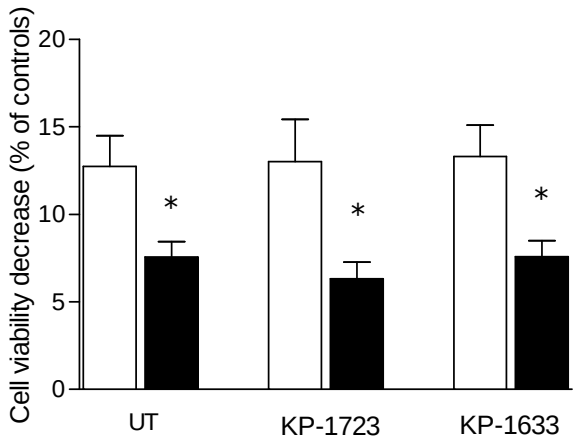

Fig. 18 *Effects of 5 μM control peptide KP-1723 or PKCε inhibitor peptide KP-1633 on cardiomyocytes viability decrease after acute MI/R, expressed as a percentage of control cells viability in the absence of MI/R. Cells were isolated from the left ventricle of rats adapted to IHH (black columns) and of normoxic animals (open columns). UT, untreated cells. Values are means ± SEM from 10 hearts in each group. *P<0.05 vs. the corresponding normoxic groups.*

4.2 Aim II

Within this aim we studied the effect of SFA, n-3 PUFA and n-6 PUFA enriched diets and IHH on the fatty acid profile of myocardial phospholipids and diacylglycerols, ischemia/reperfusion injury and protein level of PKCδ and PKCε.

4.2.1 Different lipid-enriched diets and IHH show complex pattern of influence on myocardial phospholipid and diacylglycerol fatty acid profile and concentration of conjugated diens

We compared the effects of diets enriched with SFAs, n-3 PUFAs or n-6 PUFAs. Despite the different FA composition of individual lipid diets (Table 6, chapter 3.3), the proportion of SFAs, MUFAs and total PUFAs in the myocardial phospholipids did not essentially differ between all normoxic and hypoxic diet groups (Table 7). The proportion of n-3 and n-6 PUFAs substantially differed in individual diet groups. The n-3 diet increased the proportion of n-3 PUFAs and decreased the proportion of n-6 PUFAs as compared with the SFA diet. This effect was mainly due to an accumulation of DHA (22:6n-3; by 66%) at the expense of AA (20:4n-6; decreased by 47%). The n-6 diet had an opposite effect: it reduced the proportion of n-3 PUFA in favor of n-6 PUFA as compared with the SFA diet. The DHA decrease (by 32%) and the LA (18:2n-6) increase (by 28%) substantially contributed to this effect. IHH tended to increase the proportion of n-3 PUFA (mainly due to an accumulation of DHA) and decreased that of n-6 PUFA (by a reduction of LA). In heart DAGs, SFAs and MUFAs were the most prominent fatty acids (Table 7). The proportion of DHA was higher in the n-3 diet group whereas LA was particularly enhanced in the n-6 diet group as compared with the other two dietary groups. IHH further increased the DHA or AA proportions in the n-3 or n-6 dietary groups, respectively.

Table 7 Effect of IHH and SFA, n-3 or n-6 PUFA enriched diets on the FA composition in heart phospholipids and diacylglycerols.

	Normoxia						Hypoxia					
	SFA	**SEM**	**n-3**	**SEM**	**n-6**	**SEM**	**SFA**	**SEM**	**n-3**	**SEM**	**n-6**	**SEM**
Phospholipids												
16:0	10.07	0.34	10.92	0.29	9.08§	0.24	11.55*	0.37	12.43	0.60	10.91*	0.42
18:0	26.04	0.78	25.50	1.76	26.30	1.65	25.60	0.70	25.29	1.01	26.25	1.30
18:1n-9	3.76	0.11	3.34§	0.08	3.69	0.09	3.70	0.19	3.31	0.06	3.27*	0.12
18:2n-6	20.13	1.36	20.54	0.70	25.81§	1.56	17.75	0.15	16.63*	0.36	24.43§	0.75
20:4n-6	22.08	0.20	11.61§	0.50	21.44	0.87	19.91*	0.47	11.18§	0.54	18.96*	0.51
20:5n-3	0.17	0.03	2.89§	0.18	0.04	0.01	0.20	0.04	2.74§	0.15	0.06	0.01
22:6n-3	10.41	0.39	17.31§	0.86	7.12§	0.54	12.52*	0.76	19.83§	0.88	8.91§ *	0.43
20:4n-6/22:6n-3	2.14	0.08	0.67§	0.01	3.06§	0.13	1.62*	0.07	0.56§*	0.01	2.15§*	0.09
Σ SFA	36.21	1.03	36.60	2.03	35.52	1.86	37.25	1.03	36.73	1.49	36.88	2.06
Σ MUFA	6.99	0.17	7.93§	0.18	6.14§	0.17	7.33	0.32	8.30§	0.19	5.87§	0.17
Σ n-6 PUFA	44.12	1.46	33.13§	0.97	49.77§	1.80	40.38	0.50	29.31§	0.60	46.96§	1.67
Σ n-3 PUFA	12.66	0.55	22.32§	1.05	8.54§	0.75	15.01	0.89	25.64§ *	1.04	10.26§	0.59
UI	216.49	2.83	227.46	8.85	203.27	6.94	220.92	6.61	233.91	8.38	204.20‡	5.70
Diacylglycerols												
16:0	27.97	1.80	24.77	1.19	23.86	1.90	23.53	1.45	21.44	1.37	21.11	1.53
18:0	23.76	2.46	19.51	1.78	21.37	2.12	24.83	2.18	18.97	2.23	22.44	2.32
18:1n-9	20.85	4.11	18.85	2.51	18.63	2.44	19.97	3.07	18.57	3.90	14.80	2.66
18:2n-6	6.42	0.44	8.59	1.00	14.37§	2.25	8.46	0.67	10.22	0.97	16.15§	1.62
20:4n-6	4.20	1.04	3.63	0.57	4.94	0.94	5.85	1.09	5.85	1.45	8.56*	1.69
20:5n-3	1.18	0.91	2.15	1.22	1.76	1.42	0.38	0.07	1.59§	0.33	0.54	0.23
22:6n-3	2.11	0.43	3.69§	0.61	2.04	0.22	3.29	0.44	6.01§ *	0.98	3.70	0.57
20:4n-6/22:6n-3	1.99	0.27	0.95	0.15	2.46§	0.43	1.65	0.20	1.12	0.22	2.39‡	0.35
Σ SFA	53.70	2.84	46.26	1.54	46.67	2.50	49.69	2.35	41.51	1.27	45.07	1.43
Σ MUFA	30.36	3.59	32.91	2.26	27.58	2.07	29.54	2.67	31.69	3.25	22.75	2.64
Σ n-6 PUFA	11.63	1.50	13.61	1.57	21.04§	2.82	15.81	1.50	17.25	2.05	26.53§	2.65
Σ n-3 PUFA	4.28	0.86	7.20	1.28	4.68	1.35	4.94	0.51	9.53§	1.38	5.63	0.65
UI	75.16	5.74	88.90	5.20	95.48†	5.39	85.14	5.45	102.25†	6.12	108.07†	6.57

*Σ SFA, total saturated FA; Σ MUFA, total monounsaturated FA; Σ n-3 PUFA, total n-3 polyunsaturated FA; Σ n-6 PUFA, total n-6 polyunsaturated FA. UI, unsaturation index calculated as mol % of individual unsaturated FA multiplied by the number of double bonds. Values are means ± SEM from 5 hearts in each group. *P<0.05 vs. the corresponding normoxic group; †P<0.05 vs. the SFA group; ‡P<0.05 vs. the n-3 group; §P<0.05 vs. other corresponding diet groups.*

In heart phospholipids, the (SFA+MUFA)/PUFA ratio remained unaffected by diets (Fig. 19A, B). On the other hand, this ratio closely followed that of the corresponding fed diet in heart DAGs (Fig. 19B). IHH induced a decrease of this ratio in heart DAGs but had no effect on heart phospholipids.

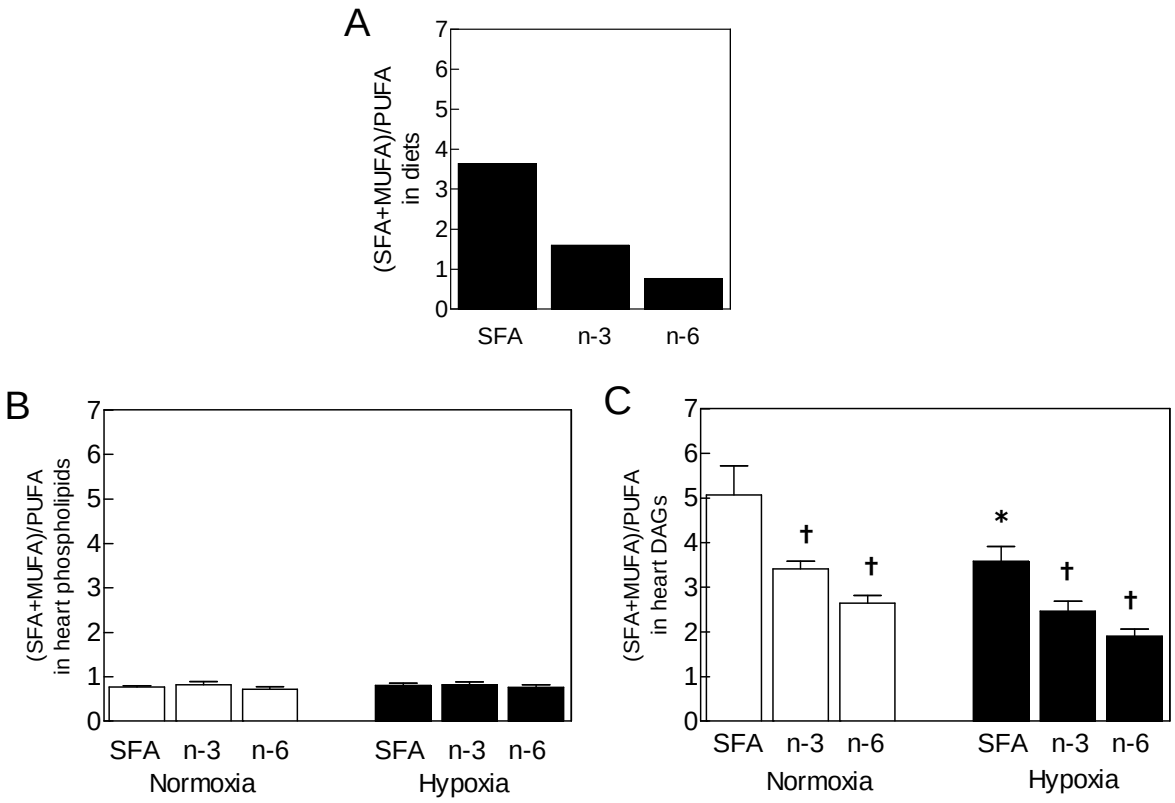

Fig. 19 *The (SFA+MUFA)/PUFA ratio in the diet (A), heart phospholipids (B) and heart 1,2-diacylglycerols (DAGs) (C) of normoxic rats and chronically hypoxic rats fed SFA, n-3 or n-6 diets. Values are means of 5 animals per group. *P<0.05 vs. the corresponding normoxic group. †P<0.05 vs. the corresponding SFA group.*

Both phospholipids and DAGs tended to reflect the n-6/n-3 ratio of the corresponding fed diet (Fig. 20). The ratio was highest in the n-6 diet group and lowest in the n-3 diet group. IHH induced a decrease of the n-6/n-3 ratio in heart phospholipids by about 23% independent of the diet groups (Fig. 20B).

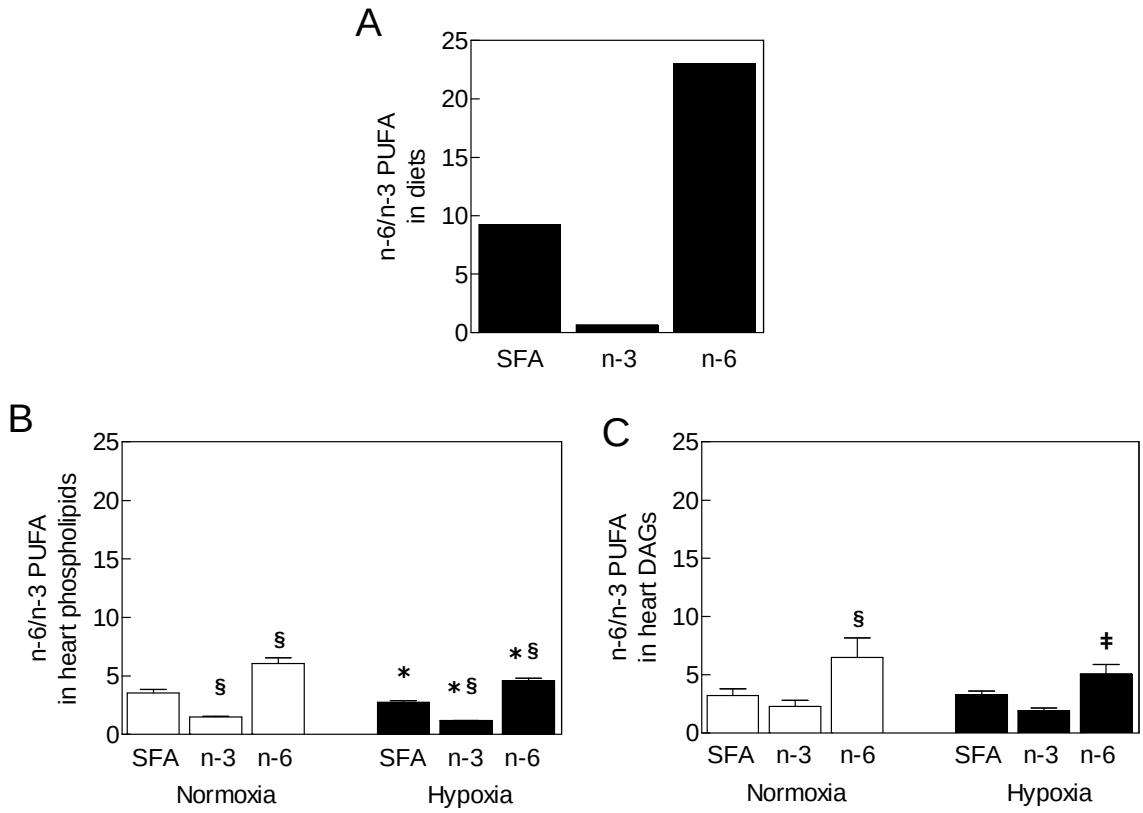

Fig. 20 *The n-6/n-3 PUFA ratio in the diet (A), heart phospholipids (B) and heart 1,2-diacylglycerols (DAGs) (C) of normoxic rats and chronically hypoxic rats fed SFA, n-3 or n-6 diets. Values are means of 5 animals per group. *P<0.05 vs. the corresponding normoxic group.* ‡*P<0.05 vs. the corresponding n-3 group.* §*P<0.05 vs. other corresponding diet groups.*

The n-3 diet increased the level of conjugated dienes in both normoxic and hypoxic hearts as compared with the corresponding SFA or n-6 dietary groups. IHH had no additional effect (Fig. 21).

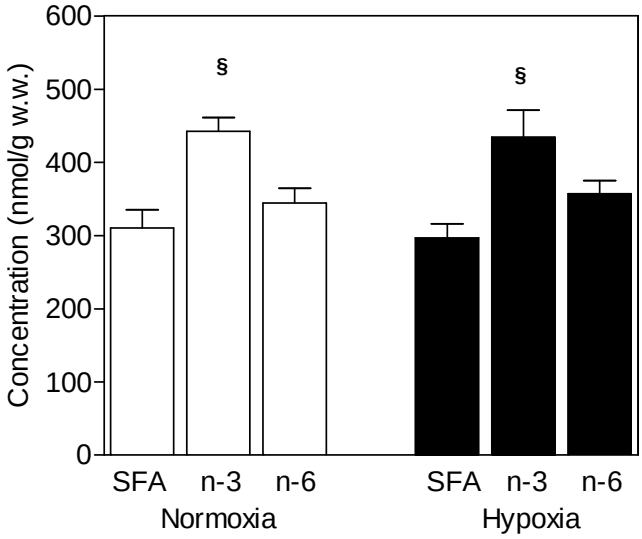

Fig. 21 *The level of conjugated dienes in the left ventricle myocardium of chronically hypoxic rats (black columns) and normoxic controls (open columns) fed SFA, n-3 or n-6 diets. w.w., wet weight. Values are mean ± SEM from 6 hearts in each group.* $^{§}P<0.05$ *vs. other corresponding diet groups.*

4.2.2 Hearts of rats fed n-3 PUFA-enriched diet have lower incidence of ventricular arrhythmias, but show larger infarct size compared to n-6 PUFA-enriched group

Neither the lipid diets nor IHH affected the values of the ischemic arrhythmia score (AS) (Table 8). Nevertheless, both in the normoxic and hypoxic groups, the given n-3 diet tended to decrease the severity of ischemic arrhythmias. The value of reperfusion AS in the normoxic n-3 group also tended to decrease compared to other diet groups. IHH decreased AS for reperfusion arrhythmias in all groups, but this effect was significant only in rats fed SFA or the n-3 diet. Reperfusion arrhythmias were almost eliminated by a combination of the n-3 diet and IHH.

Table 8 Ischemic and reperfusion arrhythmia scores: effects of lipid diets and IHH.

	Normoxia			Hypoxia		
	SFA	**n-3**	**n-6**	**SFA**	**n-3**	**n-6**
n	10	10	9	11	8	12
Ischemic arrhythmias	2.80±0.25	1.80±0.36	2.44±0.29	2.73±0.45	1.75±0.41	2.23±0.36
Reperfusion arrhythmias	2.70±0.21	1.80±0.44	2.33±0.37	2.00±0.23*	0.13±0.13*§	1.38±0.29

*n; number of animals. Ischemic arrhythmias were recorded over 20-min coronary artery occlusion. Reperfusion arrhythmias were recorded over the first 5 min of reperfusion. Dietary groups: saturated fatty acid (SFA) enriched, n-3 or n-6 polyunsaturated FA enriched diet. Values are means ± SEM. *P<0.05 vs. the corresponding normoxic group. §P<0.05 vs. other corresponding diet groups.*

The normalized area at risk (AR/LV) did not significantly differ between the groups: its mean values ranged from 40 to 42%. Normoxic rats fed the n-6 diet exhibited significantly smaller infarct area (IA) (43.6 ± 3.2% of the AR) as compared with the normoxic n-3 group (56.1 ± 3.9%). The IA/AR of the normoxic SFA group (49.3 ± 2.3%) did not significantly differ from that of other normoxic groups (Fig. 22). Adaptation to IHH had a significant infarct size-limiting effect in SFA (IA/AR 38.2 ± 2.4%) and n-3 (45.0 ± 1.8%) groups but not in the n-6 group (42.6 ± 2.5%; Fig. 22).

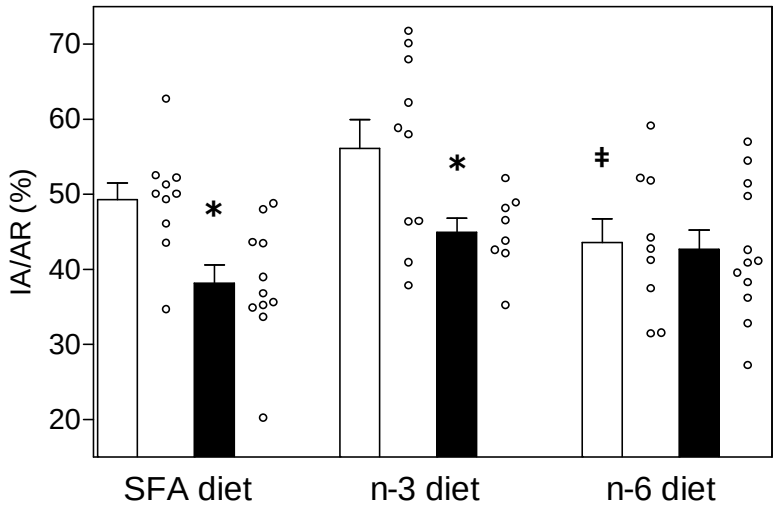

Fig. 22 *Myocardial infarct area expressed as a percentage of the area at risk (IA/AR) in chronically hypoxic rats (black columns) and normoxic controls (open columns) fed SFA, n-3 or n-6 diets. Open circles indicate individual experiments. Values are means ± SEM. *P<0.05 vs. the corresponding normoxic group, ‡P<0.05 vs. the corresponding n-3 group.*

4.2.3 Infarct size and PKCδ content in particulate fraction are inversely affected by IHH and by diets enriched in SFAs and n-3 PUFAs

Regarding diet effects, the relative protein content of PKCδ in the particulate fraction of normoxic animals fed the n-3 diet was lower as compared with the corresponding SFA and n-6 groups. IHH increased the relative protein content of PKCδ in the particulate fraction of the SFA and n-3 groups (by 40% and 82%, respectively) but not in the n-6 group. IHH increased the relative protein content of PKCδ in the cytosolic fraction of the SFA group (Fig. 23A) and significantly redistributed this isoform from cytosolic to particulate fractions only in the n-3 group (Fig. 23B).

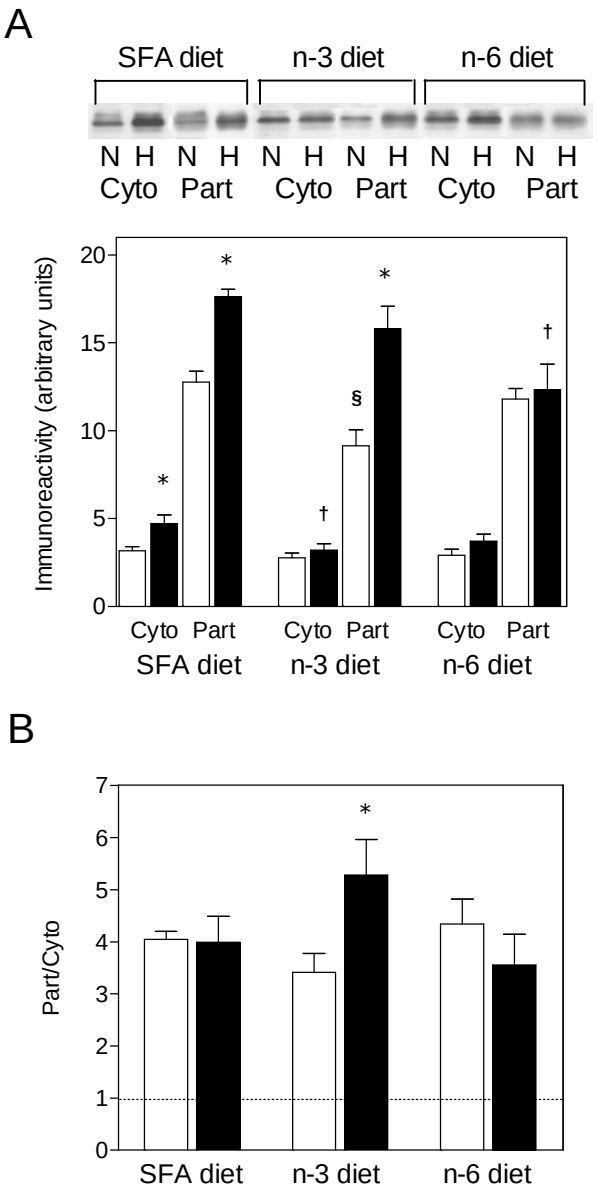

Fig. 23 *The relative protein content of PKCδ in cytosolic (Cyto) and particulate (Part) fractions (A) and its distribution between the fractions (B) from the left ventricles of chronically hypoxic rats (H; black columns) and normoxic controls (N; open columns) fed SFA, n-3 or n-6 diets. All samples compared were run on the same gel and quantified on the same membrane. The amount of protein applied to the gel was 15 μg (cytosolic fraction) or 5 μg (particulate fraction). Arbitrary units: PKCδ in each sample was quantified by densitometry measurements and expressed as the intensity of the stained band/μg of protein. Values are means ± SEM from 6 hearts in each group. *P<0.05 vs. the corresponding normoxic group, †P<0.05 vs. the corresponding SFA group, §P<0.05 vs. other corresponding diet groups.*

The content of PKCε was higher in the particulate fraction of normoxic rats fed the n-6 diet as compared with the SFA and n-3 groups. In contrast with PKCδ up-regulation, IHH did not significantly influence the amount of PKCε in the particulate fraction of the SFA and n-3 groups and it even decreased the content of this isoform in the n-6 group (by 41%). Neither lipid diets nor IHH affected the PKCε content in the cytosolic fraction (Fig. 24A). IHH tended to decrease the proportion of PKCε in the particulate fraction (expressed as a ratio of cytosolic to particulate level) but this effect did not reach statistical significance (Fig. 24B).

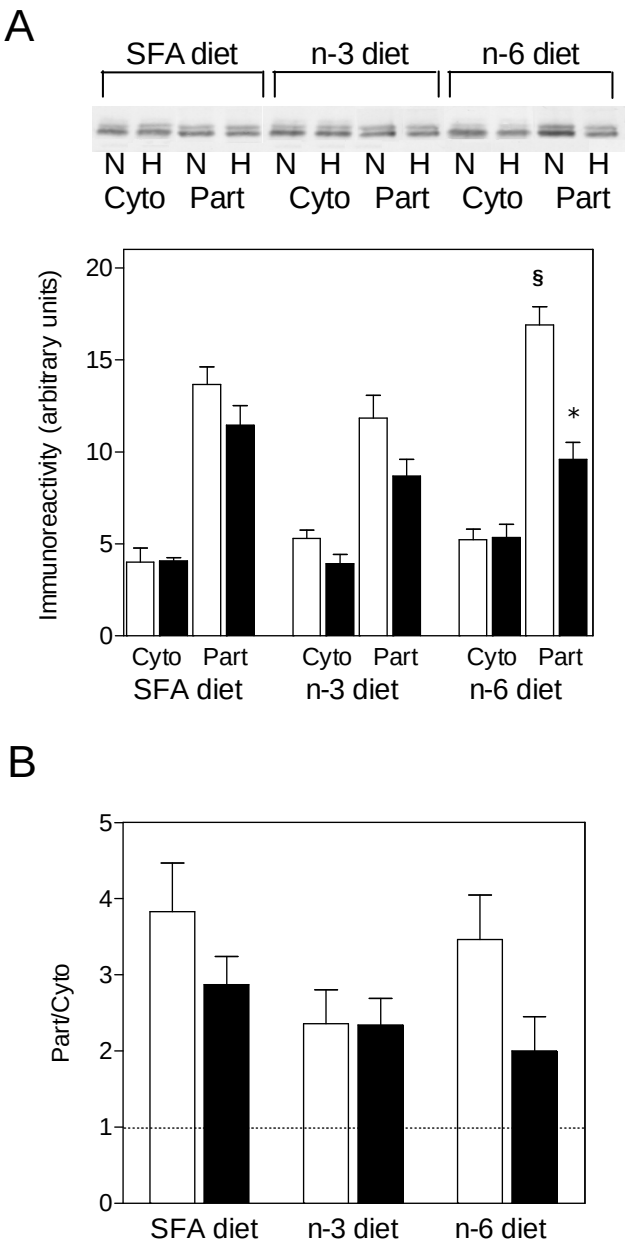

Fig. 24 *The relative protein content of PKCε in cytosolic (Cyto) and particulate (Part) fractions (A) and its distribution between the fractions (B) from the left ventricles of chronically hypoxic rats (H; black columns) and normoxic controls (N; open columns) fed SFA, n-3 or n-6 diets. All samples compared were run on the same gel and quantified on the same membrane. The amount of protein applied to the gel was 15 μg (cytosolic fraction) or 5 μg (particulate fraction). Arbitrary units: PKCε in each sample was quantified by densitometry measurements and expressed as the intensity of the stained band/μg of protein. Values are means ± SEM from 5 hearts in each group. *P<0.05 vs. the corresponding normoxic group, §P<0.05 vs. other corresponding diet groups.*

Fig. 25 presents the relationships between the mean values of PKCδ (A) or PKCε (B) relative protein contents in the myocardial particulate fraction and the mean infarct size for three normoxic and three hypoxic groups. Regression analysis demonstrated a negative linear relationship between PKCδ amount and infarct size with the correlation coefficient approaching 0.8 (Fig. 25A). Note that the hypoxic groups are shifted to the lower right portion of the regression line (smaller infarction and higher PKCδ content).

In contrast with PKCδ, regression analysis did not reveal any clear relationship between PKCε content and infarct size. Although the infarct size tended to decrease with increasing relative PKCε content within the normoxic or hypoxic groups, the hypoxic groups are shifted to the lower left portion of the graph (smaller infarction and lower PKCε content) (Fig. 25B).

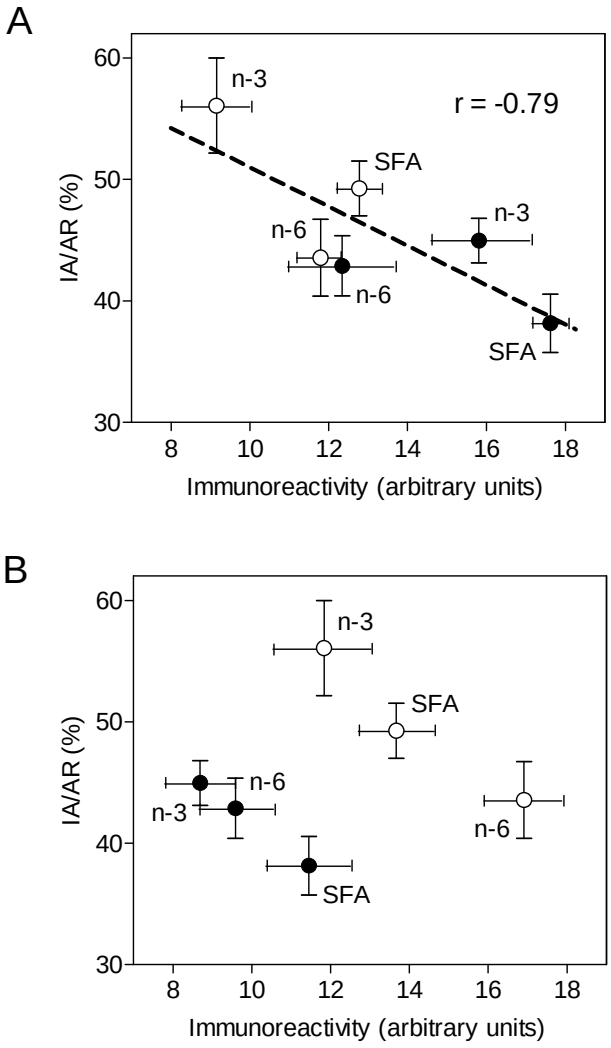

Fig. 25 *Relationships between the mean values of PKCδ (A) and PKCε (B) relative protein contents in the myocardial particulate fraction and the mean infarct area normalized to the area at risk (IA/AR) in chronically hypoxic rats (black circles) and normoxic controls (open circles) fed SFA, n-3 or n-6 diets. r, correlation coefficient.*

5 DISCUSSION

5.1 Aim I

Within this aim we studied the effect of IHH on the subcellular distribution, protein and mRNA levels of PKCδ and PKCε and their role in IHH-induced cardioprotection.

5.1.1 IHH influences PKCδ subcellular distribution, up-regulates its protein level but does not influence mRNA level

We showed that IHH up-regulated PKCδ in left ventricle, enhanced its phosphorylation on Ser643 and increased its co-localization with markers of mitochondrial and sarcolemmal membranes. Rottlerin, a PKCδ inhibitor, partially reversed the IHH-induced effects on the subcellular redistribution of PKCδ (Hlavackova et al., 2010: Supplement 1). Although the PKCδ protein was up-regulated in IHH-adapted hearts, the mRNA level of PKCδ was not changed. Similarly, 21 day exposure of rats to 5,500 m CHH did not influence the PKCδ mRNA level in left ventricular myocardium. However, the PKCδ expression was shown to transiently increase in the initial phase (after 3 days) of adaptation to CHH (Uenoyama et al., 2010). Our observation of the PKCδ protein level up-regulation due to adaptation of rats to IHH corresponds to previous reports using the same (Neckar et al., 2005; Kolar et al., 2007) or similar (Ding et al., 2004) experimental model. IHH increased the phosphorylation of PKCδ on Ser643 (a turn motif, in which phosphorylation is required to maintain the catalytic competence of the enzyme (Hauge et al., 2007)) and promoted its increased co-localization with mitochondrial and sarcolemmal membranes. Phosphorylation of PKCδ on Ser643 and its translocation to mitochondria was also observed to accompany cardioprotection induced by pharmacological preconditioning (Uecker et al., 2003). The beneficial role of PKCδ associated with its translocation to mitochondrial membrane and/or sarcolemma was confirmed by a variety of experiments using ischemic and pharmacological preconditioning (Bouwman et al., 2004; Uecker et al. 2003; Hirotani and Sadoshima, 2005; Turrell et al., 2011). The observed protective effects mediated by PKCδ were ROS-dependent and related to activation of either mitoK_{ATP} or sarcolemmal K_{ATP} (sarcK_{ATP}) channels. PKCδ is a redox-sensitive enzyme and ROS can modulate its function *via* tyrosine phosphorylation (Konishi et al., 2001). We demonstrated increased oxidative/nitrosative stress in IHH hearts by enhanced nitrotyrosine formation (Hlavackova et al., 2010: Supplement 1). It has been reported that PKC nitration increases

PKC signaling by enhancing PKC-protein complex formation; increased tyrosine nitration of PKCε enhanced PKCε binding to its anchoring protein RACK2 in rabbit cardiomyocytes (Balafanova et al., 2002). Our recent study showed that chronic antioxidant treatment during the IHH adaptation period eliminated both PKCδ up-regulation and infarct size reduction, suggesting ROS-dependence of both events (Kolar et al., 2007). Similarly to the preconditioning, the participation of mitoK_{ATP} and/or sarcK_{ATP} channels in increased ischemic tolerance of chronically hypoxic hearts was reported (Kong et al., 2001; Neckar et al., 2002a; Zhu et al., 2003). In addition, it was shown that PKCδ translocation to the sarcolemmal membrane is connected with the Na^+/Ca^{2+} exchanger-dependent cardioprotection (Bouwman et al., 2006). Therefore, it can be speculated that increased co-localization of PKCδ with sarcolemmal membrane could also play a role in IHH-induced myocardial protection.

Concerning PKCδ function in mitochondria, the d-subunit of F_1F_0 ATPase was shown as its possible target. Enhanced PKCδ expression in cardiac mitochondria and its co-immunoprecipitation with the d-subunit of F_1F_0 ATPase suggests that this putative interaction mediates inhibition of F_1F_0 ATPase or ATP synthase activities during prolonged hypoxia (Nguyen et al., 2008). In addition, Mayr et al. (2004b) postulated the essential role of PKCδ presence for a protective shift from aerobic to anaerobic metabolism induced by ischemic preconditioning. This shift from aerobic to anaerobic metabolism is also observed in IHH-adapted hearts (Bass et al., 1989). In accordance with these findings, it has been shown that PKCδ regulates the PDH complex activity, and the mitochondrial PKCδ signalosome (composed of PKCδ, p66Shc, cytochrome *c* and retinol) was identified as a mitochondrial redox state sensor, which could play an important role in the maintaince of energy homeostasis within cells (Actin-Perez et al., 2010a). Furthermore, a link between PKCδ, autophagy and cardioprotection was documented. Both autophagy and cardioprotection were abolished in rat hearts perfused with recombinant inhibitor of autophagy Tat-Atg5 (K130R) (Huang et al., 2010). Autophagy may be one way to remove damaged mitochondria under IHH conditions, when a slight decrease in protein level of oxidative phosphorylation complexes occurs (Hlavackova et al., 2010: Supplement 1). Our observation of the decrease in oxidative phosphorylation complexes is in agreement with our previous study where IHH reduced the concentration of myocardial cardiolipin (Jezkova et al., 2002), a mitochondrial inner membrane phospholipid marker. PKCδ is involved in the activation of autophagy by promoting JNK1-mediated Bcl-2

phosphorylation and dissociation of the Bcl-2/Beclin 1 complex (Chen JL et al., 2008). Beclin 1 up-regulation is associated with an increased rate of autophagy (Matsui et al., 2007). Our preliminary data showed that adaptation to IHH up-regulates mRNA level of Beclin1 and down-regulates the mRNA and protein level of its binding partner and repressor of autophagy, Bcl-2 (Kozichova and Hlavackova, unpublished data).

5.1.2 IHH-induced effect on PKCδ subcellular redistribution is disrupted by PKCδ inhibitor rottlerin

We observed that rottlerin, a PKCδ inhibitor, reversed IHH-induced PKCδ redistribution to the mitochondrial fraction without affecting the total protein amount of PKCδ in the homogenate (Hlavackova et al., 2010: Supplement 1). It was shown that acute administration of ATP-competitive inhibitor rottlerin (Gschwendt et al., 1994) before preconditioning inhibited PKCδ translocation to mitochondria (Freyer et al., 2001). Moreover, it was reported that ATP-competitive inhibitors affect redistribution of DAG-sensitive PKC isoforms (e.g. PKCδ) by altering their DAG sensitivity presumably by disrupting closed conformation of PKC (Takahashi and Namiki, 2007). Although rottlerin inhibits PKCδ more effectively than other PKC isoforms (Gschwendt et al., 1994), it is necessary to consider its possible nonspecific effects (Soltoff, 2007). Nevertheless, our immunofluorescence microscopy analysis revealed that rottlerin treatment partially reversed IHH-induced PKCδ co-localization with the sarcolemma and mitochondria. These results are in accord with the rottlerin-induced inhibition of the improved ischemic tolerance of IHH-adapted rat hearts (Neckar et al., 2005) and support the view that PKCδ plays a role in the cardioprotective mechanism of IHH. However, we are aware that these results need to be verified by using more selective PKC inhibitors. PKCδ involvement in IHH-induced cardioprotection will be studied by the use of the PKCδ inhibitor peptide, a δV1-1 analogue, which we recently obtained from KAI Pharmaceuticals Inc. A study concerning possible PKCδ targets in sarcolemmal and mitochondrial membranes is in process.

The function of PKCδ in myocardial I/R injury and its precise involvement in the mechanism of protection is still a matter of debate. One of the reasons for this ambiguity may be the fact that among all PKC isoforms, PKCδ possess the greatest flexibility in the ability to affect diverse cellular functions. This is enabled by fine regulation of its subcellular localization by means of phosphorylation of multiple serine/threonine and

tyrosine residues (Steinberg, 2004). The timing and subcellular localization of PKCδ activation appears to be a critical factor in the manifestation of either protective or detrimental effects of its activity in the heart. Whereas its activation during reperfusion leads to stimulation of pro-apoptotic pathways (Murriel et al., 2004) and exacerbation of myocardial injury (Inagaki et al., 2003), its activation well before an ischemic insult is cardioprotective (Inagaki and Mochly-Rosen, 2005). The advanced up-regulation of PKCδ in cellular membranes, as an important prerequisite of its protective action, is well fulfilled under our IHH conditions. Mayr et al. (2004a) proved PKCδ involvement in preconditioning-induced cardioprotection using PKCδ-null mice. In another study, they also showed that inhibition of PKCε resulted in compensatory phosphorylation and mitochondrial translocation of PKCδ, providing a possible explanation for the synergy of PKCδ and PKCε in cardioprotection (Mayr et al., 2009). This phenomenon can play a role under IHH conditions where PKCδ phosphorylation and its total protein level were up-regulated and PKCδ was redistributed to mitochondria while PKCε was down-regulated in rat myocardium.

5.1.3 IHH down-regulates PKCε protein level but does not affect its mRNA level and subcellular distribution

We found that adaptation to IHH decreased the total amount of PKCε in myocardium without affecting its distribution and the level of p-PKCε (Ser729) (Hlavackova et al., 2010: Supplement 1). However, studies dealing with effects of chronic hypoxia on PKCε expression, activity and subcellular distribution are rather contradictory, probably due to the diversity of the hypoxia models used (Kolar et al., 2009: Supplement 5). In previous experiments, adaptation to IHH decreased the content of PKCε protein (Kolar et al., 2007) and a similar effect was observed in adult rat myocardium prenatally exposed to hypoxic conditions (Li et al., 2004). Although we found that the total amount of the PKCε protein was decreased in hearts adapted to IHH, the level of PKCε mRNA was not changed. It was shown that exposure of H9C2 cells to 10.5% O_2 had no significant effect on the level of PKCε mRNA, while 1% O_2 down-regulated the PKCε mRNA expression (Patterson et al., 2010). PKCε gene repression was induced by prenatal exposure to 10.5% O_2 in the hearts of adult offspring. This study revealed a novel mechanism of hypoxia-derived ROS-mediated epigenetic repression of the PKCε gene (Patterson et al., 2012). Although we found no changes in PKCε subcellular distribution in

left ventricles after adaptation to IHH, partial PKCε co-localization with oxidative phosphorylation complexes in both normoxic as well as IHH hearts suggests its presence in the mitochondria. An interaction of PKCε with cytochrome *c* oxidase subunit IV associated with improved cytochrome *c* oxidase activity was reported in preconditioned rat hearts (Guo et al., 2007). In agreement with these results, we observed a tendency for IHH-induced attenuation of oxidative phosphorylation complex IV and the down-regulation of PKCε in fractions with the highest oxidative phosphorylation complex enrichment (Hlavackova et al., 2010: Supplement 1). It has been reported that PKCε protein down-regulation could be caused by PKCδ-dependent hydrophobic motif phosphorylation of PKCε on Ser729 (Rybin et al., 2003). Despite the decrease of total PKCε content in the present study (Hlavackova et al., 2010: Supplement 1), the level of the phosphorylated form of PKCε (Ser729) was not influenced by IHH, leading to an increase in p-PKCε (Ser729)/total PKCε ratio. This suggests that maintenance of the phosphorylated (active) PKCε level may be important for myocardial adaptation to IHH. Similarly, an increase in p-PKCε (Ser729)/total PKCε ratio was detected in hearts of rats adapted to IHH (5,000 m) (Wang et al., 2011). Moreover, increased phosphorylation and translocation of PKCε was observed in the hearts of neonatal rabbits under conditions of CNH (Rafiee et al., 2002). It is necessary to emphasize that PKCε is the key component of signal transduction of various forms of preconditioning (Budas et al., 2007), and the cross-regulation of PKCε and PKCδ function in cardiomyocytes has been well documented (Mayr et al., 2009; Rybin et al., 2003; Rybin et al., 2007). The work of Inagaki and Mochly-Rosen (2005) showed that the activation of PKCδ before an ischemic insult led to the activation of PKCε and consequently to cardioprotection. Despite the absence of PKCε up-regulation or subcellular redistribution by IHH observed in our study, the finding of the preserved p-PKCε level means that the potential involvement of this isoform in the protective mechanism cannot be unequivocally excluded. Therefore, we decided to explore the PKCε involvement in IHH-induced cardioprotection by using an isozyme-selective PKCε peptide inhibitor. Results of this study are discussed further.

5.1.4 PKCε peptide inhibitor KP-1633 does not affect IHH-induced protection in isolated cardiomyocytes

A prerequisite for this study was to ascertain whether isolated left ventricular myocytes (LVM) are able to maintain the protected phenotype as gained by *in vivo* IHH

adaptation. We demonstrated that the salutary effect of IHH was retained in isolated LVM, as shown by the reduced LDH release and the increased survival rate of cells exposed to MI/R, simulating acute I/R injury. These data suggest that the cell isolation procedure does not negatively interfere with myocardial protection induced by IHH, and freshly isolated myocytes are, therefore, a suitable model for the study of its underlying mechanism. A recent study of our research group (Borchert et al., 2011) has demonstrated that ventricular myocytes isolated from rats adapted to cardioprotective CNH (10% O_2, 3 wk) also retained improved resistance to injury caused by MI/R. Similarly, it has been reported that ischemic preconditioning of whole rat hearts increased the resistance of subsequently isolated ventricular myocytes to MI/R (Rodrigo and Samani, 2008).

Furthermore, we verified the activity of KP-1633 as a PKCε inhibitor. We chose the model of cardioprotection induced by exposure of the cardiac myocytes to physiologically attainable ethanol levels minutes before ischemia. This model of cardioprotection was shown to be mediated by direct activation of PKCε since the protective effect of acute ethanol exposure was blocked by an isozyme-selective PKCε inhibitor, εV1-2 (Chen et al., 1999). In our study, ethanol treatment was able to reduce LDH release from left ventricular myocytes during metabolic inhibition and KP-1633, an εV1-2-peptide analogue, blocked this protective effect.

Previous results suggested that PKC is a mediator of cardioprotection induced by IHH since the PKC general inhibitor chelerythrine administered to rat hearts 15 min before simulated ischemia abrogated the infarct size-limiting effect of IHH (Neckar et al., 2005). Moreover, in the study by Beguin et al. (2007), chelerythrine was able to remove the infarct size-limiting effect of *in vivo* intermittent hypoxic preconditioning in rats when administered before test ischemia but not before the initial hypoxic stimulus, suggesting that while PKC is an important mediator, the trigger phase of delayed protection is independent of this enzyme. Therefore, we decided to investigate the effects of PKCε inhibition by KP-1633 peptide 15 min prior to MI/R. We studied the viability and LDH release from LVM isolated from normoxic and IHH-adapted rats exposed to MI/R. Following KP-1633 administration as well as after administration of control peptide KP-1723, we observed a noticeable but insignificant decreasing trend in the post-MI/R LDH release from normoxic LVM. Neither the PKCε peptide inhibitor KP-1633 nor the control peptide KP-1723 was able to block the protective effect of IHH demonstrated as an increased survival rate of LVM from IHH-adapted rats exposed to MI/R. These

preliminary results are in line with our findings that adaptation to IHH decreased the total amount of PKCε protein and did not influence PKCε subcellular distribution in rat hearts (Hlavackova et al. 2010: Supplement 1) and support the idea that PKCε is not a key mediator of cardioprotection induced by IHH (Kolar et al., 2007; Hlavackova et al., 2007: Supplement 2). In contrast, Wang et al. (2011) found that the IHH-improved postischemic left ventricle performance in rats and I/R-induced translocation of PKCε to membranes were abrogated by perfusion of isolated rat hearts with the PKCε-selective inhibitor peptide εV1-2 (10 μM) for 5 min with a 5-min washout before ischemia. However, a straightforward interpretation of this finding may be hampered by methodological ambiguity. Wang et al. (2011) did not mention the precise εV1-2 peptide form used. In particular it was not specified whether the εV1-2 peptide was linked to the cell-permeable peptide sequence to enable the εV1-2 peptide to cross biological membranes. Also, the model of IHH used by Wang et al. (2011) was less severe (5,000 m, 4 h/day 4 wk) than our IHH model (7,000 m, 8 h/day 5-6 wk) which could be yet another reason for the discrepancy with our results. Activation of PKCε, the key component of signal transduction of preconditioning, has been proposed to be responsible for the CNH-induced cardioprotection in neonatal rabbits (Rafiee et al., 2002; 2003). In this experimental model, chelerythrine reversed translocation of PKCε from the cytosolic to the particulate fractions and abolished the protection. However, this cannot be taken as proof of the involvement of this particular isoform because chelerythrine acts as a non-selective inhibitor of all PKC isoforms. On the other hand, our experiments demonstrated that adaptation of adult rats to IHH decreased the myocardial protein level of PKCε, and this effect was not affected by interventions (antioxidant treatment, diet enriched with n-6 PUFA) that prevented the induction of protected cardiac phenotype (Kolar et al., 2007; Hlavackova et al., 2007: Supplement 2). Together with the observation, that PKCε peptide inhibiton does not abort the protective effect of IHH on cardiomyocyte survival, these data suggest that this isoform is unlikely to play a crucial role in the mediation of IHH-induced cardioprotection. The question whether the so far diverging results are attributable to the use of different animal models of hypoxia, including the protocol of hypoxic treatment, animal species, and age, are due for future research.

5.2 Aim II

Within this aim we studied the effect of SFA, n-3 PUFA and n-6 PUFA enriched diets and IHH on the fatty acid profile of myocardial phospholipids and diacylglycerols, ischemia/reperfusion injury and protein level of PKCδ and PKCε.

5.2.1 Different lipid-enriched diets and IHH show complex pattern of influence on myocardial phospholipid and diacylglycerol fatty acid profile

Dietary supplementation with saturated fats did not significantly affect the FA composition of myocardial phospholipids (Hlavackova et al., 2007: Supplement 2) as compared to the standard ST1 diet (Jezkova et al., 2002). It is important to point out that the SFA diet contained, besides a high amount of SFAs, also a similarly high proportion of MUFAs. Both n-3 and n-6 PUFA enriched diets had no effect on the myocardial content of SFAs, but slightly influenced MUFAs (the n-3 diet increased and the n-6 diet decreased the proportion of MUFAs in heart phospholipids) and, as expected, markedly changed n-3 PUFA and n-6 PUFA content in normoxic hearts (Hlavackova et al., 2007: Supplement 2). This is in agreement with the results of numerous studies showing that supplementation with fish oil (n-3 PUFAs) increased the myocardial content of n-3 PUFAs at the expense of n-6 PUFAs (Pepe and McLennan, 1996; Jude et al. 2007), whereas diets containing corn oil (n-6 PUFAs) had the opposite effects (Hock et al., 1987). Surprisingly, IHH decreased the n-6/n-3 PUFA ratio proportionally in all diet groups by approximately 23% (Hlavackova et al., 2007: Supplement 2). Independently of diet, stressful conditions such as high doses of catecholamines (Benediktsdottir and Gudbjarnason, 1988) or hyperthyroidism (Hamplova et al., 2003) increase n-3 PUFA content in heart membrane phospholipids. The shifts in the n-6 and n-3 PUFA proportions in heart phospholipids caused by diets and IHH were qualitatively similar to those observed in rats fed different PUFA-enriched diets and chronically treated with high doses of catecholamines (Benediktsdottir and Gudbjarnason, 1988). In accordance with this observation, the exposure of rats to IHH was associated with transiently increased adrenergic activity and elevated plasma levels of catecholamines (Ostadal et al., 1984). Thus, the fatty acid remodeling in phospholipids induced by IHH could be explained by stress-dependent hormonal modulation of enzyme activities involved in phospholipid acyl remodeling. Phospholipid deacylation was accelerated due to the activation of phospholipase A2 in cardiomyocytes exposed to hypoxia (Kawaguchi et al., 1991; Grynberg et al., 1988). It is

likely that this enzyme, which preferentially hydrolyses AA (20:4n-6) from the sn2 position of membrane phospholipids (Nalbone et al., 1990) and acyl-CoA synthase with preferential affinity for DHA (22:6n-3) (Bouroudian et al., 1990) could contribute to the DHA accumulation in heart phospholipids observed after IHH by the deacylation–reacylation cycle.

Our observation that DAGs comprised a high proportion of SFAs and MUFAs but a low proportion of PUFAs as compared with other lipids regardless of dietary interventions (Balkova et al., 2009: Supplement 3) is in agreement with several reports (Hamplova et al., 2005; Murase et al., 2000). The n-3 diet increased the DHA proportion in heart DAGs compared with other diets (Balkova et al., 2009: Supplement 3). This observation is in agreement with the results obtained in mice and dogs fed with a fish oil supplemented diet (Takahashi et al., 2005; Jude et al., 2007). Besides a direct interaction of DAGs with PKCs, changes induced in the membrane lipid structure by DAGs and other lipids favor the binding and activation of PKCs (Goni and Alonso, 1999). It has been shown that DAGs enriched with EPA or DHA were less efficient activators of PKC than DAGs containing AA (Madani et al., 2001). IHH increased AA in DAGs of the n-6 diet group and increasing tendency was also observed in the SFA and n-3 diet groups. This finding is in contrast with heart phospholipids where AA decreased or remained unchanged (Balkova et al., 2009: Supplement 3). The fact that IHH specifically increased the accumulation of AA in myocardial phosphatidylcholine (Jezkova et al., 2002) suggests that phosphatidylcholine could be an important source of DAGs under IHH conditions. In accordance with this view, it has been reported that DAGs generated from phosphatidylcholine by phospholipase D are involved in ischemic preconditioning (Tosaki et al., 1997). Murase et al. (2000) found an increased DAG content as well as an increased proportion of AA in the DAGs of preconditioned rat hearts, supporting the involvement of this signaling lipid in cardioprotection.

5.2.2 Hearts of rats fed n-3 PUFA-enriched diet have lower incidence of ventricular arrhythmias, but show larger infarct size compared to n-6 PUFA-enriched group

Lipid diets had distinct effects on infarct size-limitation afforded by IHH. Whereas the groups fed on SFA and n-3 PUFA rich diets were protected by IHH, the protective effect of the n-6 PUFA-enriched diet on myocardial infarction was not further enhanced by IHH. IHH decreased arrhythmias score for reperfusion arrhythmias in all groups, but this

effect was significant only in rats fed SFA or the n-3 diet. Reperfusion arrhythmias were almost eliminated by a combination of the n-3 diet and IHH (Hlavackova et al., 2007: Supplement 2). It is generally accepted that cardiac susceptibility to I/R injury can be modulated by lipid diets. In particular, numerous experimental studies demonstrated that long-term feeding of various animal species with diets enriched with PUFAs protect the heart against ischemic and reperfusion ventricular arrhythmias (Pepe and McLennan, 1996; Abeywardena and Charnock, 1995). The anti-arrhythmic influence of n-3 PUFAs appears to be superior to that of n-6 PUFAs (Isensee and Jacob, 1994). Our data support the previous findings about the anti-arrhythmic effect of diets enriched with n-3 PUFAs against both ischemic and reperfusion arrhythmias (Pepe and McLennan, 1996; Abeywardena and Charnock, 1995; Isensee and Jacob, 1994). IHH decreased the severity of reperfusion arrhythmias accordingly to previous reports (Neckar et al., 2002b); this effect was manifested in all diet groups although with different potency (Hlavackova et al., 2007: Supplement 2). The combination of the n-3 diet and IHH had a stronger protective effect on the severity of reperfusion arrhythmia than the n-3 diet alone. There is a general consensus that n-3 PUFAs, particularly EPA and DHA, exert powerful anti-arrhythmic effects (Leaf et al., 2003) resulting from their multiple actions. It was shown, that both EPA and DHA decreased cardiac susceptibility to adrenergic stimulation (Ponsard et al., 1999), prevented disturbances of membrane transport and ionic homeostasis (Pepe and McLennan, 1996; Rinaldi et al., 2002), decreased phosphoinositides production (Anderson et al., 1996), altered the fatty acid composition of DAGs and led to differential PKC activation (Jude et al., 2007). Therefore, the enrichment of EPA and DHA content in heart lipids could, as demonstrated in our study (Balkova et al., 2009: Supplement 3), contribute to the anti-arrhythmic effect of IHH observed under ischemia–reperfusion conditions that was most pronounced in the n-3 dietary group.

However, diets enriched with PUFAs seem to have much less clear effects when myocardial infarct size is determined as the major end point of I/R injury. Factors that may influence the results of dietary studies with PUFAs include animal species (Ahotupa et al., 1993)**,** age (Pepe, 2005), the duration of treatment and the relative amount of n-3 and n-6 PUFAs in the diet (L′Abbe et al., 1991). Contrary to our assumption, we observed significantly smaller infarction in rats fed the n-6 diet compared with the n-3 diet group, which exhibited the largest extent of injury (Hlavackova et al., 2007: Supplement 2). Of the three previous studies that analyzed infarct size in rats *in vivo*, two demonstrated the protective effects of either the n-6 diet (sunflower seed oil, 12%) (McLennan et al., 1985)

or the n-3 diet (fish oil, 12%) (Zhu et al., 1994), but the third one (Force et al., 1989) did not find any limitation of infarction after 6-12 weeks on 20% fish oil or corn oil diets. Similarly, several *in vitro* studies failed to detect significant cardioprotection following n-3 PUFA or n-6 PUFA supplementation (Karmazyn et al., 1987; Sergiel et al., 1998). Our observation of the weak myocardial ischemic tolerance of rats fed the n-3 diet supports the previous findings of Gudbjarnasson and Oskarsdottir (1975), who demonstrated that the n-3 diet (cod liver oil, 10%, 12 wk) aggravated isoproterenol-induced cardiac necrosis and mortality. Thus, unlike the general expectations, it seems that the effect of PUFA-enriched diets, particularly those containing n-3 PUFAs, may not necessarily have favorable effects on the cardiac susceptibility to injury. Interestingly, the thorough systematic review on cardiovascular events and total mortality found no evidence of a clear benefit of n-3 PUFAs on health (Hooper et al., 2006). The cause of aggravated injury due to the n-3 diet is unclear. One potential explanation is that highly polyunsaturated long-chain FAs, in particular n-3 PUFAs from fish oil, are extremely susceptible to peroxidation as compared with n-6 PUFAs (L′Abbe et al., 1991; Yuan and Kitts, 2003), even in the presence of added dietary antioxidants (Gonzales et al., 1992). The increased level of conjugated dienes that was observed in the hearts of rats fed the n-3 diet (Balkova et al., 2009: Supplement 3) correspond to the increased susceptibility of n-3 PUFAs to oxidative stress, compared with the n-6 PUFA class, probably due to the greater number of double bonds (Scislowski et al., 2005). We did not detect any significant effect of IHH on the myocardial level of conjugated dienes in any dietary group. This can be explained by the fact that these compounds are one of the first temporarily detectable unstable markers of lipoperoxidation (Hendra et al., 1991). It seems likely that changes in the level of conjugated dienes could have appeared at earlier stages of hypoxic adaptation. Our finding of the increased levels of conjugated dienes in the myocardium of the n-3 dietary group corresponds to the largest myocardial infarct size observed in these animals. On the other hand, the same n-3 dietary group exhibited the lowest incidence and severity of ventricular arrhythmias (Hlavackova et al., 2007: Supplement 2). This is in agreement with the suggestion of Jude´ et al. (2003) that an oxidation product of DHA could effectively protect against arrhythmias rather than DHA itself.

5.3.3 Infarct size and PKCδ content in particulate fraction are inversely affected by IHH and by diets enriched in SFAs and n-3 PUFAs

We observed that lipid diets alone had distinct effects on the subcellular distribution of PKCδ and PKCε and also modulated the IHH-induced changes of these isoforms in rat hearts (Hlavackova et al., 2007: Supplement 2). Both PKCδ and PKCε are sensitive to DAGs and PS (Steinberg, 2008). Moreover, free unsaturated FAs such as AA and LA can activate PKC in the absence of DAGs and PS (Koide et al., 1992) and affect PKC-dependent signaling pathways in cardiac cells (Huang et al., 1997; Mackay and Mochly-Rosen, 2001a). The quality of the acyl chain of DAGs also plays a role in different PKC isoform activation (Madani et al., 2001). Although PKCδ and PKCε are members of the same subgroup of PKCs, they have multiple differences in their structure and are differentially regulated (Duquesnes et al., 2011). Kashiwagi et al. (2002) showed that AA and ceramide induced different patterns of PKCε translocation as compared with PKCδ. The differential sensitivity of PKCδ and PKCε to AA is related to the subtle differences in the conserved domain (C1B). The C1B domain defines the isoform specific sensitivity of PKCs to lipid second messengers. In accordance with our data, it seems that the higher content of n-6 PUFAs (mainly AA and LA) in membranes can be associated with improved cellular signaling mediated by PKC (Huang et al., 1997; Shirai et al., 1998). We can speculate that the highest amount of PKCε in the particulate fraction of the normoxic n-6 group (Hlavackova et al., 2007: Supplement 2) is linked to increased ischemic tolerance. This view is supported by the data of Mackay and Mochly-Rosen (2001a) who demonstrated that AA protected neonatal rat cardiac myocytes from ischemic injury and selectively activated PKCε and PKCδ; the PKCε inhibitor peptide εV1-2 and the general PKC inhibitor chelerythrine both blocked the AA-induced protection.

On the other hand, we observed a lower proportion of PKCδ and PKCε in particulate fraction of normoxic hearts of the n-3 diet group (Hlavackova et al., 2007: Supplement 2), which contained higher amounts of DHA and EPA in phospholipids as compared with SFA and n-6 groups. The n-3 diet increased the DHA proportion in DAGs compared with other diets (Balkova et al., 2009: Supplement 3). These results are in accord with the finding that DAGs enriched with EPA or DHA are less efficient activators of PKCδ and PKCε than DAGs containing AA (Madani et al., 2001). Moreover, it was reported that adult porcine cardiac myocytes supplementation with EPA and DHA

decreased PKC activity in these cells as compared with cells supplemented with AA (Nair et al., 2001). Similar to our results, Jude et al. (2007) found a lower activation (translocation from cytosolic to particulate fraction) of PKCδ and PKCε in the hearts of dogs fed with fish oil supplemented diet as opposed to standard diet. Le Guennec et al. (2010) suggested that the reduction of PKCδ and PKCε translocation and of the associated activities can prevent a pro-arrhythmic inhibition of the rapidly activating delayed rectifier potassium current, which is modulated by PKCs (Wang et al., 2009), or can influence other currents also regulated by PKCs such as the L-type Ca^{2+} current (Yang et al., 2009). This hypothesis is applicable in normoxic hearts of rats fed the n-3 diet where we found a lower presence of PKCδ and PKCε in the particulate fraction together with presence of an anti-arrhythmic effect. Nevertheless, the combination of the n-3 diet and IHH increased the translocation of PKCδ to the membrane fraction and had a stronger protective effect on reperfusion arrhythmia severity than the n-3 diet alone. Moreover, we demonstrated a lower amount of PKCδ in the particulate fraction from normoxic hearts of rats fed the n-3 diet, together with the larger myocardial infarct size compared with the n-6 dietary group. Adaptation to IHH that further increased the DHA proportion in heart DAGs of the n-3 group stimulated the translocation of PKCδ to the particulate fraction and had a protective effect on infarct size. These results suggest that the impact of changes in the PUFA composition of DAGs on PKC cardioprotective activity is complex and may differ in normoxic and chronically hypoxic hearts.

IHH led to the up-regulation of PKCδ in both SFA and n-3 diet groups, but not in the n-6 diet group in compliance with the presence or absence of the infarct size-limiting effect, respectively. Unlike PKCδ, myocardial PKCε was not influenced in SFA and n-3 diet groups and down-regulated in the n-6 diet group. Regression analysis of mean values of the PKCδ relative protein content in the particulate fraction and infarct size revealed a close negative correlation between these variables. This finding provides probably the most convincing piece of evidence so far available in support of the role of PKCδ isoform in IHH-induced cardioprotection. It however still cannot be taken as an evidence of a direct causal relationship. Concerning PKCε, it may be interesting to note that the infarct size tended to decrease with increasing relative PKCε content within the normoxic or hypoxic groups, suggesting that the up-regulation of this isoform is cardioprotective. However, all hypoxic groups exhibited a parallel shift to the smaller infarction and lower PKCε content, suggesting that IHH protects the myocardium by a mechanism independent of PKCε.

These results support preliminary conclusion of our research group (Neckar et al., 2005; Kolar et al., 2007) that PKCδ, but not PKCε, plays an important role in the infarct size-limiting mechanism of IHH in adult rat hearts.

6 SUMMARY

The primary aim of this thesis was to investigate the potential involvement of PKCδ and PKCε in the mechanism of IHH-induced cardioprotection. The specific outcomes of this study were:

Aim I

- We showed that IHH up-regulated PKCδ in left ventricle, enhanced its phosphorylation on Ser643 and increased its co-localization with markers of mitochondrial and sarcolemmal membranes. PKCδ subcellular redistribution was reversed by acute treatment with its inhibitor, rottlerin. Since the infarct size-limiting effect of IHH is attenuated by rottlerin, our data further support the view that PKCδ plays a significant role in IHH-induced cardioprotection.

- We demonstrated that the salutary effect of IHH was retained in subsequently isolated left ventricular myocytes, as shown by the reduced LDH release and the increased survival rate of cells exposed to metabolic inhibition/reenergization, simulating acute ischemia/reperfusion injury.

- Adaptation to IHH decreased the PKCε total protein in the left ventricular tissue without affecting its subcellular distribution and the level of phosphorylated PKCε (Ser729). Furthermore, PKCε inhibitor peptide KP-1633 did not affect the protective effects of IHH in isolated left ventricular myocytes exposed to metabolic inhibition/reenergization, simulating acute ischemia/reperfusion injury. These findings support the idea that PKCε is not a key player in cardioprotection induced by IHH.

Aim II

- Whereas the (SFA+MUFA)/PUFA ratio in heart DAGs corresponded to the ratio in the respective diet (in the sequence SFA>n-3 PUFA>n-6 PUFA diet), heart phospholipids maintained constant content of SFAs, MUFAs and total PUFAs, independent of diet and IHH. This is the evidence of a remarkable regulatory ability of membranes to maintain a stable milieu that is necessary for a proper function of membrane proteins. On the other hand, the n-6/n-3 PUFA ratio was influenced to various extents by either the dietary PUFA supply or IHH in both heart phospholipids and DAGs.

- Lipid diets had distinct effects on infarct size-limitation afforded by IHH. Whereas the groups fed on SFA and n-3 PUFA rich diets were protected by IHH, the protective effect of the n-6 PUFA-enriched diet on myocardial infarction was not further enhanced by IHH. As the IHH-induced decrease in n-6/n-3 PUFA ratio in membranes was proportional in all groups, it seems unlikely that this response is directly involved in the mechanism of infarct size-limiting effect in IHH hearts.

- Our data demonstrate distinct effects of lipid diets on myocardial protein level and distribution of PKCδ and PKCε between cytosolic and particulate fractions in normoxic and IHH-adapted rats. The infarct size decreased with increasing relative PKCε content, which is in line with a generally accepted view that this isoform is cardioprotective. However, all hypoxic groups had smaller infarction and lower PKCε content, suggesting that IHH protects the myocardium by a mechanism independent of PKCε.

- Unlike in other diet groups, IHH did not increase myocardial ischemic tolerance and did not cause up-regulation of PKCδ protein in rats supplemented with n-6 PUFA-enriched diet. The relative content of PKCδ in myocardial particulate fraction exhibited a close negative correlation with myocardial infarct size. These results suggest that PKCδ plays an important role in the infarct size-limiting mechanism of IHH in adult rat hearts.

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8 LIST OF ABBREVIATIONS

A/R	Anoxia/Reoxygenation
AA	Arachidonic Acid
ALA	α–Linoleic Acid
ANP	Atrial Natriuretic peptide
AR	Area at Risk
AS	Arrhythmia Score
ATP	Adenosin TriPhosphate
BNIP3	Bcl-2/adenovirus E1B 19-kDa protein-Interacting Protein 3
BNP	Brain Natriuretic Peptide
CAMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cDNA	complementary DeoxyriboNucleic Acid
cGMP	cyclic Guanosine MonoPhosphate
CNH	Continuous Normobaric Hypoxia
CONT	vehicle (dimethyl sulfoxide) treated group
COX	Cytochrome *c* OXidase
C_P	Crossing point
cPKC	classical Protein Kinase C
CT	Carboxyl-terminal Tail
Cx43	Connexin 43
Cyto	Cytosolic fraction
DAG	DiAcylGlycerole
DAPI	4',6 diamidino-2-phenylindole
DGK	DiacylGlycerol Kinase
DHA	DocosaHexaenoic Acid
DMSO	DiMethyl SulfOxide
dT	deoxy-Thymine
DTT	DiThioThreitol
EDTA	EthyleneDiamineTetraacetic Acid
EET	EpoxyEicosaTrienoic acid
EGTA	Ethylene Glycol Tetraacetic Acid
eNOS	endothelial Nitric Oxide Synhase
EPA	EicosaPentaenoic Acid
ERK	Extracellular signal-Regulated Kinase
EtOH	Ethanol
FA	Fatty Acid
FGF-2	Fibroblast Growth Factor 2
G-G	Glycin-Glycin (short cleavable dipeptid linker)
GFR	Growth Factor Receptor
gp130	glycoprotein 130
GPCR	G-Protein-Coupled Receptor
GSK-3β	Glycogen Synthase Kinase-3β
HIF-1	Hypoxia-Inducible Factor 1
Hom	Homogenate
HPRT1	Hypoxanthine-guanine PhosphoRibosylTransferase 1
HSP70	Heat Shock Protein-70
HSP90	Heat Shock Protein-90
CHH	Continuous Hypobaric Hypoxia

I/R	Ischemia/Reperfusion
IA	Infarct Area
IGF-1	Insulin-like Growth Factor 1
IHH	Intermittent Hypobaric Hypoxia
IL6	Interleukin 6
INH	Intermittent Normobaric Hypoxia
iNOS	inducible Nitric Oxide Synthase
ip	intraperitoneal
JAK	JAnus Kinase
JNK	c-Jun NH_2-terminal Kinase
L-NAME	L-N^G-Nitroarginine Methyl Ester
L-NMA	N^G-Methyl-L-arginine
LA	Linoleic Acid
LDH	Lactate DeHydrogenase
LV	Left Ventricle
LVM	Myocytes isolated from the Left Ventricle
MAPK	Mitogen-Activated Protein Kinase
MI	Metabolic Inhibition
Micro	Microsomal fraction
MI/R	Metabolic Inhibition/Reenergization
Mito	Mitochondria-enriched fraction
mitoK_{ATP}	mitochondrial ATP-sensitive potassium channels
MnSOD	Manganese SuperOxide Dismutase
MPTP	Mitochondrial Permeability Transition Pores
mRNA	messenger RiboNucleic Acid
mTORC2	mammalian Target Of Rapamycin Complex 2
MUFA	MonoUnsaturated Fatty Acid
NADPH	Nicotin Amid Dinucleotide Phosphate, reduced form
NaF	sodium fluoride
NCBI	National Center for Biotechnology Information
NO	Nitric Oxide
non-RT	non-Reverse Transcriptase control
nPKC	novel Protein Kinase C
NPR	Natriuretic Peptide Receptor
Nucl	Nuclear-cytoskeletal-enriched fraction
OXPHOS	OXidative PHOSphorylation
p38	p38 mitogen-activated protein kinase
P70S6K	p70 ribsosomal S6 protein Kinase
Part	Particulate fraction
P_B	barometric pressure
PC	PhosphatidylCholine
PCR	Polymerase Chain Reaction
PDH	Pyruvate DeHydrogenase
PDK1	Phosphoinositide-Dependent Kinase-1
PDK2	Pyruvate Dehydrogenase Kinase 2
pGC	particulate Guanylyl Cyclase
PHLPP	PH domain Leucine-rich repeat Protein Phosphatase
PI	PhosphoInositide
PI3K	PhosphatidylInositol-3 Kinase

PIP_2	PhosphatidylInositol-4,5-bisPhosphate
PKA	cAMP-dependent Protein Kinase
PKC	Protein Kinase C
PKG	Protein Kinase G
PLC	PhosphoLipase C
PLD	PhosphoLipase D
PO_2	Pressure of Oxygen
PPAR	Peroxisome Proliferator-Activated Receptors
PS	PhosphatidylSerine
PUFA	PolyUnsaturated Fatty Acid
RACK	Receptor for Activated C Kinase
RISK	Reperfusion Injury Salvage Kinase
ROS	Reactive Oxygen Species
ROT	Rottlerin-treated group
SAFE	Survivor Activating Factor Enhancement
$sarcK_{ATP}$	sarcolemmal ATP-sensitive potassium channels
SDS-PAGE	Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis
SEM	Standard Error of Mean
SFA	Saturated Fatty Acid
sGC	soluble Guanylyl Cyclase
STAT3	Signal Transducer and Activator of Transcription 3
TNF-R	Tumor Necrosis Factor Receptor
TNFα	Tumor Necrosis Factor α
TTBS	Tris-Buffered Saline with Tween 20
UCN	Urocortins
UI	Unsaturation Index
UT	UnTreated
WGA	Wheat-Germ Agglutinin
wk	week

9 LIST OF PUBLICATIONS

Hlavackova M, Kozichova K, Neckar J, Kolar F, Musters RJ, Novak F, Novakova O (2010). Up-regulation and redistribution of protein kinase C-delta in chronically hypoxic heart. *Mol Cell Biochem* 345:271-282.

IF=2.168
Supplement 1

Author´s contribution: *designed research (with FK, RJM), performed research (with JN and KK), performed data analysis (with RJM and KK), wrote the article (with ON and FN).*

Hlavackova M, Neckar J, Jezkova J, Balkova P, Stankova B, Novakova O, Kolar F, Novak F (2007). Dietary polyunsaturated fatty acids alter myocardial protein kinase C expression and affect cardioprotection induced by chronic hypoxia. *Exp Biol Med (Maywood)* 232:823-832.

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Author´s contribution: *designed research (with FK, ON and FN), performed research (with JN, JJ, PB and BS), performed data analysis (with JN and PB), wrote the article (with JN).*

Balkova P, Jezkova J, **Hlavackova M**, Neckar J, Stankova B, Kolar F, Novak F, Novakova O (2009). Dietary polyunsaturated fatty acids and adaptation to chronic hypoxia alter acyl composition of serum and heart lipids. *Br J Nutr* 102:1297-1307.

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Author´s contribution: *co-designed research (with PB, ON, FN and FK), performed research (with PB, JJ, BS and JN), contributed in writing of the article (with PB, ON and FN).*

Balkova P, **Hlavackova M**, Milerova M, Neckar J, Kolar F, Novak F, Novakova O (2011). N-acetylcysteine treatment prevents the up-regulation of MnSOD in chronically hypoxic rat hearts. *Physiol Res* 60:467-474.

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Supplement 4

Author´s contribution: *discussed details of experimental design, contributed in conducting of the experiments (with PB), contributed in writing of the article (with PB).*

Book chapter

Kolar F, Novak F, Neckar J, Novakova O, **Hlavackova M**, Ostadal B, Musters RJ (2009). Role of protein kinases in chronic intermittent hypoxia-induced cardioprotection. In: *Intermittent hypoxia.* Xi L, Serebrovskaya TV (editors). Nova Science Publishers, Inc. pp. 175-191.

Supplement 5

10 SUPPLEMENTS

SUPPLEMENT 1

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Up-regulation and redistribution of protein kinase C-δ in chronically hypoxic heart

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Abstract The adaptation to chronic hypoxia confers long-lasting cardiac protection against acute ischemia–reperfusion injury. Protein kinase C (PKC) appears to play a role in the cardioprotective mechanism but the involvement of individual PKC isoforms remains unclear. The aim of this study was to examine the effects of chronic intermittent hypoxia (CIH; 7,000 m, 8 h/day) and acute administration of PKC-δ inhibitor (rottlerin, 0.3 mg/kg) on the expression and subcellular distribution of PKC-δ and PKC-ε in the left ventricular myocardium of adult male Wistar rats by Western blot and quantitative immunofluorescence microscopy. CIH decreased the total level of PKC-ε in homogenate without affecting the level of phosphorylated PKC-ε (Ser729). In contrast, CIH up-regulated the total level of PKC-δ as well as the level of phosphorylated PKC-δ (Ser643) in homogenate. Rottlerin partially reversed the hypoxia-induced increase in PKC-δ in the mitochondrial fraction. Immunofluorescent staining of ventricular cryo-sections revealed increased co-localization of PKC-δ with mitochondrial and sarcolemmal membranes in CIH hearts that was suppressed by rottlerin. The formation of nitrotyrosine as a marker of oxidative stress was enhanced in CIH myocardium, particularly in mitochondria. The expression of total oxidative phosphorylation complexes was slightly decreased by CIH mainly due to complex II decline. In conclusion, up-regulated PKC-δ in CIH hearts is mainly localized to mitochondrial and sarcolemmal membranes. The inhibitory effects of rottlerin on PKC-δ subcellular redistribution and cardioprotection (as shown previously) support the view that this isoform plays a role in the mechanism of CIH-induced ischemic tolerance.

Keywords Chronic hypoxia · Heart · Protein kinase C · Mitochondria · Cardioprotection · PKC delta up-regulation

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Introduction

It has been shown that adaptation of rats to chronic intermittent hypoxia (CIH) protects their hearts against acute ischemia–reperfusion injury. This long-lasting protective phenomenon manifests itself as a reduction in infarct size, limitation of ventricular arrhythmias, and improved recovery of contractile function [1]. Although the cardioprotective effects of CIH have been known for half a century, and several signaling pathways have been proposed to play a role [1], the detailed mechanism of improved cardiac ischemic tolerance induced by CIH still remains to be elucidated.

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In analogy to short-lived protection induced by preconditioning, we and others have shown that, for example, protein kinase C (PKC) [2, 3], phosphatidylinositol 3-kinase/Akt pathway [4], mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channels [5, 6], and reactive oxygen species (ROS) [7] are involved in the cardioprotective mechanism of CIH, although the precise interactions among these components are unclear. A link among PKC, especially the PKC-ε isoform, ROS, and mitoK_{ATP} channels has been postulated in protection induced by preconditioning [8, 9].

Whereas numerous studies have implicated individual PKC isoforms in cardioprotection induced by preconditioning, data concerning their potential role in the cardioprotective mechanism of CIH are rather limited and controversial probably due to different animal and hypoxia models [10]. In line with other reports [3, 11], the involvement of PKC in cardioprotection by chronic hypoxia is supported by the observation that chelerythrine, a general inhibitor of PKC, completely abolished the infarct size-limiting effect of CIH [2]. Several studies reported enhanced expression and/or redistribution of PKC-ε to particulate fraction under conditions of chronic hypoxia [3, 11] but our recent experiments have shown that redox-sensitive PKC-δ was up-regulated, and PKC-ε was either not affected or even moderately down-regulated by CIH in rat myocardium [2, 7, 12]. Moreover, the infarct size of rats adapted to CIH exhibited close negative correlation with PKC-δ up-regulation in the myocardial particulate fraction [12]. Chronic antioxidant treatment during the hypoxic adaptation period abolished both PKC-δ up-regulation and cardioprotective effect, indicating that these events are critically dependent on increased production of ROS associated with adaptation to CIH [7]. The involvement of PKC-δ in cardioprotection induced by CIH is further supported by our observation that rottlerin, a PKC-δ inhibitor, administered before the ischemic period, significantly attenuated the infarct size-limiting effect of CIH [2]. While these results have brought indirect evidence for the role of PKC, especially PKC-δ, in the cardioprotective mechanism of CIH, the precise localization of PKC isoforms in cellular compartments of CIH myocardium and its relevance to the improved ischemic tolerance remains to be elucidated.

The aim of this study, therefore, was to further investigate the potential involvement of PKC-δ and PKC-ε in the cardiac adaptation to CIH. We analyzed the effect of CIH on the myocardial level of total and phosphorylated forms of PKC-δ and PKC-ε. Furthermore, we studied effect of rottlerin on myocardial PKC-δ level and its detailed subcellular distribution after adaptation of rats to CIH by means of both Western blot analysis and quantitative immunofluorescence microscopy. We used the same experimental protocol under which the inhibitory effect of rottlerin on myocardial ischemic tolerance was observed [2]. Our results suggest that mitochondrial and sarcolemmal membranes are the major targets of PKC-δ in CIH-adapted hearts and support the involvement of this isoform in the cardioprotective mechanism.

Materials and methods

Animals

Adult male Wistar rats (250–280 g) were exposed to intermittent high-altitude hypoxia of 7,000 m in a hypobaric chamber for 8 h/day, 5 days a week (24–32 exposures). Barometric pressure (P_B) was lowered stepwise, so that the level equivalent to an altitude of 7,000 m ($P_B = 40.9$ kPa; $P_{O_2} = 8.5$ kPa) was reached after 13 exposures [7]. The animals were employed the day after the last hypoxic exposure. The control group of rats was kept for the same period of time at P_B and P_{O_2} equivalent to an altitude of 200 m ($P_B = 99$ kPa; $P_{O_2} = 20.7$ kPa). All animals had free access to water and a standard laboratory diet.

Animals were anesthetised with sodium pentobarbital (60 mg/kg ip, Sanofi, Montpellier, France). Rottlerin (Biomol, Plymouth Meeting, PA) was dissolved in DMSO and then diluted with saline and administered into the jugular vein in a dose of 0.3 mg/kg as a single bolus (1 ml/kg) 15 min before the excision of the heart; the final dose of DMSO was 6 µl/kg [2]. Controls were given saline (1 ml/kg) with DMSO. The animals were killed by decapitation, and their hearts were rapidly excised and perfused with Krebs–Henseleit solution containing 2,3-butanedione monoxime (20 mmol/l). Apex and the remaining part of the left ventricular (LV) free wall were dissected, weighed, snap-frozen in liquid nitrogen and stored at liquid nitrogen until use. The study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Experimental protocols were approved by the Animal Care and Use Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic. All the chemical compounds were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

Tissue fractionation and Western blot analysis

Frozen LV myocardium was pulverized at the temperature of liquid nitrogen, followed by Potter–Elvehjem homogenization as described previously [7]. The homogenization buffer for analyses of phosphorylated PKC isoforms contained phosphatases inhibitor sodium orthovanadate

(0.1 mM) and glycerol-3-phosphate (10 mM) in addition. The homogenate was centrifuged to obtain particulate (all membranes) fraction (100,000×*g* for 60 min) or nuclear–cytoskeletal-enriched (Nucl) (1,000×*g* for 10 min), mitochondria-enriched (Mito) (8,000×*g* for 10 min), microsomal (Micro) (100,000×*g* for 60 min) and cytosolic (Cyto) fractions [2, 7]. The homogenate and pellets of all fractions were re-suspended either in a homogenization buffer (non-extracted fractions; oxidative phosphorylation (OXPHOS) complex analysis) or in a homogenization buffer containing 1% Triton X-100, held on ice for 60 min and then centrifuged at 100,000×*g* for further 60 min (TX-extracted fractions; PKC analysis). Triton X-100 was also added to the cytosolic fraction to reach the final concentration of 1%.

Proteins from both TX-extracted and non-extracted fractions were separated by electrophoresis (10 or 15% SDS-PAGE) and transferred to nitrocellulose membranes (Amersham Biosciences, Freiburg, Germany). Equal protein transfer efficiency was verified by staining of membranes with Ponceau S. After blocking with 5% dry low-fat milk in Tris-buffered saline with Tween 20 (TTBS) for 60 min at room temperature, membranes were washed and probed (90 min at room temperature) with polyclonal antibodies against PKC-δ (662–673) and PKC-ε (728–737) (Research & Diagnostic Antibodies, Benicia, CA) or (overnight at 4°C) phosphorylated form of PKC-δ (p-PKC-δ) (Ser643) (Cell Signaling Technology, Beverly, MA) and p-PKC-ε (Ser729) (Upstate, Billerica, MA). After the membranes were washed with TTBS, they were incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase for 60 min at room temperature. Bands were visualized by enhanced chemiluminescence on the autoradiographic film (Amersham Biosciences), and ImageQuant software was used for quantification of the relative abundance of PKC isoforms and OXPHOS complexes. In order to ensure the specificity of PKC-δ and PKC-ε-immunoreactive proteins, prestained molar-mass protein standards, recombinant human PKC-δ and PKC-ε standards, rat brain extract, and the blocking immunizing peptides were used. In order to ensure the specificity of OXPHOS complexes-immunoreactive protein, mouse brain mitochondria (MitoSciences, Eugene, OR) were used as a positive control. From each group, one sample was run on the same gel and quantified on the same membrane. Sources for other antisera were as follows: lamin, GAPDH and Na,K-ATPase (Abcam, Cambridge, MA), the kit of monoclonal antibodies against five OXPHOS complexes from rodent mitochondria (MitoSciences).

Quantitative immunofluorescence microscopy

The subcellular redistribution of PKC-δ induced by CIH, the effect of rottlerin and the effect of CIH on myocardial nitrotyrosine content were investigated by immunofluorescent staining followed by digital imaging fluorescence microscopy. LV apex cross cryo-sections (5 μm) were incubated with a primary antibody raised against rat PKC-δ (662–673) or nitrotyrosine (Molecular Probes, Eugene, OR) and counterstained for nuclei with 4′,6-diamidino-2-phenylindole (DAPI)-containing mounting medium (Vectashield; Vector Laboratories, Burlingame, CA) and either for the sarcolemma with 10% (vol/vol) wheat-germ agglutinin (WGA; Molecular Probes) or for mitochondria with anti-OXPHOS complexes. They were then incubated with the appropriate Alexa Fluor secondary antibody conjugate (Molecular Probes) and visualized by immunofluorescence microscopy, as described by Bouwman et al. [13]. Sections were qualitatively and quantitatively analyzed with the use of imaging and analysis software (SlideBook^{TM}, version 4.1). Regions of interest from the digital images of the LV cross cryo-sections were selected in a process termed masking, a mask being a binary overlay on a digital image. Masks of segments of cross cryo-sections were created by automatically selecting standardized fluorescence thresholds of the counterstains (either sarcolemmal glycocalyx or mitochondrial OXPHOS complexes). Next, the mean intensities of fluorescence of PKC-δ or nitrotyrosine within these masks were calculated using SlideBook^{TM} imaging software. Pearson's correlation factors between channels of multiple regions of interest from at least three digital images per experimental group were derived using the SlightBook^{TM} co-localization tab.

Statistical analysis

The results are expressed as means ± SE. A one-way ANOVA and subsequent Student–Newman–Keuls test were used for comparison of differences in normally distributed variables between groups. Differences were considered as statistically significant when $P < 0.05$.

Results

Purity of subcellular fractions, distribution of OXPHOS complexes

Before PKC analysis in TX-extracted fractions, we attempted to determine the level of non-extracted subcellular fractions purity (Fig. 1). GAPDH as cytosolic and lamin as nuclear markers were distinctly detected in the corresponding fractions. Na,K-ATPase as a sarcolemmal membrane marker was detected in all particulate fractions and OXPHOS complexes as a mitochondria marker were present across all fractions analyzed. As for the effect of

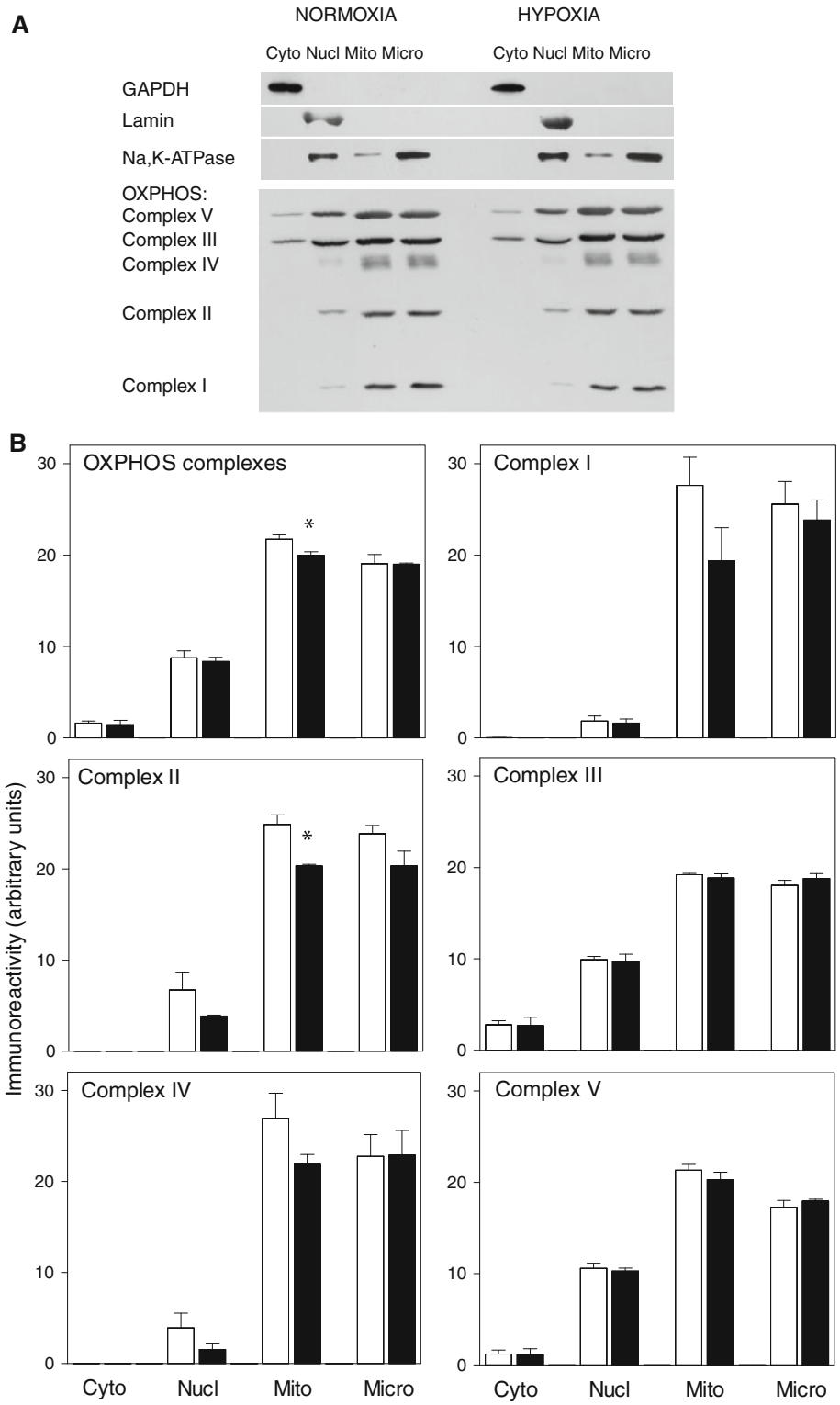

Fig. 1 Western blot analysis of fraction contamination (**a**) and mitochondrial OXPHOS complex distribution (**b**) in individual subcellular fractions. Purity of fractions was analyzed using antisera directed against marker proteins localized predominantly in the cytosol (*GAPDH*), nucleus (*lamin*), sarcolemma (*Na,K-ATPase*), and mitochondria [OXPHOS complexes: NDUFB8 (*Complex I*), SDHB (*Complex II*), core protein 2 (*Complex III*), COX I (*Complex IV*), F1α ATP synthase (*Complex V*)]. Cytosolic (*Cyto*), nuclear–cytoskeletal (*Nucl*), mitochondrial (*Mito*), and microsomal (*Micro*) non-extracted fractions from left ventricles of normoxic (*open columns*) and chronically hypoxic (*black columns*) rats were used. The amount of protein applied to the gel was 5 μg for all subcellular fractions. Values are means ± SE from three hearts in each group. * $P < 0.05$ versus the corresponding normoxic group

CIH on OXPHOS complexes, a slight decrease of the total OXPHOS complexes level in the Mito fraction was observed (Fig. 1b, upper left panel); Complexes I, II, and IV contributed to this effect (Fig. 1b, relevant panels).

Western blot analysis of PKC-ε

We have shown previously that CIH either did not influence [2] or moderately down-regulated PKC-ε in

particulate fractions [7, 12]. The possible explanation for this discrepancy is that different homogenization protocol was used and part of tissue was discarded as a debris in the study where no change of the PKC-ε level in CIH hearts was detected [2]. It was shown that, in addition to the choice of detergent and its concentration, the way of homogenization and centrifugation protocol could affect myocardial PKC isoforms distribution [14]. In this study, we confirmed that CIH decreased total level of PKC-ε in LV myocardium. We verified our Western blot results with two different antibodies, the antibody from Sigma and the antibody from Research & Diagnostic (used also for immunofluorescence microscopy analysis) and obtained comparable results. The adaptation to CIH decreased the total level of PKC-ε in homogenate and particulate fraction (by 40 and 37%, respectively) without affecting the level of p-PKC-ε (Ser729) (Fig. 2a, b). The ratio of p-PKC-ε (Ser729) to total PKC-ε was increased by CIH in homogenate and cytosolic fraction; the increase in particulate fraction did not reach statistical significance due to higher variability (Fig. 2c). The more detailed analysis of CIH effect on subcellular fractions revealed the decreased abundance of PKC-ε in the Mito and Micro fractions (Fig. 3).

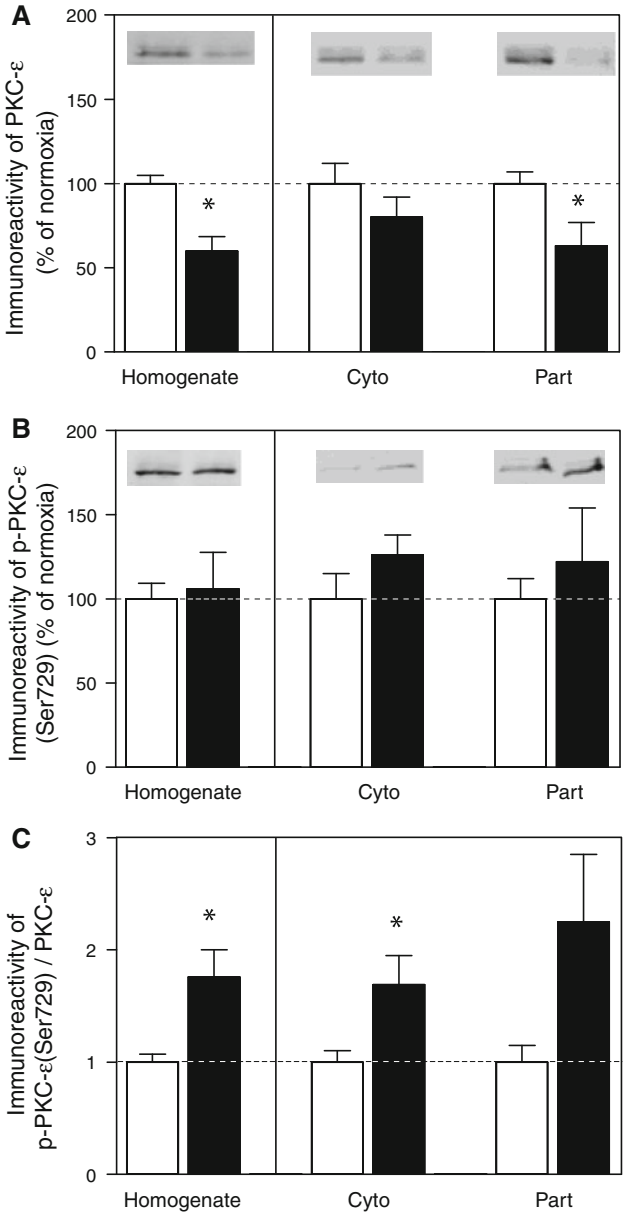

Fig. 2 Effect of CIH on the level of total PKC-ε (**a**), p-PKC-ε (Ser729) (**b**), and p-PKC-ε (Ser729) to total PKC-ε ratio (**c**) in *homogenate*, cytosolic (*Cyto*), and total particulate (*Part*) fractions from left ventricles of normoxic (*open columns*) and chronically hypoxic (*black columns*) rats, including representative Western blots. The amount of protein applied to the gel was 10 μg (*homogenate*), 15 μg (*cytosolic fraction*), or 5 μg (*particulate fraction*) for PKC-ε and 40 μg (*homogenate*), 50 μg (*cytosolic fraction*), or 40 μg (*particulate fraction*) for p-PKC-ε (Ser729). Values are means ± SE from five hearts in each group. * $P < 0.05$ versus corresponding normoxic group

Western blot analysis of PKC-δ

Chronic intermittent hypoxia increased the relative protein content of total PKC-δ as well as the level of p-PKC-δ (Ser643) in homogenate by 190 and 204%, respectively; similar changes were observed in cytosolic and particulate fractions (Fig. 4a, b). The ratio of p-PKC-δ (Ser643) to total PKC-δ was not influenced by CIH (Fig. 4c). Rottlerin had no effect on this isoform content in homogenate from either normoxic or hypoxic hearts (Fig. 5a). The detailed analysis showed that CIH up-regulated PKC-δ protein amount in all subcellular fractions with the highest increase in Mito and Micro fractions (Fig. 5b, c). Interestingly, the acute rottlerin treatment significantly decreased the relative PKC-δ protein content in the Mito fraction in favor of cytosolic and Nucl fractions of CIH-adapted hearts (Fig. 5d).

Immunofluorescence microscopy analysis

Figure 6 shows the PKC-δ redistribution pattern in response to CIH and rottlerin in LV cross cryo-sections. In the normoxic group, a diffuse staining of PKC-δ can be observed. PKC-δ was co-localized with nuclei in normoxic as well as in hypoxic hearts. CIH induced PKC-δ redistribution and increased PKC-δ co-localization with the sarcolemma (Fig. 6a, red WGA sarcolemmal glycocalyx counterstain) as well as with the mitochondria (Fig. 6b, red OXPHOS counterstain) as indicated by the increase in the yellow-orange color.

Figure 6a, c (left panel) show that the CIH-induced redistribution of PKC-δ to the sarcolemma (the mean intensity of fluorescence of PKC-δ in the sarcolemmal

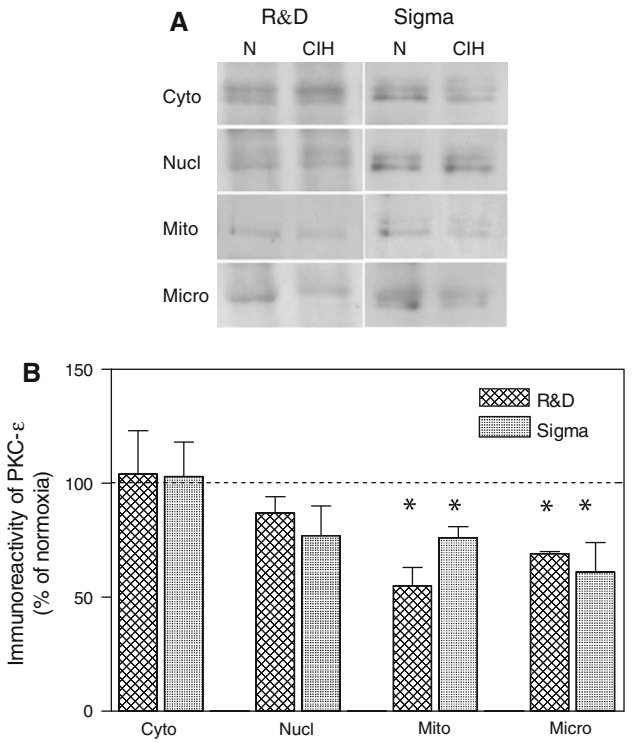

Fig. 3 Representative Western blots (**a**) showing effect of CIH on PKC-ε level in cytosolic (*Cyto*), nuclear–cytoskeletal (*Nucl*), mitochondrial (*Mito*), and microsomal (*Micro*) myocardial fractions analyzed by Research & Diagnostic (R&D) and Sigma antibodies. Quantified data (**b**) showing effect of CIH on PKC-ε level in subcellular fractions expressed as percentage of normoxic values. The amount of protein applied to the gel was 15 μg (*Cyto*), 5 μg (*Nucl*), 10 μg (*Mito*), and 5 μg (*Micro*). Values are means ± SE from four hearts in each group. * $P < 0.05$ versus corresponding normoxic group

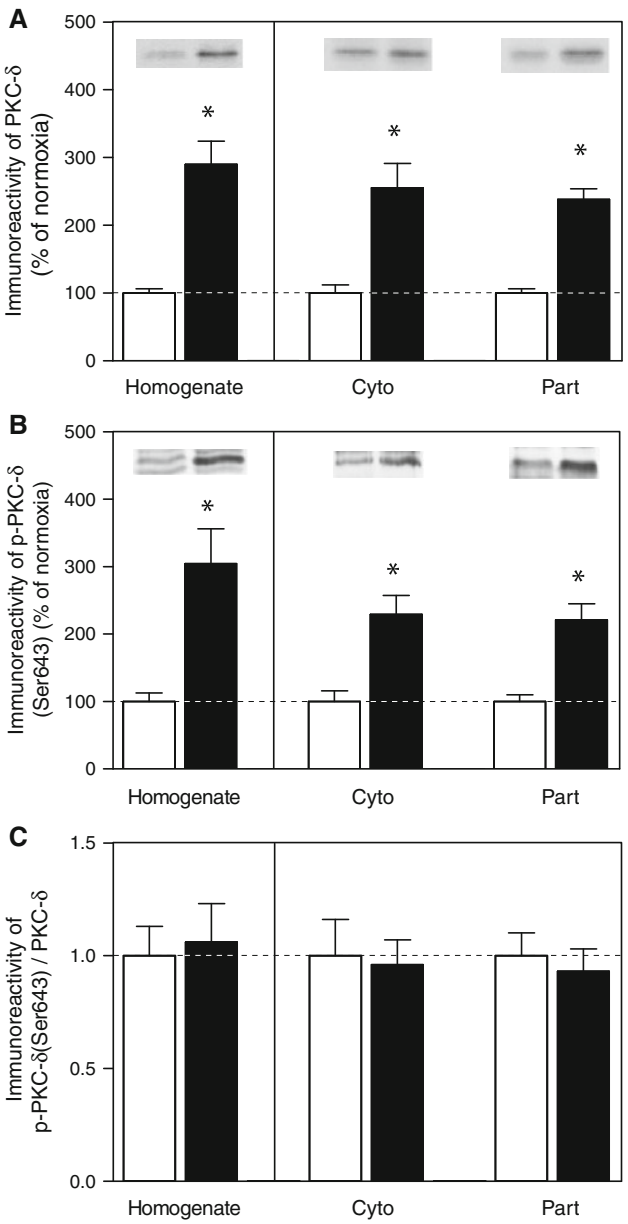

Fig. 4 Effect of CIH on the level of total PKC-δ (**a**), p-PKC-δ (Ser643) (**b**), and p-PKC-δ (Ser643) to total PKC-δ ratio (**c**) in *homogenate*, cytosolic (*Cyto*), and total particulate (*Part*) fractions from left ventricles of normoxic (*open columns*) and chronically hypoxic (*black columns*) rats, including representative Western blot. The amount of protein applied to the gel was 10 μg (*homogenate*), 15 μg (*cytosolic fraction*), or 5 μg (*particulate fraction*) for PKC-δ and 40 μg (*homogenate*), 50 μg (*cytosolic fraction*), or 40 μg (*particulate fraction*) for p-PKC-δ (Ser643). Values are means ± SE from six hearts in each group. * $P < 0.05$ versus corresponding normoxic group

mask was increased by up to 25%) was inhibited by rottlerin (the mean intensity of fluorescence of PKC-δ decreased to the normoxic value). Pearson's correlation factor between the green (PKC-δ) and the red channels (sarcolemmal WGA) was calculated to further quantify CIH-induced PKC-δ co-localization with the sarcolemmal membrane: its mean value in normoxia was 0.31 ± 0.04 and significantly increased in CIH to 0.42 ± 0.03. Rottlerin decreased its value in hypoxic tissue down to 0.27 ± 0.05. ($P < 0.05$; maximum correlation factor is 1).

In Fig. 6b, the PKC-δ co-localization with mitochondria (the red OXPHOS counterstain) is shown. In sections of hearts adapted to CIH, PKC-δ was present as a dotted-like distribution pattern with a yellow-orange color (arrows), which indicates the co-localization with the mitochondria. CIH increased the mean intensity of fluorescence of PKC-δ in the mitochondrial OXPHOS complexes mask by 19% (Fig. 6c, right panel), as well as the Pearson's correlation factor between the green (PKC-δ) and the red (OXPHOS complexes) channels. Its mean value significantly increased from 0.42 ± 0.04 in normoxic to 0.58 ± 0.06 in hypoxic tissue. Rottlerin reduced PKC-δ localization in hypoxic mitochondria, decreasing the mean intensity of PKC-δ fluorescence in the mitochondrial OXPHOS complexes mask to the normoxic control value. Rottlerin did

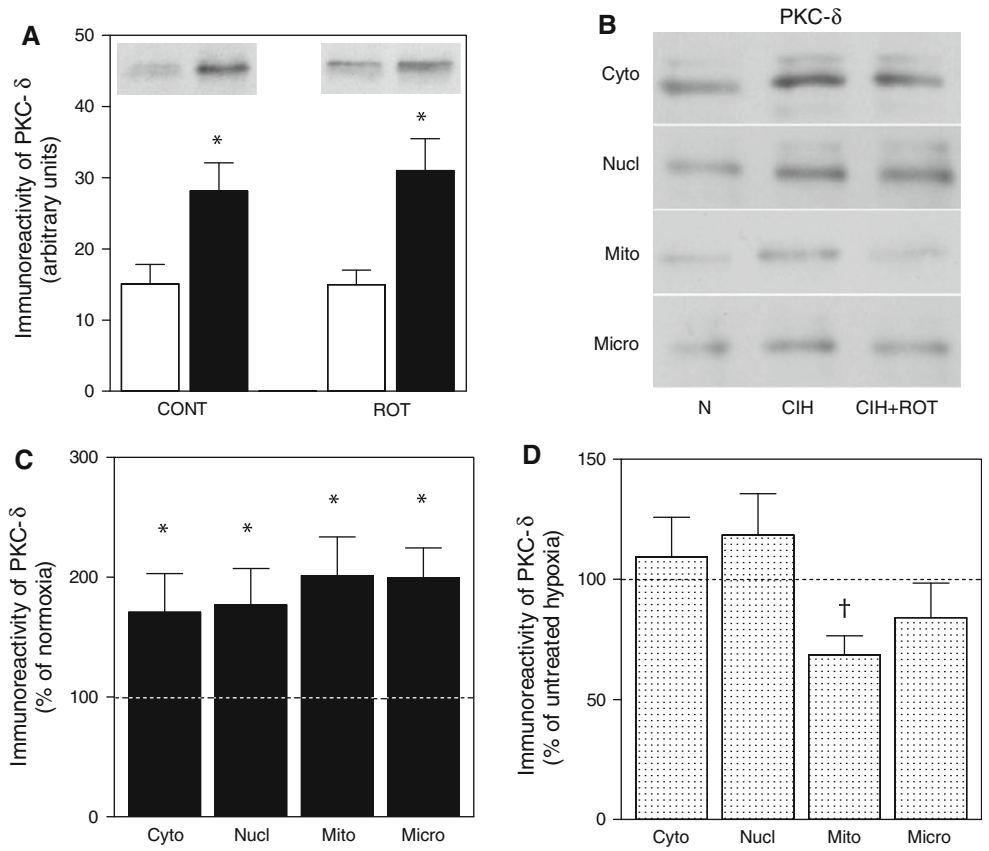

Fig. 5 Effect of CIH and rottlerin on the level of PKC-δ in homogenate from left ventricles of normoxic (*N*; *open columns*) and chronically hypoxic (*CIH*; *black columns*) rats treated by vehicle (*CONT*) or rottlerin (*ROT*), including representative Western blots (**a**). Representative Western blots (**b**) showing effect of CIH and rottlerin (*ROT*) on PKC-δ abundance in cytosolic (*Cyto*), nuclear–cytoskeletal (*Nucl*), mitochondrial (*Mito*), and microsomal (*Micro*) fractions. Quantified data (**c**) show effect of CIH on PKC-δ level in subcellular fractions expressed as percentage of normoxic values. (**d**) Effect of rottlerin on PKC-δ level in subcellular fractions from chronically hypoxic rats expressed as percentage of untreated hypoxic values. The amount of protein applied to the gel was 10 μg (*homogenate*), 15 μg (*Cyto*), 5 μg (*Nucl*), 10 μg (*Mito*), and 5 μg (*Micro*). Values are means ± SE from five hearts in each group. * $P < 0.05$ versus the corresponding normoxic group; † $P < 0.05$ versus the untreated hypoxic group

not significantly affect the PKC-δ distribution in normoxic tissue.

Unlike the PKC-δ staining in normoxic tissue, PKC-ε displayed a dotted-like distribution pattern. Partial PKC-ε co-localization with OXPHOS complexes suggests its presence in the mitochondria. Neither CIH nor rottlerin affected the PKC-ε distribution (data not shown).

Effect of CIH on nitrotyrosine formation

Figure 7a demonstrates CIH-induced formation of nitrotyrosine. CIH led to an increase in protein nitrosylation (the overall intensity of fluorescence of nitrotyrosine was increased by 24% compared to normoxia) and induced protein nitrosylation in mitochondria, nuclei, and sarcolemma (Fig. 7b). The mean Pearson's correlation factor between green (nitrotyrosine) and red channels (OXPHOS complexes) significantly increased from 0.32 ± 0.02 in normoxic tissue to 0.40 ± 0.03 in hypoxic tissue.

Discussion

The major novel finding of this study is that the up-regulation of PKC-δ in LV myocardium of CIH-adapted rats was associated with its enhanced phosphorylation on Ser643 and increased co-localization with markers of mitochondrial and sarcolemmal membranes. Rottlerin, PKC-δ inhibitor, attenuated CIH-induced effects on the expression and subcellular redistribution of PKC-δ. Concerning PKC-ε, the adaptation to CIH decreased its total abundance in myocardium without affecting its distribution and the level of p-PKC-ε (Ser729). The level of total oxidative phosphorylation complexes was slightly decreased and the formation of nitrotyrosine as a marker of oxidative stress was enhanced in CIH myocardium, particularly in mitochondria.

The studies dealing with effects of chronic hypoxia on PKC-ε expression, activity and subcellular distribution are rather controversial, probably due to the diversity of the

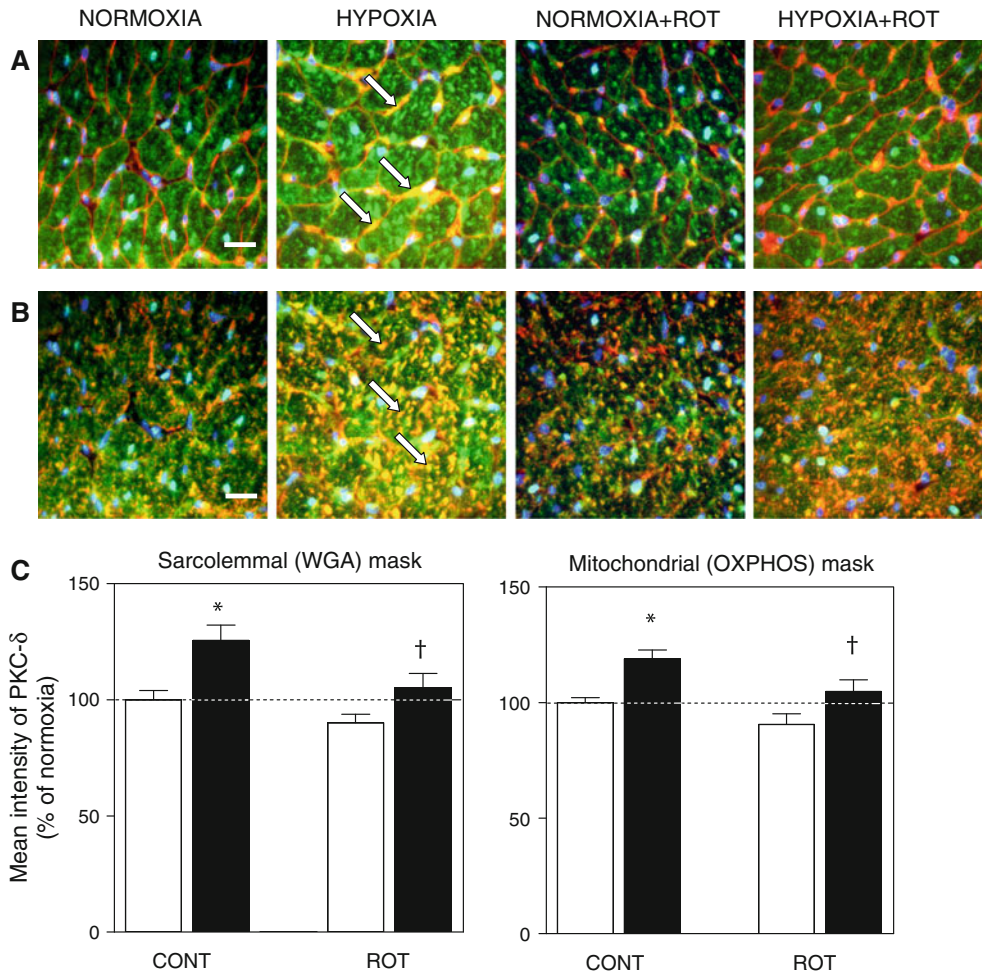

Fig. 6 PKC-δ distribution and co-localization with sarcolemma (**a**) and mitochondria (**b**) in normoxic and chronically hypoxic cross cryo-sections of left ventricles, and the effect of rottlerin (*ROT*). In all panels, *green* represents specific PKC-δ staining and *blue* indicates the nuclear 4′,6-diamidino-2-phenylindole (DAPI) staining. In panels **a**, *red* represents the wheat-germ agglutinin (WGA) staining of the sarcolemmal glycocalyx, and in panels **b**, *red* represents the OXPHOS complexes. Note the increase in the yellow-orange color in both panels, indicating increased CIH-induced co-localization of PKC-δ with sarcolemma and mitochondria (*arrows*). *Bar* represents 20 μm. (**c**) Quantification of the mean intensity of fluorescence of PKC-δ in sarcolemma (WGA staining, *left panel*) and mitochondria (OXPHOS complexes staining, *right panel*) in cryo-sections from normoxic (*open columns*) and chronically hypoxic (*black columns*) rats treated by vehicle (*CONT*) or rottlerin (*ROT*) expressed as percentage of normoxic values. Values are means ± SE from three hearts in each group. * $P < 0.05$ versus the normoxic group; † $P < 0.05$ versus the untreated hypoxic group (See online version for color pictures)

hypoxic models used [3, 10, 15, 16]. In our previous experiments, adaptation to CIH decreased PKC-ε protein amount [7] and similar effect was observed in adult rat myocardium prenatally exposed to hypoxic conditions [17]. It has been reported that PKC-ε down-regulation could be caused by PKC-δ-dependent hydrophobic motif phosphorylation of PKC-ε on Ser729 [18]. Despite the decrease of total PKC-ε content in this study, the level of phosphorylated form of PKC-ε (Ser729) was not influenced by CIH, leading to the increase in p-PKC-ε (Ser729) to total PKC-ε ratio. It suggests that the maintenance of phosphorylated (active) PKC-ε level is important for myocardial adaptation to CIH. An increased phosphorylation and translocation of PKC-ε was detected in hearts of neonatal rabbits under conditions of chronic continuous hypoxia [11]. It is necessary to emphasize that PKC-ε is the key component of signal transduction of various forms of preconditioning [19] and cross-regulation of PKC-ε and PKC-δ function in cardiomyocytes has been well documented [18, 20]. The work of Inagaki and Mochly-Rosen [21] showed that the activation of PKC-δ before ischemic insult led to the activation of PKC-ε and consequently to cardioprotection. Despite the absence of PKC-ε up-regulation or subcellular redistribution by CIH in this study, the fact that the phosphorylated PKC-ε level was preserved does not allow to unequivocally exclude the potential involvement of this isoform in the protective mechanism.

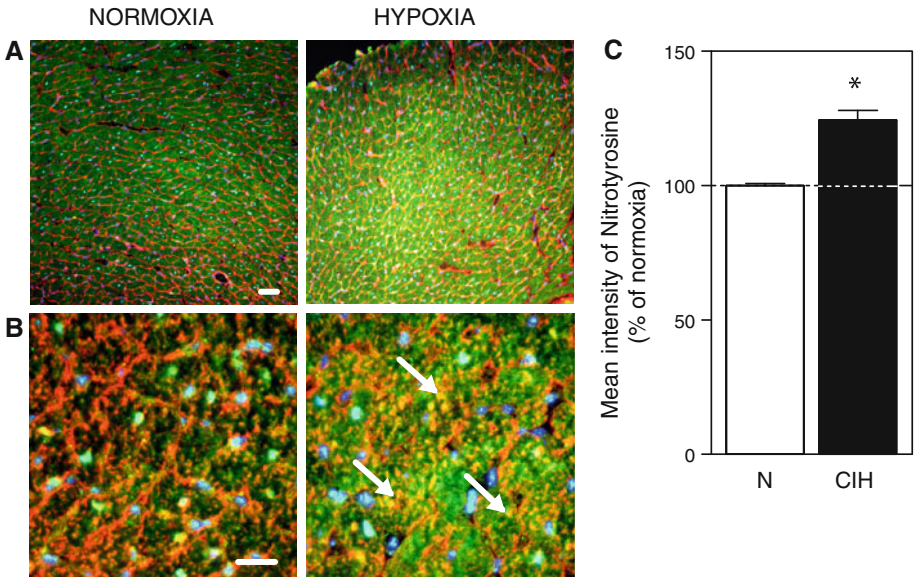

Fig. 7 Nitrotyrosine staining in left ventricular myocardium of normoxic and chronically hypoxic rats. In all panels, *green* indicates specific nitrotyrosine staining, and *blue* represents the nuclear 4′,6-diamidino-2-phenylindole (DAPI) staining. In panels **a**, *red* represents the wheat-germ agglutinin staining of the sarcolemmal glycocalyx, and in panels **b**, *red* represents the OXPHOS complexes. Note the increase in mitochondrial protein nitrosylation in hypoxia compared to normoxia (*arrows*). *Bar* represents 20 μm. (**c**) Quantification of the mean intensity of fluorescence of nitrotyrosine in cryo-sections from normoxic (*N*; *open columns*) and chronically hypoxic (*CIH*; *black columns*) rats expressed as percentage of normoxic values. Values are means ± SE from three hearts in each group. * $P < 0.05$ versus the normoxic group (See online version for color pictures)

Concerning PKC-δ, our observation of its significant up-regulation due to adaptation of rats to CIH corresponds to previous reports using the same [2, 7, 12] or similar [3] experimental models. Furthermore, we showed that CIH increased the phosphorylation of PKC-δ on Ser643 and promoted its increased co-localization with mitochondrial and sarcolemmal membranes. Phosphorylation of PKC-δ on Ser643 and its translocation to mitochondria was also observed in cardioprotection induced by pharmacological preconditioning [22]. The beneficial role of PKC-δ associated with its translocation to mitochondria and/or sarcolemma was confirmed in a variety of experiments on ischemic and pharmacological preconditioning [13, 22–25]. These protective effects of PKC-δ were ROS-dependent and related to either mitoK_{ATP} or sarcolemmal K_{ATP} (sarcK_{ATP}) channels activation. Similarly to preconditioning, the participation of sarcK_{ATP} and/or mitoK_{ATP} channels in increased ischemic tolerance of chronically hypoxic hearts was reported [5, 6, 26]. In addition, it was shown that PKC-δ translocation to sarcolemmal membrane is connected with the Na^+/Ca^{2+} exchanger-dependent cardioprotection [25]. Therefore, it can be speculated that increased co-localization of PKC-δ with sarcolemmal membrane could also play a role in CIH-induced myocardial protection.

Konishi et al. [27] reported that PKC-δ is a redox-sensitive enzyme, and ROS can modulate its function *via* tyrosine phosphorylation. This study demonstrated increased oxidative/nitrosative stress in CIH hearts by enhanced nitrotyrosine formation. Tyrosine nitration leads to the generation of antigenic epitopes on proteins, the changes in catalytic activity of enzymes [28] and could also influence their translocation, as shown, e.g., for PKC-ε [29]. Concerning the role of PKC-δ and ROS in CIH-induced cardioprotection, our recent study showed that chronic antioxidant treatment during the adaptation period eliminated both PKC-δ up-regulation and infarct size reduction, suggesting ROS-dependence of both events [7].

The observation that PKC-δ up-regulation in CIH hearts was co-localized with mitochondria prompted us to analyze OXPHOS complexes in detail. OXPHOS complexes were distributed across all subcellular fractions, though with different abundance. The OXPHOS distribution in fractions is in line with the study of Guo et al. [30], who measured immunoreactivity and activity of cytochrome *c* oxidase in the rat heart using similar homogenization and fractionation protocol. Mito and Micro fractions exhibited the highest OXPHOS enrichment. The later fraction is considered to contain mitochondrial fragments in addition to other membranes, such as sarcolemma and sarcoplasmic reticulum. The relatively high abundance of OXPHOS complexes in the Nucl fraction, which contains mainly broken cell debris, nuclei, cytoskeletal, and myofibrillar proteins, probably reflects the presence of interfibrillar mitochondria [31]. Our observation of the decrease in OXPHOS complexes is in agreement with our previous

study where CIH reduced the concentration of myocardial cardiolipin [32], a mitochondrial inner membrane lipid marker. Less severe chronic continuous hypoxia (5,000 m, 21 days) caused a global decrease in all OXPHOS complex activities [33]. Furthermore, decreased LV mitochondrial respiratory capacity in rats adapted to chronic continuous hypoxia (11% O_2, 4 weeks) was observed [34]. On the other hand, an increase in the activity of OXPHOS complexes I and III was found in the hearts of rats adapted to long-lasting high-altitude hypoxia (4,340 m, 84 days) [35]. As for the relation between PKC-ε and OXPHOS complexes, an interaction of PKC-ε with cytochrome *c* oxidase subunit IV associated with improved cytochrome *c* oxidase activity was documented in preconditioned myocardium [30, 36]. In agreement with these results, we observed a tendency to CIH-induced attenuation of OXPHOS complex IV and the down-regulation of PKC-ε in fractions with the highest OXPHOS enrichment. Concerning PKC-δ, the d-subunit of F_1F_0 ATPase was shown as its possible target in mitochondria. Enhanced PKC-δ expression in cardiac mitochondria and its co-immunoprecipitation with the d-subunit of F_1F_0 ATPase have suggested that this putative interaction mediates inhibition of F_1F_0 ATPase or ATP synthase activities during prolonged hypoxia [37]. In addition, Mayr et al. [38] postulated the necessity of PKC-δ presence for a protective shift from aerobic to anaerobic metabolism induced by ischemic preconditioning. In accordance, it has been shown that PKC-δ inhibits the pyruvate dehydrogenase complex [39], thereby likely contributing to the shift from aerobic to anaerobic metabolism observed in CIH-adapted hearts [40]. Furthermore, a link among PKC-δ, autophagy, and cardioprotection was documented. Autophagy and cardioprotection were abolished in rat hearts perfused with recombinant inhibitor of autophagy Tat-Atg5 (K130R) [41]. Autophagy may be the way for removing damaged mitochondria under CIH conditions, when the decrease in OXPHOS complexes occurs.

The function of PKC-δ in myocardial ischemia–reperfusion injury and its precise involvement in the mechanism of protection is still a matter of debate. One of the reasons of this ambiguity could be the greatest flexibility of PKC-δ among all PKC isoforms to affect various cellular functions because its subcellular localization is finely regulated by phosphorylation at multiple serine/threonine and tyrosine residues [42]. The timing and subcellular location of PKC-δ activation appears to be a critical factor in the manifestation of either protective or detrimental functions of this isoform in the heart. Whereas the activation of PKC-δ during reperfusion leads to a stimulation of pro-apoptotic pathways [43] and exacerbation of myocardial injury [44], the activation of the enzyme well before an ischemic insult is cardioprotective [21]. The advanced up-regulation of PKC-δ in cellular membranes, as an important prerequisite of its protective action, is well fulfilled under our CIH conditions. Mayr et al. [45] evidenced PKC-δ involvement in the preconditioning-induced cardioprotection using PKC-δ-null mice. In another study, they also showed that inhibition of PKC-ε resulted in compensatory phosphorylation and mitochondrial translocation of PKC-δ, providing a possible explanation for the synergy of PKC-δ and PKC-ε in cardioprotection [46]. This phenomenon can play a role under CIH conditions where PKC-δ was up-regulated and redistributed to mitochondria while PKC-ε was either not affected or even moderately down-regulated in rat myocardium [2, 7, 12].

In this study, we observed that rottlerin, a PKC-δ inhibitor, reversed CIH-induced PKC-δ redistribution to the Mito fraction without affecting the total protein amount of PKC-δ in homogenate. It was shown that acute rottlerin administration before preconditioning inhibited PKC-δ translocation to mitochondria [47] and nuclei [22]. Moreover, it was documented that ATP-competitive inhibitors, where rottlerin belongs to [48], affect redistribution of DAG-sensitive PKC isoforms (e.g., PKC-δ) by altering their DAG sensitivity perhaps by disrupting their closed conformation [49]. Although PKC-δ is inhibited by rottlerin more effectively than other PKC isoforms [48], it is necessary to consider possible non-specific effects of this compound [50]. Nevertheless, our immunofluorescence microscopy analysis revealed that rottlerin treatment partially reversed CIH-induced PKC-δ co-localization with the sarcolemma and mitochondria. These results are in line with the inhibitory effect of rottlerin on the improved ischemic tolerance of CIH-adapted rat hearts [2] and support the view that PKC-δ plays a role in the cardioprotective mechanism of CIH. However, we are aware that these results need to be verified by using more selective PKC inhibitors and searching for possible PKC-δ targets in sarcolemmal and mitochondrial membranes. The involvement of other PKC isoforms in cardioprotective effect of CIH cannot be excluded in view of the complete inhibition of cardioprotection by chelerythrine [2].

We conclude that the increase of PKC-δ expression in CIH-adapted rat hearts is localized mainly to mitochondrial and sarcolemmal membranes. CIH-induced PKC-δ subcellular redistribution was reversed by acute treatment with its inhibitor, rottlerin. Our results are in line with the view that PKC-δ mediates the cardioprotective effect of chronic hypoxia, probably via its mitochondrial and sarcolemmal target(s).

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SUPPLEMENT 2

Dietary Polyunsaturated Fatty Acids Alter Myocardial Protein Kinase C Expression and Affect Cardioprotection Induced by Chronic Hypoxia

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We examined the influence of dietary fatty acid (FA) classes on the expression of protein kinase C (PKC) δ and ε in relation to the cardioprotective effects of chronic intermittent hypoxia (CIH). Adult male Wistar rats were fed a nonfat diet enriched with 10% lard (saturated FA [SFA]), fish oil (n-3 polyunsaturated FA [n-3 PUFA]), or corn oil (n-6 PUFA) for 10 weeks. After 4 weeks on the diet, each group was divided into two subgroups that were either exposed to CIH in a barochamber (7000 m, 8 hrs/day) or kept at normoxia for an additional 5–6 weeks. A FA phospholipid profile and Western blot analysis of PKC were performed in left ventricles. Infarct size was assessed in anesthetized animals subjected to 20-min coronary artery occlusion and 3-hr reperfusion. CIH decreased the n-6/n-3 PUFA ratio in all groups by 23% independently of the initial value set by various diets. The combination of n-3 diet and CIH had a stronger antiarrhythmic effect during reperfusion than the n-3 diet alone; this effect was less pronounced in rats fed the n-6 diet. The normoxic n-6 group exhibited smaller infarctions (by 22%) than the n-3 group. CIH decreased the infarct size in n-3 and SFA groups (by 20% and 23%, respectively) but not in the n-6 group. Unlike PKCε, the abundance of PKCδ in the myocardial particulate fraction was increased by CIH except for the n-6 group. Myocardial infarct size was negatively correlated ($r = -0.79$) with the abundance of PKCδ in the particulate fraction. We conclude that lipid diets modify the infarct size–limiting effect of CIH by a mechanism that involves the PKCδ-dependent pathway. Exp Biol Med 232:823–832, 2007

Key words: chronic hypoxia; ischemia; infarction; protein kinase C; polyunsaturated fatty acids

Introduction

It is well known that the fatty acid (FA) composition in the diet modulates the tolerance of heart to ischemia/reperfusion injury. Both clinical and experimental studies have demonstrated that saturated FA (SFA) exhibit detrimental effects on ischemic hearts, unlike polyunsaturated FA (PUFA), which are believed to be protective (1, 2). It has been shown in various animal species that long-lasting intake of food enriched with n-3 or n-6 PUFA improves cardiac tolerance to acute ischemia/reperfusion injury (3–9). Beneficial effects of n-3 PUFA have also been reported by some clinical studies that demonstrated reduction of mortality related to coronary heart disease without decreasing the incidence of coronary events in patients consuming increased amounts of n-3 PUFA (10).

Improved ischemic tolerance in the heart can also be achieved by adaptation to chronic intermittent hypoxia (CIH) (11, 12). The cardioprotective effects of CIH persist for several weeks after the termination of adaptation (13–15), which is much longer than the protection induced by n-3 PUFA supplementation (7) or various forms of preconditioning (16). CIH induces a variety of adaptive changes in the myocardium that can be considered beneficial in terms of ischemic tolerance. The molecular mechanism of

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Table 1. FA Composition (Mole Percent) of the Diet Lipids in the SFA, n-3, and n-6 Groups[ab]

	SFA	n-3	n-6
14:0	1.40	5.66	0.58
16:0	24.15	17.86	12.28
16:1n-7	2.48	7.71	0.93
18:0	10.87	2.13	1.81
18:1n-9	35.50	16.48	24.70
18:1n-7	2.49	3.44	0.95
18:2n-6	19.03	15.38	53.16
18:3n-3	1.62	2.61	2.31
20:1n-9	0.96	7.31	1.03
20:5n-3	0.18	9.94	N.D.
22:5n-3	0.07	1.78	N.D.
22:6n-3	0.21	8.04	N.D.
Others	1.04	1.66	2.25
ΣSFA	36.42	25.65	14.67
ΣMUFA	41.43	34.94	27.61
Σn-6 PUFA	19.30	15.38	53.16
Σn-3 PUFA	2.08	22.70	2.31

[a] Values are means of two separate analyses.
[b] N.D., not detected; ΣSFA, total SFA; ΣMUFA, total MUFA; Σn-3 PUFA, total n-3 PUFA; Σn-6 PUFA, total n-6 PUFA.

protection by CIH is poorly understood, although several signaling pathways have been proposed to play a role (11). Our recent studies suggested that the cardioprotection afforded by CIH in rats involves protein kinase C (PKC), in particular isoform δ (17–19). Concerning lipids, Jezkova *et al*. (20) showed that repeated hypoxic exposure altered the FA profile, decreased the n-6:n-3 PUFA ratio, and increased the unsaturation index in rat heart phospholipids (PL). A similar shift of FA composition in myocardial PL occurred in animals fed a diet enriched with n-3 PUFA (6, 9, 21). It was assumed that the changes in myocardial PL FA composition induced by CIH may contribute to improved ischemic tolerance (20). Thus, the primary goal of this study was to find out whether an altered FA profile plays a role in the cardioprotective mechanism of CIH in a PKC-dependent manner. We compared the effects of diets enriched with SFA, n-3 PUFA, or n-6 PUFA on FA composition of myocardial PL, the expression and distribution of PKC isoforms δ and ε between cytosolic and particulate fractions, and the susceptibility of normoxic and chronically hypoxic rat hearts to acute ischemia/reperfusion injury induced by coronary artery occlusion. These experiments revealed that lipid diet composition is important for the manifestation of the infarct size–limiting mechanism of CIH, which involves the PKCδ-dependent pathway while the hypoxia-induced changes in n-3 and n-6 PUFA proportion in membrane PL seem unlikely to play a direct role.

Materials and Methods

Animal Model. Adult male Wistar rats (250–280 g) were fed a nonfat diet based on standard ST1 (Velaz, Prague, Czech Republic) enriched with 10% lard (SFA), fish oil (n-3 PUFA; Lehmann & Voss, Hamburg, Germany) or corn oil (n-6 PUFA; Olmühle GmbH, Bruck, Austria) for 10 weeks. The FA composition of the dietary oils is shown in Table 1. After 4 weeks on the diet, each group was divided into two subgroups that were either exposed to intermittent hypobaric hypoxia of 7000 m for 8 hrs/day 5 days/week or kept at normoxia for an additional 5–6 weeks. Barometric pressure (P_B) was lowered stepwise so that the level equivalent to an altitude of 7000 m ($P_B = 308$ mm Hg, 41 kPa; $P_{O_2} = 65$ mm Hg, 8.6 kPa) was reached after 13 exposures; the total number of exposures was 24–30 (22). The control normoxic subgroups of animals were kept for the same period of time at P_B and P_{O_2} equivalent to an altitude of 200 m ($P_B = 742$ mm Hg, 99 kPa; $P_{O_2} = 155$ mm Hg, 20.7 kPa). All animals had free access to water. The study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996).

All animal experiments commenced the day after the last hypoxic exposure. Rats to be used for biochemical analyses were anesthetized with sodium pentobarbital (60 mg/kg body wt ip; Sanofi, Montpellier, France). The right ventricle (RV) was catheterized *via* the jugular vein, and RV pressure was measured with a Gould P23Gb transducer (Statham, Hato Rey, Puerto Rico). Thereafter, hearts were rapidly excised, washed in cold (0°C) saline, and dissected into RV and left ventricle (LV) free walls and the septum. All parts were weighed, and the LVs were frozen and stored in liquid nitrogen. All the chemical compounds were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

Analysis of FA Composition. PL and their FA composition were analyzed as described previously (20). Briefly, frozen LV myocardium was pulverized and homogenized. Lipids were extracted in three consecutive steps according to the modified method of Folch *et al*. (23). The first extraction was performed in three portions of chloroform-methanol mixture (1:3, 2:1, and 2:1) in a chilled mortar. Subsequent extractions were performed in the 2:1 mixture. Saline (20% volume of extract) was added, and after vigorous shaking the lower lipid layer was dried at 40°C under a stream of nitrogen. Total PL were separated by one-dimensional thin-layer chromatography (0.5-mm silica gel H; Merck, Darmstadt, Germany) using the solvent mixture hexane-ether-acetic acid (80:20:3). Spots were observed under ultraviolet light after staining with 0.005% 2,7-dichlorofluorescein, scraped out, and stored in a nitrogen atmosphere at –20°C until the next day when methyl esters were prepared. FA methyl esters were separated with a gas chromatograph (CP 438 A; Chrompack, Middelburg, The Netherlands).

PKC Analysis. Myocardial samples were pulverized to a fine powder, followed by Potter-Elvehjem homogenization in eight volumes of ice-cold buffer composed of (in m*M*): 12.5 Tris-HCl (pH 7.4), 250 sucrose, 2.5 EGTA, 1

EDTA, 100 NaF, 5 dithiothreitol, 0.3 phenylmethylsulfonyl fluoride, 0.2 leupeptin, and 0.02 aprotinin. The homogenate was centrifuged at 100,000 *g* for 90 mins. The resulting pellet represented the particulate fraction; the supernatant was the cytosolic fraction. The pellet of particulate fraction was resuspended in homogenization buffer containing 1% Triton X-100, held on ice for 90 mins, and then centrifuged at 100,000 *g* for a further 90 mins. The resulting detergent-treated supernatant was used for immunoblot analyses. Triton X-100 was added to the cytosolic fraction to reach a final concentration of 1%. Protein content was determined according to the Lowry assay modified by Peterson (24). Detergent-treated extracts of the subcellular fractions were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on an 8% bis-acrylamide gel at 20 mA/gel for 90 mins on a Mini-Protean III apparatus (Bio-Rad, Hercules, CA); the resolved proteins were transferred to a nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany). Equal protein transfer efficiency was verified by staining with Ponceau S. After blocking with 5% dry low-fat milk in Tris-buffered saline with Tween 20 (TTBS) for 60 mins at room temperature, membranes were washed and probed with PKCδ- or PKCε-specific polyclonal primary rabbit antisera (1:8000 in TTBS) for 90 mins at room temperature. Anti-PKCδ was elicited against a synthetic peptide corresponding to the C-terminal variable (V5) region (amino acids 662–673) of rat PKCδ, and anti-PKCε was elicited against a synthetic peptide corresponding to the C-terminal variable (V5) region (amino acids 726–737) of PKCε. The membranes were washed again and incubated with secondary swine antirabbit IgG antibody labeled with horseradish peroxidase (1:4000 in TTBS; Sevapharma, Prague, Czech Republic) for 60 mins at room temperature. Before enhanced chemiluminescence (ECL), the membranes were washed and stored in TTBS for at least 2 hrs. For ECL, substrates A (luminol solution) and B (H_2O_2 solution) were prepared, mixed 1:1 just before use, and poured on the membranes. The specific signal was detected on the autoradiographic film (Amersham Biosciences). Scanning (Perfection 1240U Scanner; Epson, Meerbusch, Germany) and ImageQuant software were used for quantification of the relative abundance of individual PKC isoforms. To ensure the specificity of PKCδ- and PKCε-immunoreactive proteins, prestained molar-mass protein standards (Fluka, Buchs, Switzerland), recombinant human PKCδ and PKCε standards, rat brain extract, and the respective blocking immunizing peptides were used (18). From each group, one sample was run on the same gel and quantified on the same membrane. The amounts of protein applied to the gel was 15 μg (cytosolic fraction) and 5 μg (particulate fraction).

Infarct Size Determination and Analysis of Arrhythmias. Animals were subjected to acute myocardial ischemia/reperfusion as described previously (22). Anesthetized rats (as above) were ventilated (Columbus Instruments, Columbus, OH) *via* tracheal cannulas with room air at 68 strokes/min (tidal volume of 1.2 ml/100 g body wt). Both blood pressure in the left carotid artery and a single-lead electrocardiogram (ECG) were continually recorded and subsequently analyzed by our custom-designed software. The animals' temperatures (rectal measurements) were maintained between 36.5°C and 37.5°C by a heated table throughout the experiment. Hematocrit in the tail blood was estimated by using the capillary micromethod.

Left thoracotomy was performed and a polyester suture 6/0 (Ethicon, Edinburgh, UK) was placed around the left anterior descending coronary artery about 1–2 mm distal to its origin. After a 10-min stabilization, regional myocardial ischemia was induced by tightening the suture threaded through a polyethylene tube. Characteristic changes in the configuration of the ECG and a transient decrease in blood pressure verified the coronary artery occlusion. After a 20-min occlusion period, the ligature was released and reperfusion of previously ischemic tissue continued.

After 3 hrs of reperfusion, the hearts were arrested with 0.25 mg of verapamil (Krka, Novo Mesto, Slovenia) administered into the jugular vein. The hearts were excised and washed with 20 ml of saline through the cannulated aorta. The area at risk and the infarct size were determined as described earlier (22) by staining with 5% potassium permanganate (Lachema, Brno, Czech Republic) and 1% 2,3,5-triphenyltetrazolium chloride dissolved in 0.1 *M* phosphate buffer (pH 7.4), respectively. The hearts were cut perpendicularly to the LV long axis into slices 1-mm thick and stored overnight in 10% neutral formaldehyde solution. The day after the infarct size staining, the RV free wall was separated and both sides of the LV slices were photographed. The sizes of the infarct area (IA), area at risk (AR), and LV were determined by a computerized planimetric method. The IA was normalized to the AR (IA/AR), and the AR was normalized to the LV (AR/LV).

Severity of ventricular arrhythmias occurring during prolonged ischemic insult and the first 5 mins of reperfusion were assessed according to the Lambeth Conventions (25). Premature ventricular complexes (PVCs) occurring as singles, salvos, or tachycardia (a run of four or more consecutive PVCs) were counted separately. The incidence of ventricular tachycardia (VT) and fibrillation (VF) was also evaluated. VF lasting more than 2 minutes was considered sustained (VFs). The severity of arrhythmias in each individual heart was evaluated by means of a 5-point arrhythmia score: single PVCs were given a score of 1, salvos 2, VT 3, reversible VF 4, and VFs 5. An assigned number corresponded to the most severe type of arrhythmia observed in that heart. Scores were used for group analysis of severity of arrhythmias.

Statistical Analysis. The results are expressed as means ± SEM. Two-way analysis of variance and subsequent Student-Newman-Keul's test were used for comparison of differences in normally distributed variables

Table 2. Weight Parameters, RV Systolic Pressures, and Hematocrits of Normoxic and Chronically Hypoxic Rats Fed SFA, n-3, or n-6 Diet[a]

	n[b]	Body weight, g	LV, mg	RV, mg	Septum, mg	LV/body weight, mg/g	RV/body weight, mg/g	RV systolic pressure, mm Hg	Hematocrit, %
Normoxic									
SFA	9	414 ± 9	420 ± 17	167 ± 4	208 ± 9	1.02 ± 0.06	0.40 ± 0.01	25.5 ± 0.9	48.2 ± 0.6
n-3	9	434 ± 9	457 ± 16	179 ± 6	205 ± 9	1.05 ± 0.05	0.41 ± 0.01	27.2 ± 1.2	47.7 ± 0.6
n-6	9	430 ± 11	468 ± 18	195 ± 9	217 ± 11	1.09 ± 0.05	0.45 ± 0.02	24.4 ± 1.4	48.0 ± 0.4
Hypoxic									
SFA	10	365 ± 9*	460 ± 13	276 ± 13*	216 ± 8	1.26 ± 0.03*	0.76 ± 0.04*	42.8 ± 1.0*	78.0 ± 0.8*
n-3	7	366 ± 10*	517 ± 42	258 ± 11*	244 ± 18	1.42 ± 0.12*	0.70 ± 0.02*	37.2 ± 1.9*§	77.6 ± 0.6*
n-6	9	359 ± 6*	477 ± 23	250 ± 8*	196 ± 7	1.33 ± 0.09*	0.70 ± 0.02*	44.9 ± 2.4*	76.6 ± 0.9*

[a] Values are means ± SEM.
[b] n, number of animals.
* $P < 0.05$ versus corresponding normoxic group; § $P < 0.05$ versus other corresponding diet groups.

among the groups. Differences were considered statistically significant at $P < 0.05$.

Results

Weight Parameters, Hematocrit, and RV Pressures. Adaptation of rats to CIH led to a marked increase in hematocrit and a significant retardation of body growth compared with age-matched normoxic controls in all diet groups. The heart weights increased mainly due to hypertrophy of the right ventricles. The relative weights of the RV (RV/body weight) and LV (LV/body weight) increased in all chronically hypoxic groups by about 75% and 30%, respectively. Lipid diets had no effect on weight parameters and hematocrit in both normoxic and hypoxic rats. CIH raised the RV systolic pressure in all diet groups, but this effect was significantly less pronounced in the n-3 group (by 36%) compared with the SFA (by 67%) and n-6 (by 84%) groups (Table 2).

Table 3. Proportion of Main FA Classes (Mole Percent) in Total Myocardial PL in Normoxic and Chronically Hypoxic Rats Fed SFA, n-3, or n-6 Diet

	Normoxic		
	SFA	n-3	n-6
Normoxic			
ΣSFA	36.2 ± 1.0	36.6 ± 2.0	35.5 ± 1.9
ΣMUFA	7.0 ± 0.2	7.9 ± 0.2§	6.1 ± 0.2§
Σn-6 PUFA	44.1 ± 1.5	33.1 ± 1.0§	49.8 ± 1.8§
Σn-3 PUFA	12.7 ± 0.6	22.3 ± 1.1§	8.6 ± 0.8§
Hypoxic			
ΣSFA	37.3 ± 1.0	37.9 ± 1.6	37.3 ± 1.7
ΣMUFA	7.3 ± 0.3	8.3 ± 0.2§	5.8 ± 0.2§
Σn-6 PUFA	40.4 ± 0.5	28.9 ± 0.6*§	46.7 ± 1.4§
Σn-3 PUFA	15.0 ± 0.9	24.9 ± 1.1§	10.3 ± 0.5§

[a] Values are mean ± SEM from seven hearts in each group.
* $P < 0.05$ versus corresponding normoxic group; § $P < 0.05$ versus other diet groups.

Myocardial FA Composition. Despite different FA compositions of individual lipid diets (Table 1), the proportions of SFA, monounsaturated FA (MUFA), and total PUFA in the myocardial PL did not essentially differ among all normoxic and hypoxic diet groups (Table 3). However, in normoxic myocardium, the proportion of n-3 and n-6 PUFA substantially differed in individual diet groups. The n-3 diet increased the proportion of n-3 PUFA and decreased the proportion of n-6 PUFA compared with the SFA diet. This effect was mainly due to an accumulation of docosahexaenoic acid (DHA; 22:6 n-3, by 66%) at the expense of arachidonic acid (AA; 20:4 n-6, decreased by 47%). The n-6 diet had an opposite effect: it reduced the proportion of n-3 PUFA in favor of n-6 PUFA compared with the SFA diet. The DHA decrease (by 32%) and linoleic acid (LA; 18:2 n-6) increase (by 28%) substantially contributed to this effect. CIH tended to increase the proportion of n-3 PUFA (mainly due to an accumulation of DHA) and decreased that of n-6 PUFA (by a drop of LA) (Table 3). Consequently, the n-6:n-3 PUFA ratio declined by about 23% independent of the diet groups (Fig. 1).

Infarct Size. Table 4 summarizes the values of heart rate and mean arterial blood pressure in all groups, determined at baseline (before ischemia), at the end of test ischemia, and at the end of the 3-hr reperfusion. No significant differences were found in the baseline values of heart rate among the groups; heart rates were significantly lower at the end of reperfusion in each group compared with corresponding baseline values. CIH tended to increase the mean arterial pressure in all groups; significantly higher values were recorded at the end of reperfusion.

The normalized area at risk (AR/LV) did not significantly differ among the groups: its mean values ranged from 40% to 42%. Normoxic rats fed the n-6 diet exhibited significantly smaller infarct areas (43.6% ± 3.2% of the AR) as compared with the normoxic n-3 group (56.1% ± 3.9%). The IA/AR of the normoxic SFA group (49.3% ±

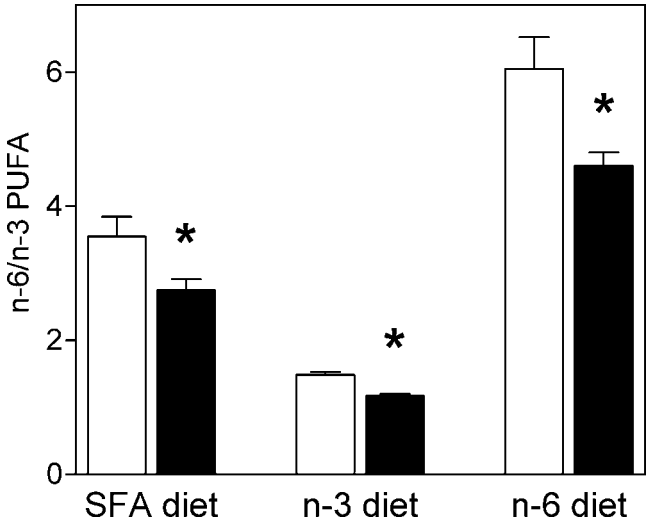

Figure 1. The n-6:n-3 PUFA ratio in total myocardial phospholipids of chronically hypoxic rats (black columns) and normoxic controls (open columns) fed SFA, n-3, or n-6 diet. Values are means ± SEM from seven hearts in each group. *$P < 0.05$ versus corresponding normoxic group.

2.3%) did not significantly differ from that of the other normoxic groups (Fig. 2). Adaptation to CIH had a significant infarct size–limiting effect in the SFA (IA/AR 38.2% ± 2.4%) and n-3 (45.0% ± 1.8%) groups but not in the n-6 group (42.6% ± 2.5%; Fig. 2).

Table 4. Heart Rate and Mean Arterial Blood Pressure After Stabilization (Baseline), at the End of 20-Min Coronary Artery Occlusion and at the End of the 3-Hr Reperfusion in Normoxic and Chronically Hypoxic Rats Fed SFA, n-3, or n-6 Diet[a]

	Baseline	Ischemia	Reperfusion
Heart rate, bpm			
Normoxic			
SFA	456 ± 8	452 ± 9	402 ± 11**
n-3	438 ± 11	439 ± 13	369 ± 11**
n-6	441 ± 12	451 ± 8	389 ± 10**
Hypoxic			
SFA	436 ± 6	431 ± 5	397 ± 8**
n-3	437 ± 6	432 ± 6	398 ± 5**
n-6	420 ± 8	411 ± 9*	384 ± 10**
Blood pressure, mm Hg			
Normoxic			
SFA	103 ± 7.1	104 ± 6.7	97 ± 7.7
n-3	109 ± 7.6	112 ± 7.9	90 ± 7.7
n-6	104 ± 8.4	110 ± 5.1	94 ± 7.2
Hypoxic			
SFA	128 ± 5.5	128 ± 6.1	134 ± 5.6*
n-3	131 ± 2.8	134 ± 3.3	131 ± 4.2*
n-6	121 ± 6.6	128 ± 6.8	127 ± 7.3*

[a] Values are mean ± SEM from 8–12 animals in each group.
* $P < 0.05$ versus corresponding normoxic group; ** $P < 0.05$ versus baseline.

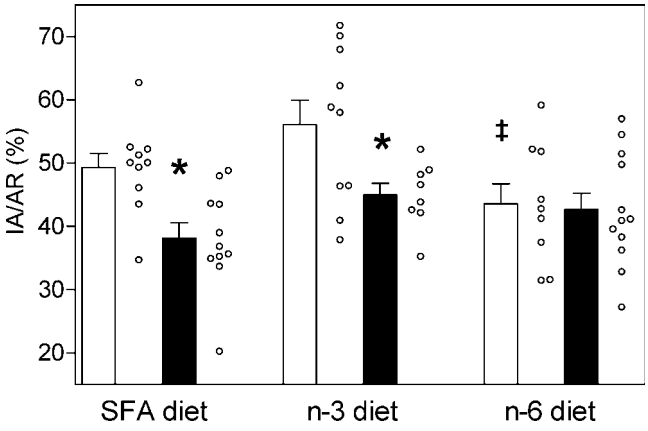

Figure 2. Myocardial IA expressed as a percentage of the AR (IA/AR) in chronically hypoxic rats (black columns) and normoxic controls (open columns) fed SFA, n-3, or n-6 diet. Open circles indicate individual experiments. Values are means ± SEM. *$P < 0.05$ versus corresponding normoxic group; ‡$P < 0.05$ versus corresponding n-3 group.

Ventricular Arrhythmias. Neither lipid diet nor CIH affected the values of ischemic arrhythmia score (AS) (Table 5). Nevertheless, both normoxic and hypoxic groups fed the n-3 diet tended to have decreased severity of ischemic arrhythmias. The value of reperfusion AS in the normoxic n-3 group also tended to exhibit decreases compared with the other diet groups. CIH decreased AS for reperfusion arrhythmias in all groups, but this effect was significant only in rats fed the SFA or n-3 diet. Reperfusion arrhythmias were almost eliminated by a combination of the n-3 diet and CIH (Table 5). In this study, no sustained VF was observed in any group.

Expression and Distribution of PKC Isoforms. As for diet effects, the abundance of PKCδ in the particulate fraction of normoxic animals fed the n-3 diet was lower compared with the corresponding SFA and n-6 groups. CIH increased the relative protein content of PKCδ

Table 5. AS Over 20-Min Coronary Artery Occlusion (Ischemic Arrhythmias) and Over the First 5 Mins of Reperfusion (Reperfusion Arrhythmias) in Normoxic and Chronically Hypoxic Rats Fed SFA, n-3, or n-6 Diet

	n	Ischemic arrhythmias	Reperfusion arrhythmias
Normoxic			
SFA	10	2.80 ± 0.25	2.70 ± 0.21
n-3	10	1.80 ± 0.36	1.80 ± 0.44
n-6	9	2.44 ± 0.29	2.33 ± 0.37
Hypoxic			
SFA	11	2.73 ± 0.45	2.00 ± 0.23*
n-3	8	1.75 ± 0.41	0.13 ± 0.13*§
n-6	12	2.23 ± 0.36	1.38 ± 0.29

[a] Values are mean ± SEM.
* $P < 0.05$ versus corresponding normoxic group; § $P < 0.05$ versus other corresponding diet groups.

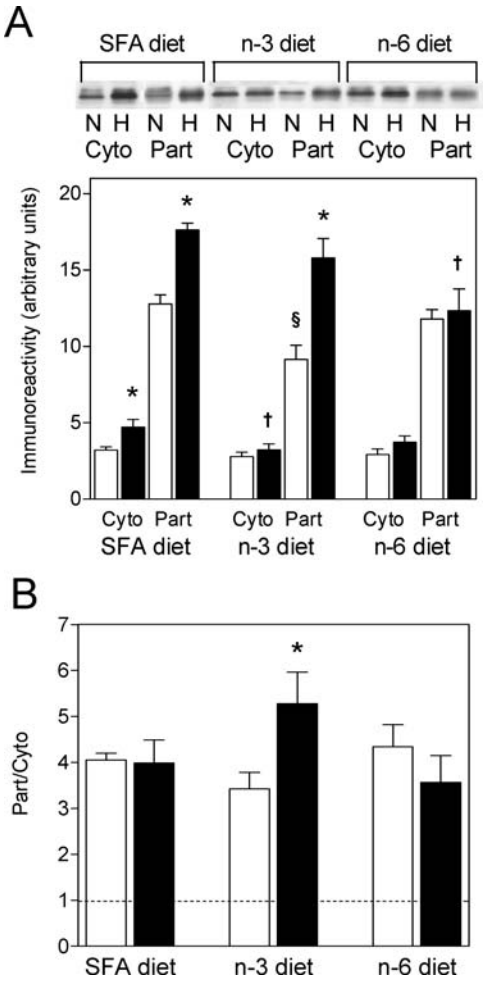

Figure 3. (A) Expression of PKCδ in cytosolic (Cyto) and particulate (Part) fractions and (B) its distribution between the fractions from the myocardium of chronically hypoxic rats (H; black columns) and normoxic controls (N; open columns) fed SFA, n-3, or n-6 diet. All samples compared were electrophoresed on the same gel and quantified on the same membrane. The amount of protein applied to the gel was 15 μg (Cyto) or 5 μg (Part). Values are means ± SEM from six hearts in each group. *$P < 0.05$ versus corresponding normoxic group; †$P < 0.05$ versus corresponding SFA group; §$P < 0.05$ versus other diet groups.

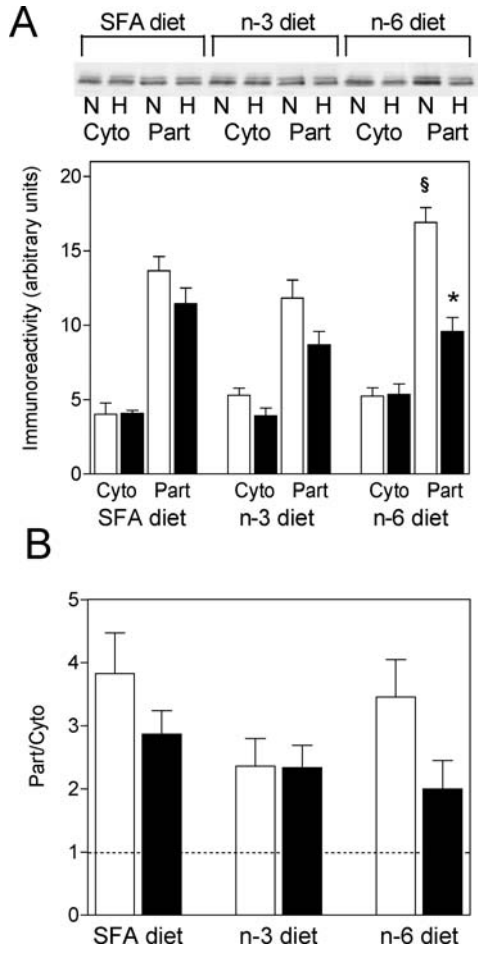

Figure 4. (A) Expression of PKCε in Cyto and Part fractions and (B) its distribution between the fractions from the myocardium of chronically hypoxic rats (H; black columns) and normoxic controls (N; open columns) fed SFA, n-3, or n-6 diet. All samples compared were electrophoresed on the same gel and quantified on the same membrane. The amount of protein applied to the gel was 15 μg (Cyto) or 5 μg (Part). Values are means ± SEM from 5 hearts in each group. *$P < 0.05$ versus corresponding normoxic group; §$P < 0.05$ versus other diet groups.

in the particulate fraction of the SFA and n-3 groups (by 40% and 82%, respectively) but not in the n-6 group. CIH increased the relative protein content of PKCδ in the cytosolic fraction of the SFA group (Fig. 3A) and significantly redistributed this isoform from cytosolic to particulate fractions in the n-3 group only (Fig. 3B).

The abundance of PKCε was higher in the particulate fraction of normoxic rats fed the n-6 diet compared with the SFA and n-3 groups. In contrast with PKCδ up-regulation, CIH did not significantly influence the abundance of PKCε in the particulate fraction of the SFA and n-3 groups, and it even decreased the content of this isoform in the n-6 group (by 41%). Neither lipid diet nor CIH affected the PKCε content in the cytosolic fraction (Fig. 4A). CIH tended to decrease the proportion of PKCε in the particulate fraction in the SFA and n-6 groups (expressed as a ratio of cytosolic to particulate level), but this effect did not reach statistical significance (Fig. 4B).

Figure 5 presents relationships between the mean values of PKCδ (A) or PKCε (B) relative content in the myocardial particulate fraction and the mean infarct size for three normoxic and three hypoxic groups. Regression analysis demonstrated a negative linear relationship between PKCδ abundance and infarct size with the correlation coefficient approaching 0.8 (Fig. 5A). Note that the hypoxic groups were shifted to the lower right portion of the regression line (smaller infarction and higher PKCδ content).

Compared with PKCδ, the relationship between PKCε abundance and infarct size differed considerably. Although the infarct size decreased with increasing relative PKCε content within the normoxic or hypoxic groups, the hypoxic

groups were shifted to the lower left portion of the graph (smaller infarction and lower PKCε content) (Fig. 5B).

Discussion

Myocardial FA Composition and General Effects of CIH and Diets. Dietary supplementation with saturated fats did not significantly affect the FA composition of myocardial PL as compared with the standard ST1 diet (20). It is important to point out that the SFA diet, besides a large amount of SFA, also contained a similarly high proportion of MUFA. Both PUFA diets had no effect on the myocardial content of SFA, only slightly influenced MUFA, and as expected, markedly changed n-3 PUFA and n-6 PUFA in normoxic hearts. This is in agreement with the results of numerous studies showing that supplementation with fish oil (n-3 PUFA) increased the myocardial content of n-3 PUFA at the expense of n-6 PUFA (9, 21, 26), whereas diets containing corn oil (n-6 PUFA) had the opposite effect (27, 28). Surprisingly, CIH decreased the n-6:n-3 PUFA ratio proportionally in all diet groups. This means that different PUFA loads offered by lipid diets that resulted in substantially altered compositions of myocardial PL apparently did not affect the additional PUFA remodeling induced by CIH. The observed decrease in the n-6:n-3 PUFA ratio most likely resulted from the adaptation of the deacylation-reacylation cycle to chronically hypoxic conditions that may be viewed as an important mechanism of membrane protection against oxidative stress (29).

We did not observe any effect of diets on basic heart weight parameters or hemodynamics in normoxic animals. CIH-induced adaptive responses were not affected by diet composition, except for the increase in RV systolic pressure, which was significantly less pronounced in rats fed the n-3 diet. This finding is in agreement with the previous observation of Archer *et al*. (30) and suggests that a fish oil diet can partially protect against the development of hypoxic pulmonary hypertension.

Effects of CIH and Diets on Ischemia/Reperfusion Injury. It is generally accepted that cardiac susceptibility to ischemia/reperfusion injury can be modulated by lipid diets. In particular, numerous experimental studies have demonstrated that long-lasting feeding of various animal species by diets enriched with PUFA protect the heart against ischemic and reperfusion ventricular arrhythmias (3, 5, 9, 31). The antiarrhythmic influence of n-3 PUFA appears to be superior to that of n-6 PUFA (32). Our data support previous findings about the antiarrhythmic effect of a diet enriched with n-3 PUFA against both ischemic and reperfusion arrhythmias (9, 31, 32). CIH decreased the severity of reperfusion arrhythmias as previous reports indicated (13, 22, 33); this effect manifested itself in all diet groups, although with different potencies. A combination of the n-3 diet and CIH had a stronger protective effect on reperfusion arrhythmia severity than n-3 diet alone, suggesting independent additive actions.

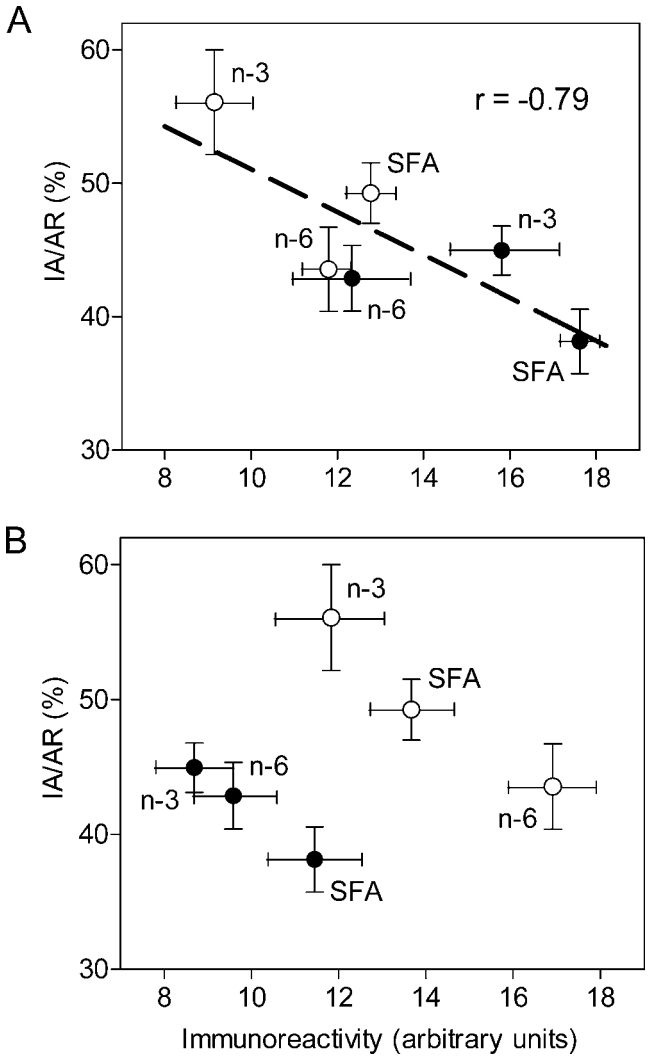

Figure 5. Relationships between the mean values of (A) PKCδ and (B) PKCε relative content in the myocardial Part and the mean IA normalized to the AR (IA/AR) in chronically hypoxic rats (black circles) and normoxic controls (open circles) fed SFA, n-3, or n-6 diet. r indicates correlation coefficient.

However, diets enriched with PUFA seem to have much fewer clear effects when myocardial infarct size is set as the major end point of ischemia/reperfusion injury. Factors that may influence the results of dietary studies with PUFA include animal species (34), age (35), duration of treatment, and relative amounts of n-3 and n-6 PUFA in the diet (36, 37). Against our expectations, we observed significantly smaller infarction in rats fed the n-6 diet compared with the n-3 diet group, which exhibited the largest extent of injury. Of the three previous studies that analyzed infarct size in rats *in vivo*, two demonstrated protective effects of either the n-6 diet (sunflower seed oil; 12%) (4) or the n-3 diet (fish oil; 12%) (7); however, the third one (28) did not find any limitation of infarction after 6–12 weeks on fish oil or corn oil diets (20%). Similarly, several *in vitro* studies failed to detect significant cardioprotection following n-3 or n-6 PUFA supplementation (38,

39). Our observation of weak myocardial ischemic tolerance of rats fed the n-3 diet supports the previous findings of Gudbjarnason and Oskarsdottir (40), who demonstrated that an n-3 diet (cod liver oil; 10%; 12 weeks) aggravated isoproterenol-induced cardiac necrosis and mortality. It seems, therefore, that the impact of diets enriched with PUFA (particularly n-3 PUFA) on cardiac susceptibility to injury is not always favorable as previously thought. Interestingly, a recent thorough systematic review on cardiovascular events and total mortality found no evidence of a clear benefit of n-3 PUFA on health (41).

The cause of aggravated injury due to an n-3 diet is unclear. One potential explanation is that highly polyunsaturated long-chain FA, in particular n-3 PUFA from fish oil, are extremely susceptible to peroxidation compared with n-6 PUFA (36, 42), even in the presence of added dietary antioxidants (43). Thus, we can speculate that the marked enrichment of membranes with n-3 PUFA in rats fed the diet supplemented with 10% fish oil increased myocardial susceptibility to oxidative stress induced by ischemia/reperfusion that resulted in larger infarction compared with the n-6 group.

The major result of this study is that lipid diets had distinct effects on infarct size limitation afforded by CIH. Whereas less tolerant diet groups (SFA and n-3) were protected by CIH, the protective effect of the n-6 diet on myocardial infarction was not further enhanced by adaptation. As the decrease in the n-6:n-3 PUFA ratio in membranes induced by CIH was the same in all groups (i.e., independent of the level set by diets), it seems unlikely that this adaptive response is directly involved in the mechanism of the infarct size–limiting effect in chronically hypoxic hearts.

Effects of CIH and Diets on PKC. We showed previously that PKCδ plays a role in the cardioprotective mechanism of CIH (18), and both PKCδ up-regulation and protection depend on the level of oxidative stress during the adaptation period (17). The present study demonstrated for the first time that lipid diets alone have distinct effects on the expression and subcellular distribution of PKCδ and PKCε and also modulate the hypoxia-induced changes of these isoforms.

Isoforms δ and ε belong to the group of novel PKC. Both PKCδ and PKCε are sensitive to diacylglycerols (DAG) and phosphatidylserine (PS) (44). Several other lipid second messengers have been shown to activate PKC *in vitro* and *in vivo*. *cis*-Unsaturated FA, such as AA, LA, and oleic acid, can activate PKC in the absence of DAG and PS (45, 46). It has been demonstrated that free *cis*-unsaturated FA could affect PKC-dependent signaling pathways in cardiac (47–49) and other cell types (50, 51). Moreover, the quality of the acyl chain of DAG also plays a role in different PKC isoform activation (52, 53). Despite PKCδ and PKCε overall structural similarities, these isoenzymes are differentially regulated (54). Kashiwagi *et al.* (55) showed that AA and ceramide induced different patterns of PKCε translocation compared with PKCδ. The differential sensitivity of PKCδ and PKCε to AA is related to the subtle differences in the conserved domain (C1B). The C1B domain defines the isoform-specific sensitivity of PKC to lipid second messengers. In line with our data, it seems that the higher content of n-6 PUFA (mainly AA) in membranes can be associated with improved cellular signaling mediated by PKC (45, 49, 51). We can speculate that the highest amount of PKCε in the particulate fraction of the normoxic n-6 group is linked to increased ischemic tolerance. This view is supported by the data of Mackay and Mochly-Rosen (56), who demonstrated that AA selectively activates PKCε in neonatal rat cardiac myocytes and this activation could lead to protection.

On the other hand, we observed a lower expression of PKC isoforms in normoxic myocardium of the group fed the n-3 diet, which contained higher amounts of DHA and eicosapentaenoic acid (EPA) in PL compared with the SFA and n-6 groups. This is in agreement with studies showing that n-3 PUFA can decrease the activity of PKC *in vitro* (57, 58). Similarly, a fish oil diet inhibited redistribution of PKCε but not PKCδ from the cytosol to the membrane fraction in hypertrophic mouse hearts (59). EPA and DHA supplementation decreased PKC activity in cardiac cells compared with cells supplemented with AA (60). In addition, we cannot exclude that the decreased expression of PKC isoforms observed in the n-3 diet group was due to the effect of n-3 PUFA on gene expression of these proteins. It has been shown that PUFA could be important regulators of gene expression in various tissue types (61–63), but any data on direct effects of n-3 PUFA on PKC genes have not yet been generated.

In addition to the fact that various lipid diets influence PKC properties through the quality of lipid-signaling molecules, membranes containing different levels of FA, such as SFA, MUFA, and PUFA, differentially affect the propensity of membranes to form nonlamellar phases (64). Nonlamellar phases regulate PKC translocation to membranes (65), and this mechanism should also be taken into account as potentially contributing to the observed effects of lipid diets.

CIH led to the up-regulation of PKCδ in both the SFA and n-3 diet groups but not in the n-6 diet group in compliance with the presence or absence, respectively, of the infarct size–limiting effect. Unlike PKCδ, myocardial PKCε was down-regulated in all three hypoxic groups. Regression analysis of the mean values of the PKCδ relative content in the particulate fraction and infarct size revealed a close negative correlation between these variables; all hypoxic groups were located in the lower right portion of the graph (smaller infarction and higher PKCδ content). These results further support our previous conclusion (17, 18) that PKCδ plays an important role in the infarct size–limiting mechanism of CIH in adult rat hearts. Concerning PKCε, the infarct size also decreased with increasing relative PKCε content within the normoxic or hypoxic

groups, which is in agreement with a generally accepted view that this isoform is cardioprotective. However, all values of the hypoxic groups exhibited a parallel shift to the lower left portion of the graph (smaller infarction and lower PKCε content), suggesting that CIH protects the myocardium by a mechanism independent of PKCε.

In conclusion, our data demonstrated distinct effects of lipid diets on myocardial ischemic tolerance and the expression and subcellular distribution of PKC isoforms δ and ε in normoxic and chronically hypoxic rats. These data support the view that lipid diet composition is important for the manifestation of the infarct size–limiting mechanism of CIH, which involves the PKCδ pathway, while the hypoxia-induced changes in n-3 and n-6 PUFA proportions in membrane PL seem unlikely to play a direct role.

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SUPPLEMENT 3

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Dietary polyunsaturated fatty acids and adaptation to chronic hypoxia alter acyl composition of serum and heart lipids

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The effects of dietary supplementation with fat of different fatty acid profile and chronic intermittent hypoxia (CIH) on the fatty acid composition of serum and heart lipids were analysed. Adult male Wistar rats were fed a standard non-fat diet enriched with 10 % of lard, fish oil (*n*-3 PUFA) or maize oil (*n*-6 PUFA) for 10 weeks. After 4 weeks on the diets, each group was divided in two subgroups, either exposed to CIH in a barochamber (7000 m, twenty-five exposures) or kept at normoxia. In normoxic rats, the fish oil diet increased the level of conjugated dienes. The *n*-6:*n*-3 PUFA ratio in serum TAG, phospholipids (PL), cholesteryl esters (CE) and heart TAG, PL and diacylglycerols (DAG) followed the ratio in the fed diet (in the sequence maize oil > lard > fish oil). In heart TAG, PL and DAG, 20 : 4*n*-6 and 18 : 2*n*-6 were replaced by 22 : 6*n*-3 in the fish oil group. The main fatty acid in CE was 20 : 4*n*-6 in the lard and maize oil groups whereas in the fish oil group, half of 20 : 4*n*-6 was replaced by 20 : 5*n*-3. CIH further increased 20 : 5*n*-3 in CE in the fish oil group. CIH decreased the *n*-6:*n*-3 PUFA ratio in serum CE, heart TAG, PL and DAG in all dietary groups and stimulated the activity of catalase in the maize and fish oil groups. In conclusion, PUFA diets and CIH, both interventions considered to be cardioprotective, distinctly modified the fatty acid profile in serum and heart lipids with specific effects on conjugated diene production and catalase activity.

Dietary *n*-3 and *n*-6 PUFA: Chronic hypoxia: Serum and heart lipids: Antioxidative enzymes: Conjugated dienes: Cardioprotection

Lipids are important dietary components and changes in their quality may cause significant alterations in the fatty acid composition and properties of cardiac membranes. Changes in the composition of membrane lipids can account for nutritional benefits exerted by certain fatty acids through the so-called 'membrane lipid-therapy'[1]. Many experimental and clinical studies have shown that dietary lipids can influence the incidence and severity of CHD. While MUFA and PUFA (*n*-3 and *n*-6 classes) usually display beneficial effects, SFA may have an adverse impact on the heart[2–4]. Both classes of PUFA collectively protect the heart against ischaemia–reperfusion injury but *n*-3 PUFA are considered more potent in their cardioprotective activity[5]. Dietary PUFA play a key role in cardiac protection by modulating serum and tissue lipid composition and in this regard affecting metabolic and signal-transduction pathways[6]. Chronic intermittent hypoxia (CIH) is another protective phenomenon that increases cardiac tolerance to all major damaging consequences of ischaemia–reperfusion[7]. Our recent results suggest that the signalling pathway involving protein kinase C (PKC) and reactive oxygen species plays a role in the mechanism of CIH-induced cardioprotection[8]. Moreover, we have shown that CIH induces the remodelling of cardiac membranes leading to the increase in the *n*-3 PUFA proportion in phospholipids (PL)[9].

The present study was prompted by results of our recent experiments[10] combining two systemic interventions that can improve cardiac ischaemic tolerance: dietary supplementation with *n*-3 or *n*-6 PUFA and adaptation to CIH. The *n*-6 and *n*-3 PUFA-enriched diets as well as CIH protected against arrhythmias but had distinct effects on myocardial infarct size induced by coronary artery occlusion. The *n*-6:*n*-3 PUFA ratio in the heart PL was altered in accordance with the fatty acid composition of the lipid diet while CIH decreased this ratio to the same extent, independent of dietary fatty acid profile. The main purpose of the present study was to analyse in detail the effects of lipid diets and CIH on acyl composition of the main lipid classes in rat serum and heart. For the first time we demonstrated that, under these conditions, the individual classes of transport and membrane lipids markedly differ in their ability to maintain fatty acid composition. In line

Abbreviations: CE, cholesteryl ester; CIH, chronic intermittent hypoxia; DAG, diacylglycerol; GPX, glutathione peroxidase; PKC, protein kinase C; PL, phospholipid; SOD, superoxide dismutase.

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with the increased susceptibility of *n*-3 PUFA to oxidative stress, myocardial markers of lipid peroxidation increased in fish oil-supplemented rats.

Materials and methods

Animal model

Adult male Wistar rats (weight 250–280 g) were fed a non-fat standard ST1 diet (Velaz, Czech Republic) enriched with either 10 % by weight of lard, fish oil (*n*-3 PUFA; Löhmann & Voss, Germany) or maize oil (*n*-6 PUFA; Olmühle GmbH, Austria) for 10 weeks. The composition of the non-fat standard ST1 diet was as follows (per kg): 240 g crude protein; 37·2 g non-nutrient fibres; 65·1 g ash; 240 g N substances; antioxidants (butylhydroxytoluene, ethoxyquin, butylhydroxyanisol); 4500 μg (15 000 IU) vitamin A; 25 μg (1000 IU) vitamin D; 107·9 mg vitamin E; 0·34 mg Se; 22·6 mg Cu; 13·2 g Ca; 8·7 g P; 1·8 g Na; Myco ad (adsorbent of mycotoxins). The fatty acid composition of the diets enriched with lard, fish oil or maize oil is shown in Table 1; the term 'enriched' is omitted hereafter for the sake of simplicity. After 4 weeks on the diets, each group was divided into two subgroups that were either exposed or not to chronic intermittent (hypobaric) hypoxia corresponding to 7000 m for 8 h/d, 5 d/week. In the exposed subgroups, barometric pressure (P_B) was lowered stepwise so that the level equivalent to an altitude of 7000 m ($P_B = 41$ kPa; $PO_2 = 8{\cdot}6$ kPa) was reached after thirteen exposures; the total number of exposures was twenty-five[8]. The control subgroups of animals were kept for the same period of time at P_B and PO_2 equivalent to an altitude of 200 m ($P_B = 99$ kPa; $PO_2 = 20{\cdot}7$ kPa). All animals had free access to water. The day after the last hypoxic exposure, rats were anaesthetised by sodium pentobarbitone (60 mg/kg body weight, intraperitoneally; Sanofi, France).

Table 1. Fatty acid composition (mol %) of the diet lipids
(Means of two separate analyses)

	Lard	Fish oil	Maize oil
14 : 0	1·40	5·66	0·58
16 : 0	24·15	17·86	12·28
16 : 1*n*-7	2·48	7·71	0·93
18 : 0	10·87	2·13	1·81
18 : 1*n*-9	35·50	16·48	24·70
18 : 1*n*-7	2·49	3·44	0·95
18 : 2*n*-6	19·03	15·38	53·16
18 : 3*n*-3	1·62	2·61	2·31
20 : 1*n*-9	0·96	7·31	1·03
20 : 4*n*-6	0·10	0·49	0·05
20 : 5*n*-3	0·18	9·94	ND
22 : 5*n*-3	0·07	1·78	ND
22 : 6*n*-3	0·21	8·04	ND
20 : 4*n*-6:22 : 6*n*-3 ratio	0·47	0·06	ND
Σ SFA*	36·42	25·65	14·67
Σ MUFA*	41·43	34·94	27·61
Σ *n*-6 PUFA*	19·30	15·38	53·16
Σ *n*-3 PUFA*	2·08	22·70	2·31
Unsaturation index†	86·86	180·37	140·86

ND, not detected.
*Σ SFA, Σ MUFA, Σ *n*-6 PUFA and Σ *n*-3 PUFA comprise of all fatty acids analysed.
†The unsaturation index is calculated as mol % of individual unsaturated fatty acids multiplied by the number of double bonds.

After collecting the blood samples, the hearts were rapidly excised, washed in cold (0°C) saline and dissected into the right and left ventricular free walls and the septum. Heart regions were weighed and the left ventricles were frozen and stored in liquid N_2. All of the chemicals were purchased from Sigma (St Louis, MO, USA), unless otherwise indicated. The study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). The experimental protocol was approved by the Animal Care and Use Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic.

Fatty acid composition

Serum (1 ml) was dissolved in 21 ml of a chloroform–methanol mixture (2:1, v/v) and shaken in a pear-shaped flask. The serum protein precipitate was removed by filtration. Then 10 ml of a chloroform–methanol–water mixture (3:48:47, by vol.) was added and after a vigorous shaking the lower lipid layer was separated and dried at 40°C under a stream of N_2. Individual lipids, i.e. PL, TAG and cholesteryl esters (CE), were separated by one-dimensional TLC (0·5 mm Silica Gel H; Merck, Darmstadt, Germany) using the solvent mixture hexane–diethyl ether–acetic acid (85:15:1, by vol.), detected by 2,7 dichlorofluorescein (0·005 % in methanol), scraped out and stored in an N_2 atmosphere at −20°C. On the next day, fatty acid methyl esters were prepared and separated by GC[11].

Heart lipids and their fatty acid composition were analysed as described previously[9]. Briefly, frozen left ventricular myocardium was pulverised and homogenised. Lipids were extracted according to Folch *et al.*[12] and dried at 40°C under a stream of N_2. The one-dimensional TLC of PL, TAG and diacylglycerols (DAG) as well as further procedures were the same as with the serum samples.

Antioxidative enzyme activities

Frozen left ventricular myocardium was pulverised to a fine powder at the temperature of liquid N_2, followed by Potter-Elvehjem homogenisation in 8 volumes of ice-cold buffer composed of (mmol/l): 12·5 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris)-HCl (pH 7·4); 250 sucrose; 2·5 ethylene glycol tetra-acetic acid; 1 EDTA; 100 NaF; 5 dithiothreitol; 0·3 phenylmethylsulfonyl fluoride; 0·2 leupeptin; 0·02 aprotinin.

Catalase activity was measured by the method of Aebi[13]. The rate of H_2O_2 decomposition was monitored spectrophotometrically.

Glutathione peroxidase (GPX) activity was determined by the indirect procedure described by Paglia & Valentine[14]. Oxidised glutathione was produced by GPX reaction and immediately reduced by NADPH in the presence of glutathione reductase. The rate of NADPH consumption was recorded as a measure of oxidised glutathione formation.

Total superoxide dismutase (SOD) activity was determined by the modified nitroblue tetrazolium method in homogenates[15]. The xanthine–xanthine oxidase reaction was utilised to generate a superoxide flux. Nitroblue tetrazolium reduction by superoxide anions to blue formazan was measured spectrophotometrically.

Conjugated dienes

The extraction of lipids was carried out by the method of Folch *et al.* as described above[12]. The dry lipid residue was redissolved in cyclohexane and the tissue content of conjugated dienes was measured spectrophotometrically[16].

Statistical analysis

The results are expressed as mean values with their standard errors. Two-way ANOVA and subsequent Student–Newman–Keuls tests were used for comparison of differences in normally distributed variables between groups. Differences were considered as statistically significant when $P<0{\cdot}05$.

Results

Fatty acid composition of serum and heart lipids

Table 2 presents fatty acid composition of serum lipids. The fatty acid profiles and values of the unsaturation index in serum TAG of normoxic dietary groups closely followed those of the corresponding fed diets (Table 1). The proportion of SFA and MUFA decreased in favour of total PUFA in the

Table 2. Effect of diets and chronic hypoxia on the fatty acid composition in serum lipids
(Mean values with their standard errors for five animals per group)

	Normoxia						Hypoxia					
	Lard		Fish oil		Maize oil		Lard		Fish oil		Maize oil	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
TAG												
16:0	29·02	0·39	23·67§	0·82	17·27§	0·59	30·57	0·48	23·83§	0·38	21·23§*	0·73
18:0	5·43	0·37	2·20†	0·09	1·67†	0·12	5·61	0·36	2·42†	0·24	1·72†	0·06
18:1*n*-9	37·34	0·47	23·79†	0·81	24·45†	0·65	39·20	0·63	24·24§	0·77	27·69§*	0·49
18:2*n*-6	16·03	0·27	15·21	0·73	44·10§	1·30	12·72*	0·98	14·88	0·69	38·73§*	1·06
20:4*n*-6	1·27	0·08	0·62§	0·02	3·98§	0·33	0·88	0·17	0·68	0·06	2·34§*	0·26
20:5*n*-3	0·24	0·01	6·01§	0·61	0·25	0·05	0·13	0·03	6·15§	0·68	0·14	0·03
22:6*n*-3	0·83	0·05	10·04§	0·54	0·73	0·13	0·50	0·09	9·22§	0·78	0·31	0·04
20:4*n*-6:22:6*n*-3 ratio	1·54	0·11	0·06	0·004	6·12§	0·92	1·91	0·25	0·07§	0·01	7·62§	0·74
Σ SFA‖	35·61	0·53	27·77§	0·79	19·63§	0·61	37·24	0·40	28·08§	0·44	23·59§*	0·74
Σ MUFA‖	43·49	0·49	35·24§	1·17	27·55§	0·80	46·47	1·29	36·31§	1·02	32·03§*	0·40
Σ *n*-6 PUFA‖	18·52	0·21	16·49	0·76	50·57§	1·08	14·83*	1·32	16·23	0·75	42·97§*	0·73
Σ *n*-3 PUFA‖	2·36	0·10	20·48§	1·15	2·24	0·24	1·44	0·22	19·36§	1·59	1·40	0·10
Unsaturation index¶	95·75	1·15	180·57§	4·13	150·38§	2·37	86·06	3·60	175·02§	6·39	131·17§*	1·11
Phospholipids												
16:0	20·30	0·25	24·48†	0·82	23·17†	0·79	22·49*	0·45	28·14§*	0·59	22·38	0·34
18:0	23·86	0·78	20·01†	0·76	19·55†	1·26	24·93	2·67	18·34†	1·24	19·05†	0·70
18:1*n*-9	6·27	0·25	5·64	0·50	5·06	0·56	8·14*	0·48	6·58§	0·23	5·32§	0·26
18:2*n*-6	23·01	0·73	19·35	1·79	22·97	1·33	22·82	0·33	18·56§	1·13	28·40§*	0·52
20:4*n*-6	19·47	1·15	11·89§	2·85	20·24	1·46	14·49*	0·26	6·89§	0·36	18·83§	0·58
20:5*n*-3	0·23	0·03	5·91§	1·50	0·64	0·50	0·24	0·05	8·02§	0·15	0·08	0·02
22:6*n*-3	2·30	0·16	4·42§	0·77	2·51	0·45	2·28	0·09	5·46§	0·37	1·94	0·09
20:4*n*-6:22:6*n*-3 ratio	8·61	0·54	2·69	2·10	8·06	1·94	6·52*	0·21	1·26§	0·12	9·71§	0·76
Σ SFA‖	44·49	0·70	42·34	0·81	43·24	1·77	44·90	0·37	46·05	1·42	42·72	1·42
Σ MUFA‖	8·39	0·22	9·90	1·12	8·40	1·06	10·56*	0·55	11·72	0·31	7·51§	0·31
Σ *n*-6 PUFA‖	44·05	0·74	35·42	4·06	45·84	2·40	42·07	0·45	27·56§	0·97	47·62	1·46
Σ *n*-3 PUFA‖	3·07	0·16	11·97§	2·31	3·26	0·99	2·98	0·11	14·66§	0·40	2·37	0·08
Unsaturation index¶	154·46	4·06	171·28†	1·30	161·46	4·80	142·28*	1·50	160·24*	4·30	152·12	6·44
Cholesteryl esters												
16:0	8·57	0·67	10·71	0·72	7·53‡	0·59	9·83	0·33	11·05	0·39	9·33	1·40
18:0	1·03	0·18	1·36	0·34	0·82	0·27	1·17	0·11	1·23	0·14	1·42	0·59
18:1*n*-9	7·57	0·23	8·11	0·25	8·14	1·16	8·92	0·40	7·90	0·11	12·71	4·68
18:2*n*-6	26·52	0·80	22·31§	1·70	28·34	0·68	27·65	1·20	19·03§	0·47	29·43	1·60
20:4*n*-6	50·15	1·64	24·37§	5·82	49·81	2·62	44·84	2·00	16·55§	0·27	40·88	5·36
20:5*n*-3	0·54	0·13	23·41§	5·72	0·36	0·13	0·53	0·18	33·74§*	0·54	0·23	0·05
22:6*n*-3	0·72	0·02	1·62§	0·24	0·50	0·04	0·86	0·06	1·62§	0·11	0·61	0·09
20:4*n*-6:22:6*n*-3 ratio	70·00	0·88	20·14§	9·78	100·97§	7·87	53·09	3·24	10·47§	0·99	68·97*	9·39
Σ SFA‖	10·18	0·86	13·01	0·98	9·08‡	1·12	11·87	0·32	13·33	0·37	11·52	1·85
Σ MUFA‖	10·84	0·62	14·06	1·07	10·77	2·02	12·70	0·58	14·49	0·33	15·83	5·32
Σ *n*-6 PUFA‖	77·46	1·30	47·34§	7·53	79·30	3·23	73·68	0·82	36·44§	0·35	71·38	6·88
Σ *n*-3 PUFA‖	1·53	0·12	25·50§	5·96	1·11	0·17	1·73	0·13	35·73§*	0·57	1·26	0·12
Unsaturation index¶	274·68	5·38	286·47	6·52	274·99	9·42	259·85	4·86	301·16§	1·92	247·73	19·49

* Mean value was significantly different from that of the corresponding normoxic group ($P<0{\cdot}05$).
† Mean value was significantly different from that of the corresponding lard dietary group ($P<0{\cdot}05$).
‡ Mean value was significantly different from that of the corresponding fish oil dietary group ($P<0{\cdot}05$).
§ Mean value was significantly different from those of the other dietary groups ($P<0{\cdot}05$).
‖ Σ SFA, Σ MUFA, Σ *n*-6 PUFA and Σ *n*-3 PUFA comprise of all fatty acids analysed.
¶ The unsaturation index is calculated as mol % of individual unsaturated fatty acids multiplied by the number of double bonds.

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following sequence: lard > fish oil > maize oil dietary groups. The high abundance of 18 : 1*n*-9 was the characteristic feature of the lard dietary group whereas the maize oil group was rich in 18 : 2*n*-6. In contrast to the fed diets, 20 : 4*n*-6 appeared in serum TAG in the lard and particularly in the maize oil dietary groups. The highest unsaturation index of the fish oil group was in agreement with the markedly higher *n*-3 PUFA proportion in this group compared with other dietary groups. CIH increased SFA and MUFA proportions due to the rise of 16 : 0 and 18 : 1*n*-9 and decreased the *n*-6 PUFA proportion due to the fall of 18 : 2*n*-6 and 20 : 4*n*-6 in the maize oil dietary group, resulting in lowered unsaturation index values. Regarding the lard group, CIH decreased the 18 : 2*n*-6 proportion only. No effect of CIH on serum TAG was observed in the fish oil group.

The dominant fatty acids in serum PL of the lard and maize oil dietary groups were 16 : 0, 18 : 0, 18 : 2*n*-6 and 20 : 4*n*-6 in nearly equimolar proportions while for the fish oil group, a half of 20 : 4*n*-6 was substituted by 20 : 5*n*-3 and 22 : 6*n*-3. CIH increased the abundance of 16 : 0 and 18 : 1*n*-9 and decreased 20 : 4*n*-6 in serum PL of the lard group with a qualitatively similar but less pronounced effect in the fish oil group;

Table 3. Effect of diets and chronic hypoxia on the fatty acid composition in heart lipids
(Mean values with their standard errors for five animals per group)

	Normoxia						Hypoxia					
	Lard		Fish oil		Maize oil		Lard		Fish oil		Maize oil	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
TAG												
16 : 0	27·39	0·76	22·74†	0·52	19·84†	1·42	27·61	1·56	21·16†	1·58	17·83†	0·81
18 : 0	10·78	0·42	4·94†	0·22	5·99†	0·73	10·71	0·69	4·38†	0·22	5·14†	0·73
18 : 1*n*-9	34·9	0·80	25·80†	0·87	25·59†	0·78	32·40	1·08	23·18†*	0·57	25·46†	0·51
18 : 2*n*-6	13·58	0·26	16·59§	0·65	37·83§	1·22	12·92	0·42	15·39§	0·38	39·93§	1·01
20 : 4*n*-6	1·46	0·11	0·73	0·05	2·28§	0·30	1·76	0·16	0·78§	0·05	2·08	0·36
20 : 5*n*-3	0·07	0·01	1·36§	0·17	0·03	0·01	0·11	0·02	1·68§	0·13	0·15	0·07
22 : 6*n*-3	1·57	0·18	8·01§	1·01	1·08	0·20	2·99*	0·38	12·84§*	1·30	1·87	0·52
20 : 4*n*-6:22 : 6*n*-3 ratio	0·97	0·06	0·10§	0·01	2·38§	0·25	0·63*	0·06	0·06§*	0·01	1·28§*	0·10
Σ SFA‖	38·82	0·78	29·72†	0·61	26·66†	2·06	39·33	1·09	27·23†	1·93	23·72†	0·58
Σ MUFA‖	42·33	0·83	39·52	1·18	29·22§	0·86	39·47	1·10	36·86	0·95	29·30§	0·41
Σ *n*-6 PUFA‖	16·01	0·32	18·05	0·63	41·86§	1·21	16·24	0·59	17·06	0·39	43·62§	0·71
Σ *n*-3 PUFA‖	2·83	0·20	12·69§	1·59	2·24	0·28	4·94	0·58	18·82§*	1·84	3·34	0·69
Unsaturation index¶	93·76	1·99	147·76§	7·00	130·91§	4·17	104·58*	3·87	179·11§*	9·83	140·37§	4·01
Phospholipids												
16 : 0	10·07	0·34	10·92	0·29	9·08§	0·24	11·55*	0·37	12·43	0·60	10·91*	0·42
18 : 0	26·04	0·78	25·50	1·76	26·30	1·65	25·60	0·70	25·29	1·01	26·25	1·30
18 : 1*n*-9	3·76	0·11	3·34§	0·08	3·69	0·09	3·70	0·19	3·31	0·06	3·27*	0·12
18 : 2*n*-6	20·13	1·36	20·54	0·70	25·81§	1·56	17·75	0·15	16·63*	0·36	24·43§	0·75
20 : 4*n*-6	22·08	0·20	11·61§	0·50	21·44	0·87	19·91*	0·47	11·18§	0·54	18·96*	0·51
20 : 5*n*-3	0·17	0·03	2·89§	0·18	0·04	0·01	0·20	0·04	2·74§	0·15	0·06	0·01
22 : 6*n*-3	10·41	0·39	17·31§	0·86	7·12§	0·54	12·52*	0·76	19·83§	0·88	8·91§*	0·43
20 : 4*n*-6:22 : 6*n*-3 ratio	2·14	0·08	0·67§	0·01	3·06§	0·13	1·62*	0·07	0·56§*	0·01	2·15§*	0·09
Σ SFA‖	36·21	1·03	36·60	2·03	35·52	1·86	37·25	1·03	36·73	1·49	36·88	2·06
Σ MUFA‖	6·99	0·17	7·93§	0·18	6·14§	0·17	7·33	0·32	8·30§	0·19	5·87§	0·17
Σ *n*-6 PUFA‖	44·12	1·46	33·13§	0·97	49·77§	1·80	40·38	0·50	29·31§	0·60	46·96§	1·67
Σ *n*-3 PUFA‖	12·66	0·55	22·32§	1·05	8·54§	0·75	15·01	0·89	25·64§*	1·04	10·26§	0·59
Unsaturation index¶	216·49	2·83	227·46	8·85	203·27	6·94	220·92	6·61	233·91	8·38	204·20‡	5·70
Diacylglycerols												
16 : 0	27·97	1·80	24·77	1·19	23·86	1·90	23·53	1·45	21·44	1·37	21·11	1·53
18 : 0	23·76	2·46	19·51	1·78	21·37	2·12	24·83	2·18	18·97	2·23	22·44	2·32
18 : 1*n*-9	20·85	4·11	18·85	2·51	18·63	2·44	19·97	3·07	18·57	3·90	14·80	2·66
18 : 2*n*-6	6·42	0·44	8·59	1·00	14·37§	2·25	8·46	0·67	10·22	0·97	16·15§	1·62
20 : 4*n*-6	4·20	1·04	3·63	0·57	4·94	0·94	5·85	1·09	5·85	1·45	8·56*	1·69
20 : 5*n*-3	1·18	0·91	2·15	1·22	1·76	1·42	0·38	0·07	1·59§	0·33	0·54	0·23
22 : 6*n*-3	2·11	0·43	3·69§	0·61	2·04	0·22	3·29	0·44	6·01§*	0·98	3·70	0·57
20 : 4*n*-6:22 : 6*n*-3 ratio	1·99	0·27	0·95	0·15	2·46§	0·43	1·65	0·20	1·12	0·22	2·39‡	0·35
Σ SFA‖	53·70	2·84	46·26	1·54	46·67	2·50	49·69	2·35	41·51	1·27	45·07	1·43
Σ MUFA‖	30·36	3·59	32·91	2·26	27·58	2·07	29·54	2·67	31·69	3·25	22·75	2·64
Σ *n*-6 PUFA‖	11·63	1·50	13·61	1·57	21·04§	2·82	15·81	1·50	17·25	2·05	26·53§	2·65
Σ *n*-3 PUFA‖	4·28	0·86	7·20	1·28	4·68	1·35	4·94	0·51	9·53§	1·38	5·63	0·65
Unsaturation index¶	75·16	5·74	88·90	5·20	95·48†	5·39	85·14	5·45	102·25†	6·12	108·07†	6·57

* Mean value was significantly different from that of the corresponding normoxic group ($P<0·05$).
† Mean value was significantly different from that of the corresponding lard dietary group ($P<0·05$).
‡ Mean value was significantly different from that of the corresponding fish oil dietary group ($P<0·05$).
§ Mean value was significantly different from those of the other dietary groups ($P<0·05$).
‖ Σ SFA, Σ MUFA, Σ *n*-6 PUFA and Σ *n*-3 PUFA comprise of all fatty acids analysed.
¶ The unsaturation index is calculated as mol % of individual unsaturated fatty acids multiplied by the number of double bonds.

the unsaturation index decreased in both groups. In the maize oil dietary group, the increase in the proportion of 18 : 2*n*-6 was observed after adaptation to CIH.

In serum CE, the proportions of individual SFA and MUFA were very low as compared with the other lipids analysed. The distinguishing feature of CE was the high level of 20 : 4*n*-6 in

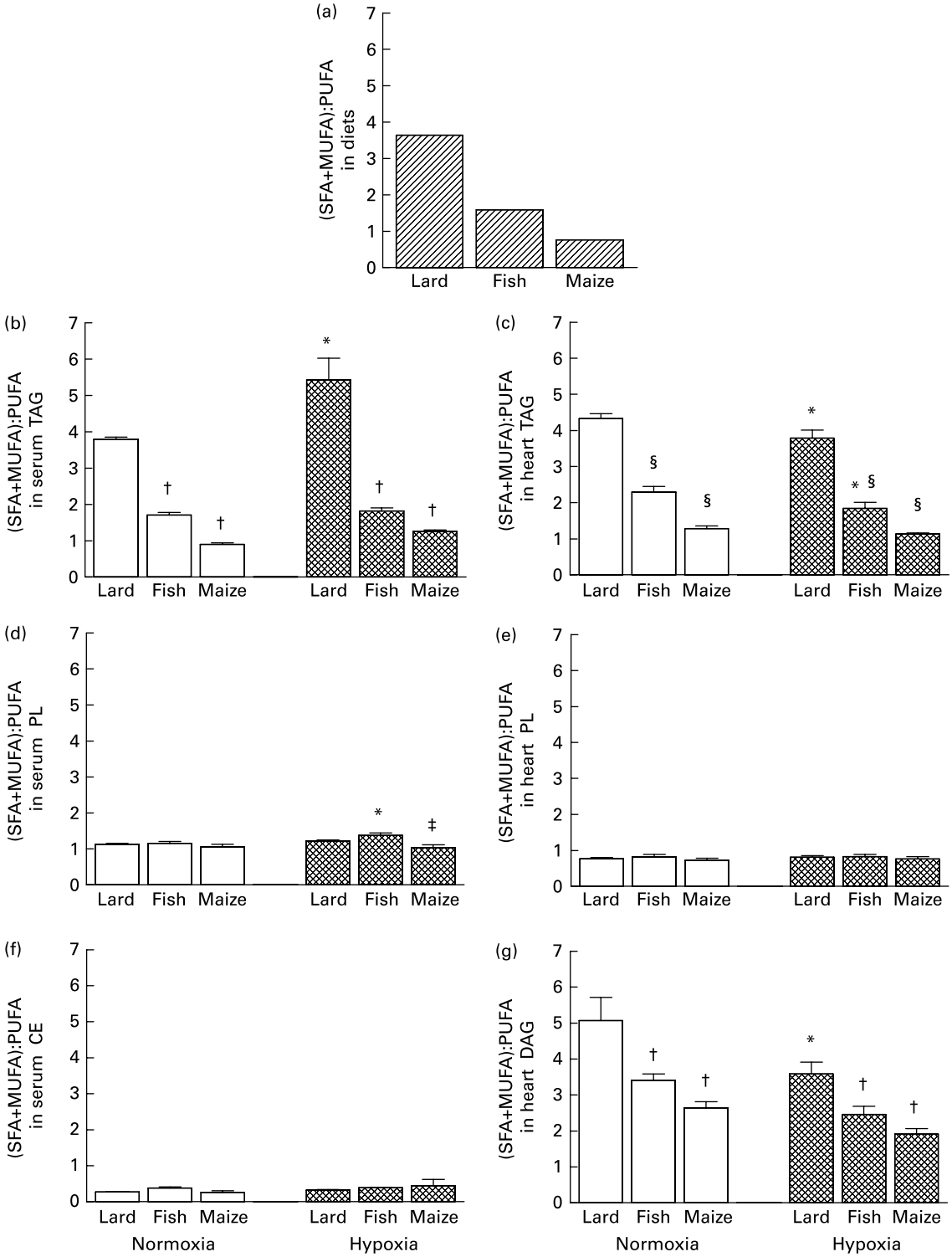

Fig. 1. The (SFA + MUFA):PUFA ratio in the diet (a), serum TAG (b), heart TAG (c), serum phospholipids (PL) (d), heart PL (e), serum cholesteryl esters (CE) (f) and heart diacylglycerols (DAG) (g) of normoxic rats and chronically hypoxic rats fed a lard, fish oil or maize oil diet. Values are means of five animals per group, with standard errors represented by vertical bars. * Mean value was significantly different from that of the corresponding normoxic group ($P<0{\cdot}05$). † Mean value was significantly different from that of the corresponding lard dietary group ($P<0{\cdot}05$). ‡ Mean value was significantly different from that of the corresponding fish oil dietary group ($P<0{\cdot}05$). § Mean value was significantly different from those of the other dietary groups ($P<0{\cdot}05$).

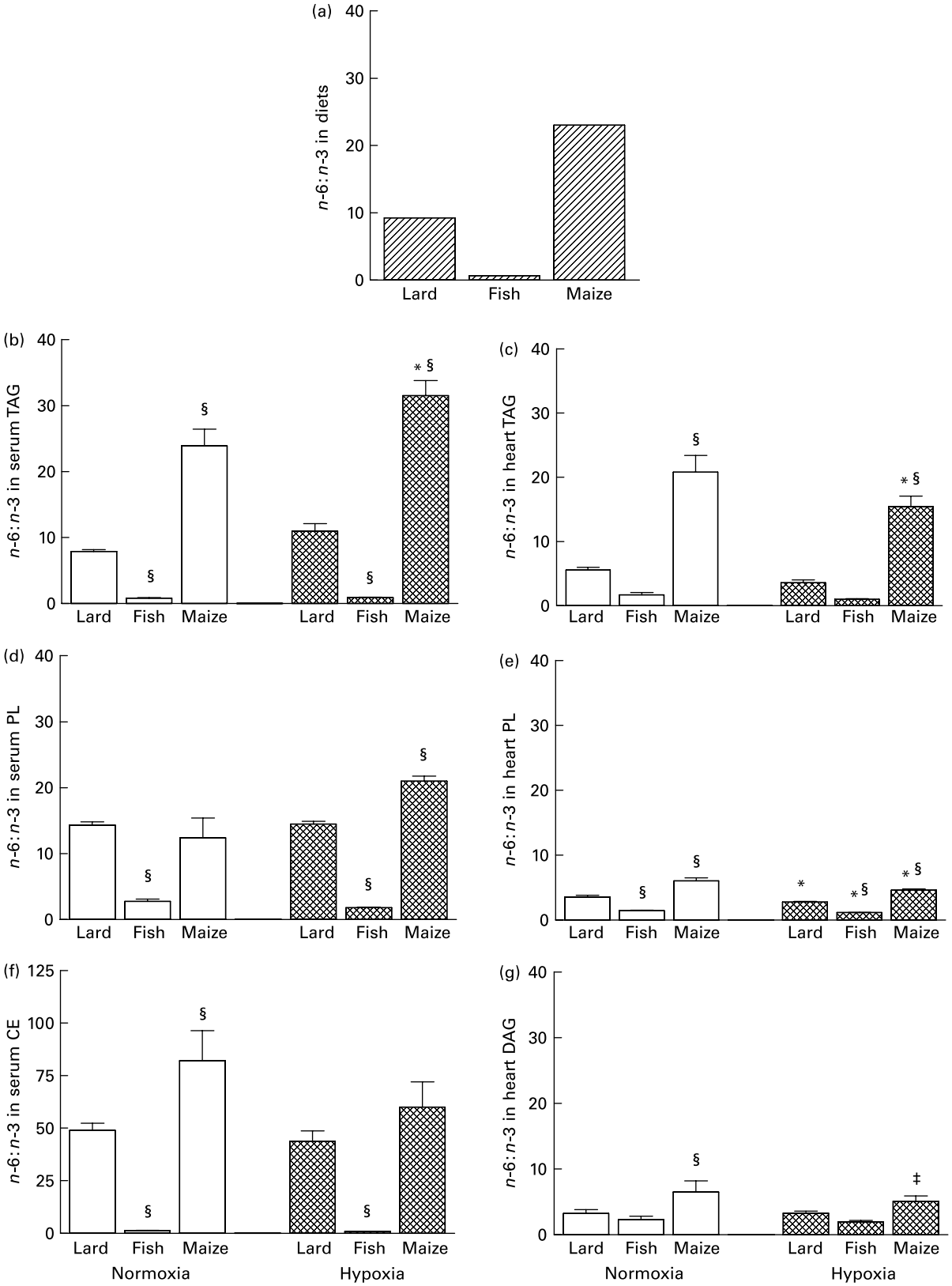

Fig. 2. The *n*-6:*n*-3 PUFA ratio in the diet (a), serum TAG (b), heart TAG (c), serum phospholipids (PL) (d), heart PL (e), serum cholesteryl esters (CE) (f) and heart diacylglycerols (DAG) (g) of normoxic rats and chronically hypoxic rats fed a lard, fish oil or maize oil diet. Note that the vertical axis of the graph of serum CE (f) has a different scale. Values are means of five animals per group, with standard errors represented by vertical bars. * Mean value was significantly different from that of the corresponding normoxic group ($P<0{\cdot}05$). ‡ Mean value was significantly different from that of the corresponding fish oil dietary group ($P<0{\cdot}05$). § Mean value was significantly different from those of the other dietary groups ($P<0{\cdot}05$).

Table 4. Level of conjugated dienes and antioxidative enzyme activities in myocardial homogenates of normoxic and chronically hypoxic rats fed a lard, fish oil or maize oil diet

(Mean values with their standard errors for six hearts per group)

	Normoxia						Hypoxia					
	Lard		Fish oil		Maize oil		Lard		Fish oil		Maize oil	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Conjugated dienes (nmol/g, w/w)	310	25	442§	19	344	20	297	19	435§	37	358	17
Catalase activity (U/mg protein)	19·6	2·1	18·2	1·3	14·9	1·1	20·0	1·2	25·6*	2·1	23·3*	2·5
GPX activity (U/mg protein)	1·31	0·20	1·13	0·18	0·87	0·09	1·30	0·11	1·06	0·17	1·26	0·15
SOD activity (U/mg protein)	243	43	248	37	205	29	209	13	214	15	182	15

GPX, glutathione peroxidase; SOD, superoxide dismutase.
* Mean value was significantly different from that of the corresponding normoxic group ($P<0·05$).
§ Mean value was significantly different from those of the other dietary groups ($P<0·05$).

the lard and maize oil dietary groups, representing about 50 % of total fatty acids. The fish oil diet replaced 18 : 2*n*-6 and 20 : 4*n*-6 by the equimolar proportion of 20 : 5*n*-3 and 22 : 6*n*-3. CIH further increased 20 : 5*n*-3 at the expense of 18 : 2*n*-6 and 20 : 4*n*-6 in the fish oil dietary group.

Table 3 presents the fatty acid composition of heart lipids. Both PUFA dietary groups had a lower proportion of SFA (16 : 0, 18 : 0) and a higher unsaturation index in heart TAG as compared with the lard group. The MUFA proportion was the lowest and that of *n*-6 PUFA the highest one in the maize oil group, compared with other dietary groups. The fish oil group exhibited a higher proportion of *n*-3 PUFA compared with other dietary groups. CIH increased 22 : 6*n*-3 and the unsaturation index in the lard and fish oil groups. The 20 : 4*n*-6:22 : 6*n*-3 ratio was decreased by CIH in all dietary groups.

In heart PL of the fish oil group, the accumulation of 20 : 5*n*-3 and 22 : 6*n*-3 at the expense of 20 : 4*n*-6 was observed as compared with the other two dietary groups. Adaptation to CIH increased 16 : 0, 22 : 6*n*-3 and decreased 20 : 4*n*-6 proportions in the lard and maize oil dietary groups, while it decreased 18 : 2*n*-6 and increased total *n*-3 PUFA proportions in the fish oil group. The 20 : 4*n*-6:22 : 6*n*-3 ratio was decreased by CIH in all dietary groups.

In heart DAG, SFA and MUFA were the most prominent fatty acids. The proportion of 22 : 6*n*-3 was higher in the fish oil group whereas 18 : 2*n*-6 was particularly enhanced in the maize oil group as compared with the other two dietary groups. CIH further increased the 22 : 6*n*-3 or 20 : 4*n*-6 proportions in the fish oil or maize oil dietary groups, respectively.

In serum TAG and heart TAG and DAG, the (SFA + MUFA):PUFA ratio (Fig. 1) closely followed that of the corresponding fed diet. On the other hand, this ratio remained unaffected by diets in serum PL and CE, as well as in heart PL. The (SFA + MUFA):PUFA ratio was considerably lower in serum PL, serum CE and heart PL as compared with other lipid classes due to the relatively high proportion of total PUFA. CIH increased the (SFA + MUFA):PUFA ratio in serum TAG of the lard group and in serum PL of the fish oil group. In contrast, CIH induced a decrease of this ratio in heart TAG and DAG but had no effect in serum CE and heart PL.

All lipid classes tended to reflect the *n*-6:*n*-3 ratio of the corresponding fed diet (Fig. 2). The ratio was the highest in the maize oil dietary group and the lowest in the fish oil group. Nevertheless, the difference in these ratios among the groups was less pronounced in the heart PL and DAG as compared with other lipids. CIH induced a decrease of the *n*-6:*n*-3 ratio in all lipid classes, except for serum TAG.

Conjugated dienes and antioxidative enzymes

The fish oil diet increased the level of conjugated dienes in both normoxic and hypoxic myocardium as compared with the corresponding lard or maize oil dietary groups. CIH had no additional effect (Table 4). However, CIH enhanced catalase activity in the hearts of rats fed both PUFA diets compared with the lard group. Activities of SOD and GPX were affected by neither lipid diets nor CIH (Table 4).

Discussion

Effect of diets and chronic intermittent hypoxia on the fatty acid composition in serum and heart lipids

In agreement with other studies[17], we confirmed that the *n*-6:*n*-3 PUFA ratio in all serum and heart lipids was shifted in favour of the predominant PUFA class in the diet. CIH induced a decrease of the *n*-6:*n*-3 PUFA ratio due to the 22 : 6*n*-3 enrichment at the expense of 20 : 4*n*-6 in all heart lipids, independent of the diet. This effect was the most consistent in heart PL. Whereas the (SFA + MUFA):PUFA ratio in serum and heart TAG and DAG reflected the ratio of the corresponding diet, in serum PL and CE and heart PL it was kept relatively constant regardless of dietary interventions or CIH.

The primary role of TAG is the transport of fatty acids into tissues providing a store of energy. In the present study, fatty acid profiles of serum and heart TAG closely followed those of the diets in line with the fact that samples were collected from fed rats, i.e. chylomicrons were the predominant form of circulating TAG-rich lipoproteins. It is generally accepted that fatty acids derived from the hydrolysis of TAG-rich chylomicrons may be the primary source of fatty acids utilised by the heart, particularly in the fed state[18].

CE represent the transport form of cholesterol in the blood that is supplied to the heart, which has low cholesterol biosynthetic capacity[19]. The present results show that nearly a half of the overall fatty acid content in CE of the lard and maize oil

groups comprises 20 : 4*n*-6 while in the fish oil group this PUFA is replaced to a large extent by 20 : 5*n*-3. It has been reported that 20 : 5*n*-3 is the preferred substrate both for lysophosphatidylcholine acyltransferase, leading to the formation of phosphatidylcholine in the rat liver[20], and for plasma lecithin:cholesterol acyltransferase which transfers PUFA from plasma phosphatidylcholine to CE[21]. This may explain the partial replacement of 20 : 4*n*-6 with 20 : 5*n*-3 in plasma CE of the fish oil dietary group in agreement with results of Garg *et al.* [22]. CIH further increased the incorporation of 20 : 5*n*-3 into CE of the fish oil group, probably due to the enhanced supply of 20 : 5*n*-3 from serum PL (i.e. phosphatidylcholine).

Serum and heart PL contained substantially higher proportions of 18 : 0 and 20 : 4*n*-6 than the diets. It has been reported that a large proportion of 18 : 0 and 20 : 4*n*-6 is incorporated into PL due to the preferential affinity of acyltransferases and transacylases for these fatty acids during the fatty acid remodelling process associated with PL *de novo* synthesis in the rat liver[23]. In the fish oil group, 20 : 5*n*-3 was preferentially incorporated into serum PL and CE whereas the accumulation of 22 : 6*n*-3 was favoured in all heart lipids despite nearly equimolar proportions of these two *n*-3 PUFA in the diet. The preferential incorporation of 22 : 6*n*-3 into myocardial lipids has been reported in other studies on rodents[24] and human subjects[25]. CIH increased the accumulation of 22 : 6*n*-3 at the expense of 20 : 4*n*-6 in myocardial PL of the lard and maize oil groups. However, in the fish oil group, CIH stimulated the accumulation of 22 : 6*n*-3 in heart PL at the expense of 18 : 2*n*-6 without any compensatory effect on 20 : 4*n*-6. It is evident that CIH-adapted myocardium tends to preserve the relatively low level of 20 : 4*n*-6 in PL that was already reduced by the fish oil diet.

The shifts in the *n*-6 and *n*-3 PUFA proportions in heart PL caused by diets and CIH were qualitatively similar to those observed in rats fed different PUFA-enriched diets and chronically treated with high doses of catecholamines[26]. In line with this observation, exposure of rats to CIH was associated with transiently increased adrenergic activity and elevated plasma levels of catecholamines[27,28]. Thus, the fatty acid remodelling in PL induced by CIH could be explained by stress-dependent hormonal modulation of enzyme activities involved in PL acyl remodelling. PL deacylation was accelerated due to the activation of phospholipase A2 in cardiomyocytes exposed to hypoxia[29,30]. It is likely that this enzyme which preferentially hydrolyses 20 : 4*n*-6 from the sn2 position of membrane PL[31] and acyl-CoA synthase with preferential affinity for 22 : 6*n*-3[32] could contribute to the 22 : 6*n*-3 accumulation in heart PL by the deacylation–reacylation cycle observed after CIH.

Our observation that DAG comprised a high proportion of SFA and MUFA but a low proportion of PUFA as compared with other lipids regardless of dietary interventions is in agreement with several reports[33–36]. The fish oil diet increased the 22 : 6*n*-3 proportion in DAG compared with other diets. This observation is in line with the results obtained in fish oil-supplemented mice and dogs[37,38]. DAG are important endogenous lipid activators of PKC[39]. Besides a direct interaction of this lipid with PKC, changes induced in the membrane lipid structure by DAG and other lipids favour the binding and activation of PKC[40]. It has been shown that DAG enriched with 20 : 5*n*-3 or 22 : 6*n*-3 were less efficient activators of PKC than DAG containing 20 : 4*n*-6[41]. Judé *et al.* [38] observed a lower activation of PKC δ and PKC ε in the hearts of fish oil-supplemented dogs than in those fed the standard diet. In line with the results mentioned above, Hlavackova *et al.* demonstrated a lower abundance of PKC δ in the particulate fraction from normoxic hearts of rats fed the fish oil diet, together with the larger myocardial infarct size compared with the maize oil dietary group[10]. On the other hand, adaptation to CIH that further increased the 22 : 6*n*-3 proportion in heart DAG of the fish oil group stimulated the expression of PKC δ and had a protective effect on infarct size. These results suggest that the impact of changes in the PUFA composition of DAG on PKC activation or inactivation in connection with cardiac ischaemic tolerance is complex and may differ in normoxic and chronically hypoxic hearts. CIH also increased 20 : 4*n*-6 in DAG of the maize oil group and tended to increase it in the lard and fish oil groups in contrast with heart TAG and PL where 20 : 4*n*-6 either decreased or remained unchanged. The fact that CIH specifically increased the accumulation of 20 : 4*n*-6 in myocardial phosphatidylcholine[9] offers the explanation that phosphatidylcholine could be an important source of DAG under the CIH conditions. In accordance with this assumption, it has been reported that DAG generated from phosphatidylcholine by phospholipase D is involved in ischaemic preconditioning[42,43]. Murase *et al.* [35] found an increased DAG content as well as an increased proportion of 20 : 4*n*-6 in DAG of preconditioned rat hearts, supporting the involvement of this signalling lipid in the cardioprotection.

There is a general consensus that *n*-3 PUFA, particularly 20 : 5*n*-3 and 22 : 6*n*-3, exert powerful anti-arrhythmic effects[44] resulting from their multiple actions. It was shown, for example, that both 20 : 5*n*-3 and 22 : 6*n*-3 decreased cardiac susceptibility to adrenergic stimulation[45], prevented disturbances of membrane transport and ionic homeostasis[46,47], decreased phosphoinositides production[48], altered the fatty acid composition of DAG and led to differential PKC activation[38]. Therefore, the enrichment of 20 : 5*n*-3 and 22 : 6*n*-3 in serum and heart lipids demonstrated in the present study could contribute to the anti-arrhythmic effect of CIH observed under ischaemia–reperfusion conditions that was most pronounced in the fish oil group[10].

Effect of diets and chronic intermittent hypoxia on myocardial redox status

The increased level of conjugated dienes in the myocardium of fish oil-supplemented rats is in agreement with the increased susceptibility of *n*-3 PUFA to oxidative stress, compared with the *n*-6 PUFA class, probably due to the greater presence of double bonds[49]. Conjugated dienes are one of the primary metabolic intermediate products of lipoperoxidation[50,51]. Our finding of increased levels of conjugated dienes in the myocardium of the fish oil dietary group corresponds to the largest myocardial infarct size observed in these animals. On the other hand, the same fish oil group exhibited the lowest incidence and severity of ventricular arrhythmias[10]. This is in line with the suggestion of Judé *et al.* [52] that an oxidation product of 22 : 6*n*-3 could effectively protect against arrhythmias rather than 22 : 6*n*-3 itself.

Despite the increased formation of conjugated dienes in the fish oil group, we did not notice any change in the activities of SOD, GPX or catalase. Likewise, Nageswari *et al.* [53] did not observe any effect of a fish oil diet on antioxidative enzyme activities in rat hearts despite the increased level of hydroperoxides. In contrast, Diniz *et al.* [54] have shown that both *n*-3 and *n*-6 PUFA diets decreased the activities of SOD and catalase and increased the activity of GPX in rat hearts. Under similar experimental conditions, a decrease in activities of myocardial SOD and GPX was detected in another study[55]. This inconsistency of available data regarding the effects of lipid dietary supplementation may be due to the variability of experimental models, fatty acid composition of diets and their intake level.

Recently we demonstrated that CIH was associated with increased oxidative stress as evidenced by decreased myocardial reduced:oxidised glutathione ratio and elevated concentration of lipofuscin-like pigments[8]. The present study did not detect any significant effect of CIH on the myocardial level of conjugated dienes in any dietary group. This can be explained by the fact that these compounds are one of the first temporarily detectable unstable markers of lipoperoxidation. It seems likely that changes in the level of conjugated dienes could have appeared at earlier stages of hypoxic adaptation. CIH increased the activity of catalase in the hearts of the fish oil and maize oil groups as compared with the lard-fed group. This suggests that oxidative stress associated with CIH was probably higher in both PUFA dietary groups than in the lard group. In line with our observation, CIH enhanced the activity of catalase in the hearts of rats kept on a standard diet[56]. On the other hand, myocardial activities of catalase and GPX did not differ between normoxic and chronically hypoxic young rats whereas the SOD activity was decreased in the hypoxic group[57]. Although total SOD and GPX remained unchanged in the myocardial homogenate of CIH animals, we cannot exclude that their activities increased in specific subcellular compartments, such as mitochondria, that are involved in reactive oxygen species generation.

We conclude that both dietary interventions as well as CIH had systemic effects leading to distinct changes in the fatty acid profile in serum and heart lipids. In particular, membrane PL maintained SFA, MUFA and total PUFA proportions constant independently of diet and CIH. This is the evidence of a remarkable regulatory ability of membranes to maintain a stable milieu that is necessary for a proper function of membrane proteins. On the other hand, the *n*-6:*n*-3 PUFA ratio was influenced to various extents by either the dietary PUFA supply or CIH in all lipid classes. These changes may affect myocardial adaptive responses under various physiological and pathophysiological conditions, such as those associated with ischaemia–reperfusion and oxidative stress.

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P. B. measured enzyme activities and participated in drafting the manuscript; J. J. was responsible for lipid analysis; M. H. measured the level of conjugated dienes; J. N. and F. K. performed animal experiments and tissue sampling and participated in preparation of the manuscript; B. S. was responsible for fatty acid analysis; O. N. and F. N. were responsible for interpretation of the results and preparation of the manuscript.

There are no conflicts of interest.

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SUPPLEMENT 4

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N-acetylcysteine Treatment Prevents the Up-Regulation of MnSOD in Chronically Hypoxic Rat Hearts

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Summary

Chronic intermittent hypoxia (CIH) is associated with increased production of reactive oxygen species that contributes to the adaptive mechanism underlying the improved myocardial ischemic tolerance. The aim was to find out whether the antioxidative enzyme manganese superoxide dismutase (MnSOD) can play a role in CIH-induced cardioprotection. Adult male Wistar rats were exposed to intermittent hypobaric hypoxia (7000 m, 8 h/day, 25 exposures) (n=14) or kept at normoxia (n=14). Half of the animals from each group received *N*-acetylcysteine (NAC, 100 mg/kg) daily before the hypoxic exposure. The activity and expression of MnSOD were increased by 66 % and 23 %, respectively, in the mitochondrial fraction of CIH hearts as compared with the normoxic group; these effects were suppressed by NAC treatment. The negative correlation between MnSOD activity and myocardial infarct size suggests that MnSOD can contribute to the improved ischemic tolerance of CIH hearts.

Key words

Chronic hypoxia • MnSOD • Ischemia/reperfusion • Myocardial infarction • Cardioprotection

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Introduction

It has been shown that reactive oxygen species (ROS) formed under stress conditions, such as exercise, hyperthermia or various forms of preconditioning, may enhance the tolerance of the heart to ischemia-reperfusion injury by stimulation of cellular antioxidant defence systems that maintain optimal redox balance (Hoshida *et al*. 2002). The main source of ROS in myocardium is the respiratory chain of the inner mitochondrial membrane where manganese superoxide dismutase (MnSOD; SOD2), the key antioxidative enzyme, is also localized (Starkov *et al.* 2008). Several signalling pathways involving ROS as a second messenger in conjunction with the induction of MnSOD gene expression have been identified (Rogers *et al*. 2001, Hussain *et al*. 2004). MnSOD is an inducible homotetrameric protein, synthesized in the cytoplasm as a precursor and imported post-translationally in the mature form into the mitochondrial matrix (Wispe *et al*. 1989). MnSOD catalyzes the dismutation of two superoxide radicals, generating hydrogen peroxide and molecular oxygen (McCord *et al.* 1969). This enzyme is most likely of crucial importance for cell viability as mice lacking MnSOD die from dilated cardiomyopathy within the first days after the birth (Li *et al*. 1995).

Adaptation to chronic intermittent hypoxia (CIH) is known to protect the heart against acute ischemia/reperfusion injury. The mechanism underlying induction of the long-lasting protected phenotype of CIH adapted hearts is not precisely understood (Kolář *et*

al. 2004). It has been proposed that ROS generated during the adaptation period are important components of a signalling pathway leading to this form of cardioprotection as chronic treatment of rats with an antioxidant eliminated the infarct size-limiting effect of CIH (Kolář *et al*. 2007). The main goal of our study was, therefore, to find out whether CIH affects the activity and expression of MnSOD in rat heart mitochondria and whether these changes depend on ROS production. Levels of pyruvate dehydrogenase (PDH) in mitochondrial and cytosolic fractions were measured as a marker of mitochondrial integrity. We showed that CIH led to the up-regulation and activation of MnSOD, and these effects were absent in animals chronically treated with the antioxidant N-acetylcysteine (NAC) during the adaptation period. Moreover, the negative correlation between MnSOD activity and myocardial infarct size suggests that this enzyme can contribute to the improved ischemic tolerance of CIH-adapted hearts.

Materials and Methods

Animal model

Adult male Wistar rats were exposed to intermittent hypobaric hypoxia of 7000 m for 8 h/day, 5 days a week, as described earlier (Kolář *et al*. 2007). Barometric pressure (P_B) was lowered stepwise, so that the final level equivalent to the altitude of 7000 m (P_B=306.8 mm Hg; PO_2=63.8 mm Hg) was reached after 13 exposures; the total number of exposures was 25. The control group of animals was kept at normoxia for the same period of time. One-half of the rats from each group received NAC (dissolved in saline and neutralized with sodium hydroxide) subcutaneously in a dose of 100 mg/kg daily before each hypoxic exposure and the remaining rats received the same volume of saline (2 ml/kg) in the corresponding manner. Rats were fed standard laboratory diet and had free access to water. All rats were employed on the next day after the last hypoxic exposure and sacrificed by decapitation. Hearts were rapidly excised, washed in cold saline and left ventricular (LV) walls with the septum were dissected, weighed and used for analysis. The study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The experimental protocol was approved by the Animal Care and Use Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic.

Table 1. Expression of common housekeeping proteins.

	Normoxia	**Hypoxia**	
Actin			42 kDa
	45.9±2.5	54.3±2.6*	
Calsequestrin			55 kDa
	43.9±3.2	56.0±3.2*	
GAPDH			36 kDa
	35.8±4.4	64.1±4.4*	
β-tubulin			50 kDa
	43.4±3.0	56.8±3.2*	

Representative Western blots are shown. Values are means ± S.E.M. from 6 hearts in each group (arbitrary units). *P<0.05 vs. normoxic group.

Isolation of mitochondrial and cytosolic fractions

LV with septum were chopped by scissors in ice-cold medium (10 vol of medium to 1 vol of tissue) consisting of (in mM) 20 Tris-HCl, 250 sucrose, 2.5 EGTA, 5 EDTA, 100 NaF, 5 dithiothreitol, 0.3 phenylmethylsulfonyl fluoride, 0.2 leupeptin, and 0.02 aprotinin (pH 7.4) and then homogenized by using a Potter-Elvehjem homogenizer with a loose-fit Teflon pestle. The homogenate was centrifuged at 800 × *g* for 20 min. The supernatant was centrifuged at 8,000 × *g* for 10 min. The mitochondrial pellet was washed (1 ml) twice and finally resuspended in the homogenization buffer. Supernatant was then centrifuged at 100,000 × *g* for 60 min to obtain the cytosolic fraction. All steps were performed at 4 °C. Samples for the measurement of MnSOD activity were sonicated using three 10-s bursts. Mitochondrial fraction for immunoblot analyses was extracted with 1 % Triton X-100 on ice for 60 min, the resulting detergent-treated supernatant was used. Triton X-100 was also added to the cytosolic fraction to reach the final concentration of 1 %. Protein content was determined according to Lowry modified by Peterson (Peterson *et al*. 1977). Neither hypoxia nor NAC influenced the protein yields in mitochondrial and cytosolic fractions (data not shown).

SDS-PAGE and Western blot analysis

Mitochondrial extracts and cytosolic fractions were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on 15 % bis-acrylamide gel at

Table 2. Body weight and heart weight parameters.

Parameter	Normoxia	Normoxia + NAC	Hypoxia	Hypoxia + NAC
n	10	10	10	9
BW, g	380 ± 6	369 ± 7	340 ± 7*	332 ± 12*
RVW, mg	174 ± 6	180 ± 8	270 ± 11*	247 ± 17*
LVW+SW, mg	601 ± 13	582 ± 18	677 ± 29*	632 ± 26*
RVW/BW, mg/g	0.459 ± 0.019	0.487 ± 0.019	0.795 ± 0.028*	0.743 ± 0.034*
LVW+SW/BW, mg/g	1.584 ± 0.021	1.576 ± 0.033	1.994 ± 0.070*	1.908 ± 0.055*

n, number of animals; NAC, N-acetylcysteine-treated groups; BW, body weight; RVW, right ventricular weight; LVW, left ventricular weight; SW, septum weight; RVW/BW, relative RV weight; LVW+SW/BW, relative LV+S weight; *P<0.05 versus corresponding normoxic group.

30 mA/gel for 90 min on a Mini-Protean III apparatus (Bio-Rad, Hercules, CA); the resolved proteins were transferred to a nitrocellulose membrane (Amersham Biosciences, Germany). After blocking with 5 % dry low-fat milk in Tris-buffered saline with Tween 20 (TTBS) for 60 min at room temperature, membranes were washed and probed with rabbit anti-MnSOD polyclonal antibody (Stressgen Bioreagents, Victoria, Canada) and mouse monoclonal antibody to PDH E1β subunit (Molecular Probes, Oregon, USA). The membranes were washed again and incubated with the secondary swine anti-rabbit and anti-mouse (Sevapharma, Prague, Czech Republic; 1:4,000 in TTBS) antibodies, respectively, labelled with horseradish peroxidase for 60 min at room temperature. To ensure the specificity of immunoreactive protein, MnSOD standard (Stressgen Bioreagents, Victoria, Canada) were used. The samples from each experimental group were run on the same gel and quantified on the same membrane. The analysis of each heart sample was repeated at least three times and normalized to total protein.

For comparative quantification of the amount of monitored protein in Western blot analysis, an appropriate housekeeping protein as an internal control is usually used. We examined the effect of CIH on expression of four common housekeeping proteins: actin (Sigma-Aldrich, St. Louis, USA), calsequestrin (Abcam, Cambridge, USA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam, Cambridge, USA), and β-tubulin (Abcam, Cambridge, USA) in heart homogenate. Data in Table 1 show that the expression of all these proteins increased significantly after the adaptation to CIH. On that ground, we used the total protein concentration as the most suitable referential value, because it was not altered under our experimental conditions. Obviously, the choice of a housekeeping protein as an internal standard in chronic experiments should always be carefully validated.

Measurement of MnSOD activity

Total superoxide dismutase (SOD) activity was determined by the modified nitroblue tetrazolium method (Elstner *et al*. 1983, Spitz *et al*. 1989) based on the generation of superoxide radicals from xanthine oxidase reaction that react further with nitroblue tetrazolium dye. Reduction of nitroblue tetrazolium by superoxide anion was measured spectrophotometrically at 540 nm (28 °C). Chloroform-ethanol extracts were then used to determine the SOD activity. The assay contained the following reagents: 0.1 mM phosphate buffer (pH 7.8), 4 mg/ml bovine serum albumin, 2 mg/ml nitroblue tetrazolium, and 1 mM xanthin. MnSOD activity was measured in the presence of 5 mM NaCN, the selective inhibitor of copper-zinc SOD.

Statistical analysis

The results are expressed as means ± S.E.M. Two-way ANOVA and subsequent Student-Newman-Keuls test were used for comparison of differences in parametric variables between the groups. Differences were assumed as statistically significant when $P<0.05$.

Results

Weight parameters

Adaptation of rats to CIH led to body growth retardation, pronounced hypertrophy of the right ventricle and moderate hypertrophy of the left ventricle. Treatment with NAC had no effect on body and heart weight parameters (Table 2).

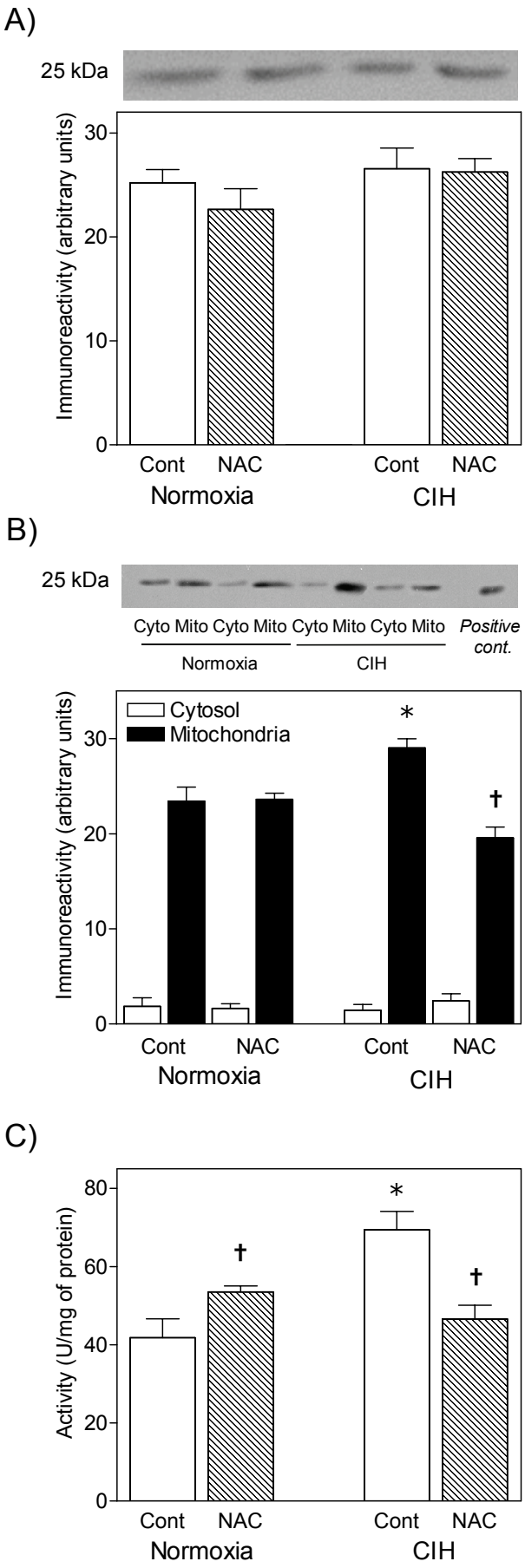

Fig. 1. Level of MnSOD in homogenate (**A**), mitochondrial and cytosolic fractions (**B**) and enzyme activity in the mitochondrial fraction (**C**) from the myocardium of control (Cont) and *N*-acetylcysteine-treated (NAC) rats adapted to CIH and of normoxic animals. Sample loading was normalized to equal protein concentration in individual samples (homogenate 5 μg, cytosol 15 μg, mitochondria 5 μg). Representative Western blots are shown. For details see *Materials and Methods*. Values are means ± S.E.M. from 6 hearts in each group. **P*<0.05 vs. corresponding normoxic group, †*P*<0.05 vs. corresponding untreated group.

Activity and expression of MnSOD

Neither CIH nor NAC treatment influenced the protein abundance of MnSOD in myocardial homogenate (Fig. 1A). On the other hand, both the activity and protein abundance of the enzyme were increased in the mitochondrial fraction of CIH hearts compared with the normoxic values (Fig. 1B, C). These effects of CIH were prevented by NAC. Interestingly, NAC treatment increased the MnSOD activity but not the expression in the mitochondrial fraction from normoxic tissue.

The low levels of MnSOD and PDH in cytosolic fractions from all experimental groups testify to good mitochondrial integrity. The protein abundance of PDH, as a mitochondrial marker, was decreased in homogenates of CIH hearts (Fig. 2A), but its abundance in the mitochondrial and cytosolic fractions was affected by neither CIH nor NAC treatment (Fig. 2B).

Correlation between MnSOD activity and infarct size

Relationship between the mean values of MnSOD activity in myocardial mitochondrial fraction and the mean infarct size normalized to the area at risk is presented in Fig. 3. Regression analysis demonstrated a negative linear relationship between MnSOD activity and infarct size with a correlation coefficient 0.93. Infarct size data were taken from our previous study (Kolář *et al*. 2007) using the same protocols of CIH and NAC treatment.

Discussion

In this study, we demonstrated that adaptation of adult rats to CIH significantly increased the activity and protein abundance of MnSOD in the mitochondrial fraction of LV myocardium but did not influence the expression of MnSOD in myocardial homogenate. This result is in line with our recent study which did not find any effect of CIH on the activity of MnSOD in myocardial homogenate (Kolář *et al*. 2007). The absence

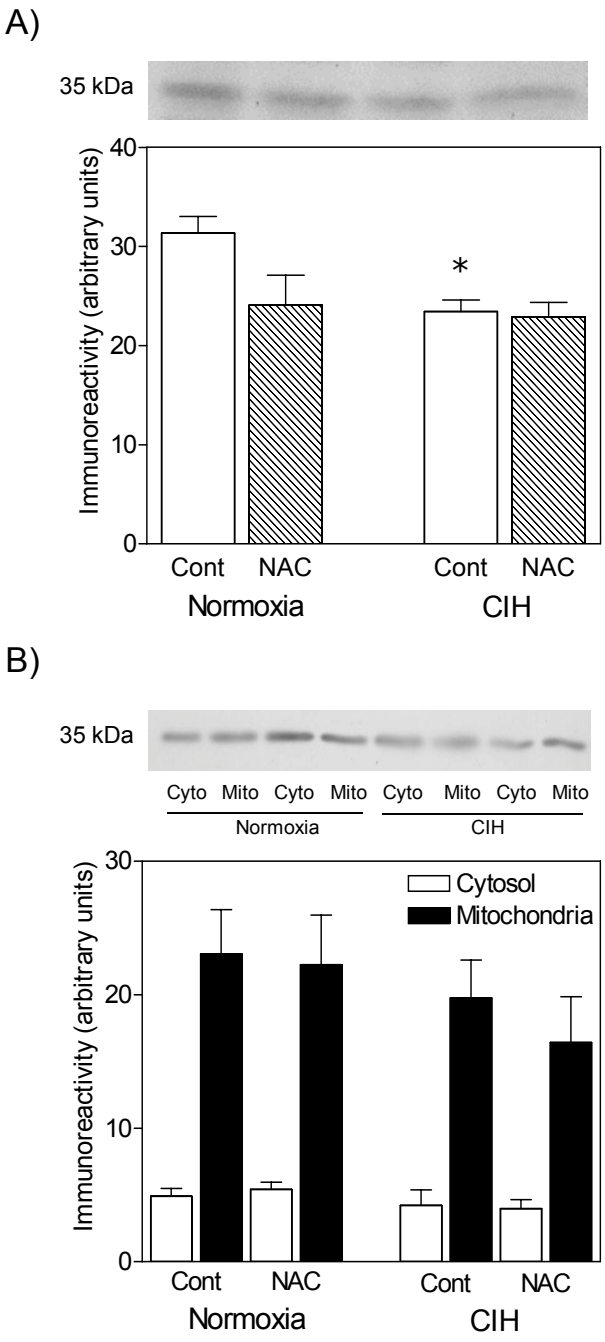

Fig. 2. Level of PDH in homogenate (**A**) and in cytosolic and mitochondrial fractions (**B**) from the myocardium of control (Cont) and *N*-acetylcysteine-treated (NAC) rats adapted to CIH and of normoxic animals. Sample loading was normalized to equal protein concentration in individual samples (homogenate 10 μg, cytosol 15 μg, mitochondria 8 μg). Representative Western blots are shown. Values are means ± S.E.M. from 6 hearts in each group. **P*<0.05 vs. corresponding normoxic group, †*P*<0.05 vs. corresponding untreated group.

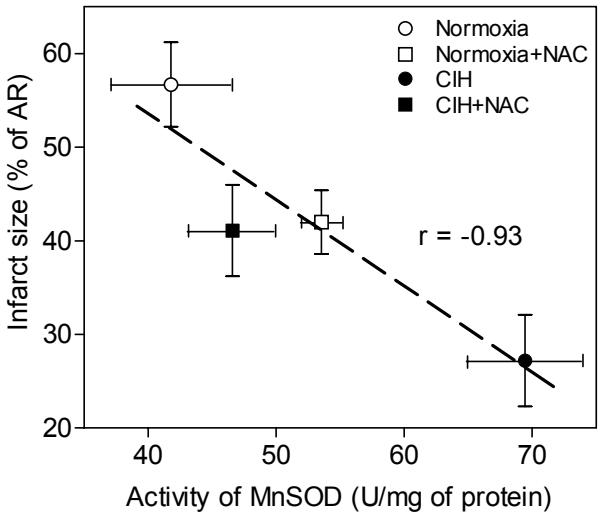

Fig. 3. Relationship between the mean values of MnSOD activity in myocardial mitochondrial fraction and the mean infarct size normalized to the area at risk (AR) in control and *N*-acetylcysteine-treated (NAC) rats adapted to CIH and in normoxic animals. Infarct size data were taken from our previous study (Kolář *et al.* 2007). Myocardial infarction was induced by a 20-min coronary artery occlusion and 3-h reperfusion in open-chest pump-ventilated animals and delineated by staining with triphenyltetrazolium chloride and potassium permanganate. r – correlation coefficient

of any effect on MnSOD in homogenate despite the significant up-regulation of the enzyme demonstrated in mitochondria can be possibly explained by a decrease in total mitochondrial mass caused by severe CIH. A decreased PDH level in the myocardial homogenate from CIH rats, shown in the present study, supports this view. Accordingly, Nouette-Gaulain *et al.* (2005) reported that CIH caused a global decrease in all OXPHOS complex activities in rat LV myocardium. Moreover, we showed previously that CIH reduced concentration of a mitochondrial inner membrane lipid marker cardiolipin in rat LV homogenate (Ježková *et al.* 2002).

The effects of CIH on MnSOD activity and protein abundance in the mitochondrial fraction were prevented by antioxidant NAC treatment during the adaptation period. These results conform to our recent observation that NAC attenuated the ROS-dependent cardioprotection induced by CIH (Kolář *et al.* 2007). However, at variance with our preliminary conclusion, the close negative correlation between MnSOD activity and myocardial infarct size suggests that this enzyme could possibly play a role in the improved mitochondrial ROS-buffering capacity and ischemic tolerance of chronically hypoxic hearts. Increased MnSOD activity was demonstrated also in the hearts of rats adapted to moderate hypoxia during early postnatal life (Zhu *et al.* 2004).

It is generally accepted that MnSOD represents the first line of cell defence against mitochondria-derived ROS and is involved in cardioprotection induced by

various stimuli (Hoshida *et al*. 2002). The MnSOD-inducing pathway in myocardial preconditioning involves ROS signalling as demonstrated in a number of studies (Lecour *et al*. 2006, Chen *et al.* 2008). The activity and expression of MnSOD were increased in a ROS-dependent manner in various forms of delayed preconditioning, and its important protective role against ischemia/reperfusion injury has been well established (Yamashita *et al.* 1999). It has been also reported that heat stress-induced preconditioning increased the expression of MnSOD mRNA in rats (Das *et al*. 1995) and total SOD activity in pig hearts (Liu *et al.* 1992). MnSOD elevation in the myocardium is supposed to be involved in the mechanism of cardioprotection induced by exercise (Hamilton *et al*. 2001). In agreement with these studies, Chen *et al*. (1998) demonstrated that overexpression of MnSOD in transgenic mice limited the infarct size.

Whereas numerous stimuli have been proposed to participate in the MnSOD up-regulation (Kiningham *et al*. 2004, Li *et al*. 1998, Oberley *et al*. 1987), no specific ROS-dependent transduction pathway leading to nuclear MnSOD gene induction has been described in the myocardium. Storz *et al*. (2005) suggested that the induction of the MnSOD gene with the subsequent increase in mitochondrial MnSOD expression in HeLa cells required transcription factor NF-κB initiated by ROS-activated protein kinase D. Other investigators demonstrated a dramatic elevation of mRNA and protein level of MnSOD induced by tumor necrosis factor-α and interleukin-1β in human skin fibroblasts and in mouse kidney, thymus, spleen and bone marrow tissues (Masuda *et al*. 1988, Wong *et al.* 1988). Interestingly, chronic hypoxia increased the level of pro-inflammatory cytokines in fetal guinea pig hearts (Oh *et al*. 2008) and in rat carotid bodies (Lam *et al*. 2008). PKC has been proposed as a further important player in the signaling pathway leading to MnSOD induction because PKC inhibitors prevented the up-regulation of MnSOD in the human adenocarcinoma cell line (Das *et al*. 1998). Accordingly, we observed that the induction of PKC-δ during the adaptation of rats to CIH was ROS-dependent because it was eliminated by NAC treatment (Kolář *et al.* 2007).

In the present study, NAC treatment completely prevented the increase in MnSOD activity and expression in mitochondria induced by CIH. It has also been reported that NAC abrogated the elevation of heat-stress protein 32 as another protective intracellular stress-responsive protein induced by the hypoxic stimulus (Borger *et al*. 1998). Heat-stress proteins in connection with MnSOD play a role in delayed cardiac preconditioning (Bolli *et al.* 2000). Suzuki *et al.* (2002) suggested that the rise in MnSOD activity is connected with the heat-stress protein 72-induced cardioprotection. In addition, heat-stress protein 72 acts as chaperone in the MnSOD maturation and its incorporation into mitochondria (Hoshida *et al*. 2002, Voos *et al.* 1993).

Interestingly, NAC treatment increased the activity of MnSOD in normoxic hearts without affecting its expression. Using the same experimental protocol, we observed a smaller infarct size in NAC-treated normoxic rats compared with untreated ones (Kolář *et al*. 2007). Menon *et al.* (2007) demonstrated that increased MnSOD activity was due to antioxidant properties of NAC. In addition, the restoration of MnSOD activity by NAC reduced oxidative stress and attenuated the development of myocardial dysfunction in diabetic rats (Xia *et al*. 2006). It appears likely that NAC acts as indirect antioxidant protecting MnSOD activity by preventing nitration of critical tyrosine residues in its active site (Barreiro *et al*. 2006, Navarro-Antolin *et al.* 2007, MacMillan-Crow *et al*. 1999).

It is known that MnSOD eliminates ROS more effectively in intact mitochondria than in those that are damaged (Raha *et al*. 2000). We therefore analyzed additional mitochondrial marker, PDH in mitochondrial and cytosolic fractions to confirm the integrity of isolated mitochondria and their vulnerability to disruption during the isolation procedure. Low levels of MnSOD and PDH in the cytosolic fraction from all experimental groups indicate good preservation of mitochondria during their isolation in this study. Neither CIH nor NAC treatment affected the sensitivity of the mitochondrial inner membrane to disruption.

In conclusion, the up-regulation and activation of mitochondrial MnSOD in close correlation with the reduction of myocardial infarct size suggest that this enzyme can contribute to the mechanism of CIH-induced tolerance against ischemia/reperfusion injury. Blunting of these effects by treatment with NAC supports the view that ROS-dependent signaling during hypoxic adaptation plays an important role in this form of cardioprotection.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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SUPPLEMENT 5

Chapter 7

ROLE OF PROTEIN KINASES IN CHRONIC INTERMITTENT HYPOXIA-INDUCED CARDIOPROTECTION

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ABSTRACT

Ischemic heart disease, in particular its acute form – myocardial infarction, is still a leading cause of morbidity and mortality worldwide despite recent advances in its therapy. Search for efficient protective strategies against myocardial ischemia/reperfusion injury is, therefore, one of the major tasks of both clinical and experimental cardiology. It has been well documented that adaptation to chronic hypoxia can improve cardiac tolerance to all major manifestations of injury caused by acute oxygen deprivation. However, the complex molecular mechanism underlying this long-lasting protective effect remains poorly understood. Several cascades of protein kinases could play an important role in signal transduction through protein phosphorylation leading to the protected cardiac phenotype. The purpose of this chapter is to summarize available experimental data concerning the effects of various forms and protocols of chronic hypoxia on myocardial expression, subcellular distribution and activity of protein kinases, and to evaluate the current evidence indicating their involvement in the cardioprotective mechanism.

INTRODUCTION

Continuous ATP production by mitochondrial oxidative phosphorylation is of essential importance for the maintenance of high-energy turnover of adult mammalian myocardium. Adequate supply of oxygenated blood *via* coronary circulation is, therefore, the basic requirement for cardiomyocyte function and viability. The heart is highly susceptible to acute oxygen deprivation leading rapidly to lethal myocyte injury, the extent of which is an important determinant of survival in patients with acute myocardial infarction. Until now, no drug has been specifically approved for the reduction of infarct size in these patients [Bolli, 2007]. As myocardial infarction has been a major cause of morbidity and mortality worldwide, the search for effective protective strategies that may reduce the extent of injury by improving the cardiac ischemic tolerance remains to be the major challenge for both clinical and experimental cardiology.

In the past two decades, a considerable attention has been paid to various forms of preconditioning, a short-lived adaptive defense phenomenon [Murry et al., 1986], which markedly increases myocardial intrinsic capability to cope with acute oxygen deprivation. Preconditioning exerts powerful and reproducible protective effects against all major manifestations of acute ischemia/reperfusion insult. Understanding the molecular mechanism of this phenomenon is a basic prerequisite for its potential therapeutic exploitation. Although a considerable progress has been achieved in this ambitious task [reviewed by Yellon & Downey, 2003; Bolli, 2007; Liehm et al., 2007; Halestrap et al., 2007], detailed roles of various triggering substances, signal transduction pathways and effector molecules still remain insufficiently resolved. Despite the fact that preconditioning represents the most powerful way to increase cardiac ischemic tolerance, its protective effects last only for short time. In its classical form, the protection disappears usually within a couple of hours following a preconditioning stimulus, and its delayed phase may last up to about 3 days [Kuzuya et al., 1993]. This could be considered as one of the serious practical limitations for an introduction of preconditioning-based therapy into clinic for patients at risk of infarction.

Several attempts have been made to overcome this limitation by maintaining a protected phenotype of the heart for a prolonged period of time. For example, Peart and Gross [2004; 2006] were able to utilize knowledge derived from preconditioning studies and induce longer-lasting myocardial protection by chronic treatment of animals with opioids. Similarly, repeated administration of an activator of mitochondrial ATP-sensitive K^+ channels (K_{ATP}) maintained rat hearts in a constant preconditioned state [Wang et al., 2007]; this approach in which protective mimetics are administered repeatedly is sometimes referred to as chronic preconditioning. Another attractive approach is prophylactic gene therapy based on transferring to the myocardium the gene(s) responsible for the salvaging effect of delayed preconditioning, such as inducible nitric oxide (NO) synthase (iNOS). It was demonstrated that a selective increase in iNOS activity *via* gene transfer in mice led to a persistent limitation of myocardial infarct size for at least several weeks [Li et al., 2006]. In light of these novel intriguing findings, yet another, historically the first recognized way of inducing and maintaining protected cardiac phenotype via a long-term adaptation to various environmental stressors such as chronic hypoxia, has not been adequately appreciated.

CARDIOPROTECTION BY CHRONIC HYPOXIA

Chronic myocardial hypoxia occurs in diverse clinical settings such as chronic obstructive lung disease or cyanotic congenital heart defects, and it is also naturally encountered in fetuses and in populations living in high altitude environments. Since late 1950s, both epidemiological observations in humans and the subsequent experimental studies on animals kept in hypobaric or normobaric hypoxia chambers for a prolonged period of time have demonstrated that chronically hypoxic myocardium is more tolerant to ischemia/reperfusion injury than normoxic myocardium [reviewed by Ostadal & Kolar, 2007]. The reduced size of myocardial infarction, improved postischemic recovery of contractility and lower incidence of life-threatening ventricular arrhythmias represent major protective endpoints of chronic hypoxia. Compared to the transient character of preconditioning, these cardioprotective effects may persist weeks or even months after the cessation of hypoxic exposure [Faltova et al., 1987; Neckar et al., 2004] making this phenomenon promising for potential therapeutic purposes. Nevertheless, the molecular mechanism of protection by chronic hypoxia was rarely investigated until recently. It was found that chronic hypoxia utilizes essentially the same pool of endogenous protective pathways as preconditioning, although with different efficiency [Neckar et al., 2002]. Unlike the early phase of preconditioning, chronic hypoxia not only activates these signaling pathways, but also affects the expression of their components and of other proteins involved in maintaining oxygen homeostasis via transcription factors such as hypoxia-inducible factor 1 (HIF-1α) [Semenza, 2004]. The list of signals and protective molecules that have been shown to participate in the protective mechanism of chronic hypoxia includes, among others, reactive oxygen species (ROS), NO, catecholamines, opioids, angiotensin II, erythropoietin, both sarcolemmal and mitochondrial K_{ATP} channels, mitochondrial permeability transition pore, Na^+/Ca^{2+} exchanger, sarcoplasmic reticulum Ca^{2+}-ATPase, phospholamban, ryanodine receptors, antioxidant enzymes, and pro- and antiapoptotic factors [Kolar & Ostadal, 2004; Manukhina et al., 2006; Ostadal & Kolar, 2007; Zaobornyj et al., 2007; Essop 2007]. Obviously, this list is far from complete and the precise roles played by these and other molecules in the complex protective network of chronic hypoxia remain obscure.

It has been well established that protein kinases could play an important role in signal transduction through protein phosphorylation leading to the protected cardiac phenotype of preconditioned hearts [Yellon & Downey, 2003; Ravingerova et al., 2003]. The purpose of this chapter is to collect the available experimental data concerning effects of various forms and protocols of chronic hypoxia on myocardial expression, subcellular distribution and activation of protein kinases, and to summarize current evidence indicating their involvement in the cardioprotective mechanism. We focus on *in vivo* models of chronic intermittent hypobaric and normobaric hypoxia (IHH and INH, respectively) but several studies using continuous normobaric hypoxia (CNH) are also included for completeness. The reason is that conventional hypoxic chambers usually do not allow feeding of animals and maintenance without opening and the resultant unplanned reoxygenation [Morel et al., 2006]. Thus, some of the previously reported continuous hypoxia models were, in fact, having included some brief and infrequent periods of normoxia. On the other hand, the reports dealing with sleep apnea, hypoxic preconditioning or cell models of subacute hypoxia are out of the scope of this chapter. These studies are discussed in details in other chapters of this book.

FAMILY OF PROTEIN KINASES

Phosphorylation of proteins catalysed by kinases is one of the posttranslational covalent modifications that represent the most common mechanism by which the protein function can be altered. Approximately one-third of eucaryotic proteins can be phosphorylated on serine, threonine, or tyrosine sites, and these phosphorylation events are crucial for the control of cellular function. Reversible phosphorylation/dephosphorylation is also firmly established fundamental mechanism for regulating the activity of most of the members of the mammalian protein kinase family. One of the most general, and the best understood forms, is phosphorylation at the activation loop, a segment nearby the active site of both serine/threonine and tyrosine kinases that must be phosphorylated for catalytic activity [reviewed by Patwardhan & Miller, 2007].

Protein kinases A (PKA; cAMP-dependent protein kinase), B (PKB; Akt), C (PKC), and G (PKG; cGMP-dependent protein kinase) are major players in cellular signal transduction whose biological function depends on a series of ordered phosphorylation events. This cascade phosphorylation of PKA, PKB, PKC and PKG is triggered by phosphoinositide-dependent kinase-1 (PDK-1). PDK-1 is important in cell signalling by providing the activating phophorylation for a majority of serine/threonine protein kinases [Toker & Newton, 2000; Mora et al., 2004]. The structure of kinase domains of PKA/PKB/PKC/PKG are highly conserved with more than 40% overall sequence identity. All these kinases share similar key phosphorylation sites in activation loop, in turn motif and in hydrophobic motif at C-terminal extension of kinase moieties. However they vary in the nature of the regulatory moiety. PKA has regulatory moiety as a separate polypeptide, whereas the regulatory determinats are on the same polypeptide chain as kinase core for PKB and PKC [Newton, 2003]. PKA and PKG are activated by cyclic nucleotides and they need specific kinase anchoring proteins providing their localization into discrete cellular compartment [Dodge-Kafka et al., 2006]. PKC and PKB are target proteins for lipid signaling molecules e.g. diacylglycerols and phosphoinositides, generated from membrane glycerophospholipids by activation of phospholipases and/or lipid kinases. These signaling lipids are key molecules for recruitment of protein kinases to the membrane where are fully active and further regulated by interaction with wide variety of other proteins [Hawkins et al., 2006; Kheifets & Mochly-Rosen, 2007].

The mitogen-activated protein kinase (MAPK) cascades also play a pivotal role in many cellular functions and convey signals in the form of phosphorylation events. MAPKs are phosphorylated by MAP kinase kinases (MAPKKs) and phosphorylate various targets such as MAPK-activated protein kinases (MAPKAPKs) or transcription factors. MAPKs are dephosphorylated and inactivated by several MAPK-phosphatases (MKPs). There are four subgroups in the MAPK family: ERK (extracellular signal-regulated kinase), p38, JNK/SAPK (c-Jun NH_2-terminal kinase) and ERK5. ERK is activated mainly by mitogenic stimuli, whereas p38 and JNK/SAPK are activated mainly by stress stimuli or inflammatory cytokines. ERK5 is activated by growth factors, osmotic stress and oxidative stress. These protein kinases mediate variable responses, such as gene expression, cell proliferation, differentiation, apoptosis etc., depending on the cell type. There are two main mechanisms regulating the signal transduction in the MAPK cascades: the docking interaction and the scaffolding one. The docking interaction is accomplished through specific conserved regions

on MAPKs with appropriate MAPK-interacting molecules. Scaffolding interaction generally requires a third molecule to tether enzymes and substrates [Tanoue & Nishida, 2003].

Protein Kinase C

PKC is a class of serine/threonine kinases with N-terminal regulatory and C-terminal catalytic regions, consisting of at least 11 isozymes, which are divided into three groups. The members of a conventional group (α, $\beta_{1/2}$ and γ) are dependent on Ca^{2+}, 1,2-diacylglycerol (DAG) and phosphatidylserine (PS) for their activation; novel PKC isoforms (δ, ε, η and θ) are Ca^{2+}-independent but require both DAG and PS; atypical isoforms (ζ and ι/λ) require PS but neither Ca^{2+} nor DAG. PKC α, δ, ε and ζ are the most abundant isoforms in the heart [Rybin et al., 1994]. PKC was the first kinase studied in a setting of myocardial ischemia/reperfusion. Since the first experimental evidence of its involvement in ischemic preconditioning [Ytrehus et al, 1994], many studies convincingly demonstrated that PKC plays a key role in various forms of innate and acquired cardioprotection. It is, therefore, not surprising that PKC attracted more attention than other kinases also in studies of protective signal transduction pathways of chronic hypoxia.

Effects of Chronic Hypoxia on PKC

Table 1 summarizes available experimental data that describe changes in myocardial expression, subcellular distribution and activity of PKC isoforms induced by chronic hypoxia. In order to avoid any misinterpretation caused by PKC involvement in hypertrophic growth of the right ventricle of chronically hypoxic animals, only data concerning left ventricular myocardium are listed. Obviously, their analysis is complicated by various experimental models and protocols of hypoxic adaptation as well as by species and age of animals used. In adult rats, 6 weeks of IHH corresponding to an altitude of 5000 m led to an increased abundance of PKC isoforms α, δ and ε in the myocardial particulate fraction [Ding et al. 2004]. Cataldi et al. [2004] focused on PKC α and demonstrated its increased phosphorylation after 12 days of INH (10% O_2). In contrast, two other reports did not show any significant effect of intermittent hypoxia at comparable intensity either on the PKC activity and content in total tissue homogenate [Rouet-Benzineb et al., 1999] or on the content and distribution of PKC isoforms α, δ, ε and ζ in cellular fractions [Morel et al., 2003]. In our studies using more severe model of IHH (7000 m), we detected an increased abundance of PKC δ in myocardial particulate fraction compared to normoxic controls. This effect was dependent on oxidative stress associated with intermittent hypoxia as it was completely prevented by chronic antioxidant treatment of animals during the hypoxic adaptation period [Kolar et al., 2007]. Since PKC activity and function are influenced by quantity and quality of lipid signaling molecules and by lipid membrane composition [Giorgione et al., 1998; Madani et al., 2001], we also examined the effect of altered phospholipid fatty acid (FA) profile, caused by various lipid diets, on myocardial PKC expression in chronically hypoxic rats. While IHH markedly upregulated PKC δ in animals fed diets enriched with either saturated or

n-3 polyunsaturated FA (PUFA), this effect was absent in the n-6 PUFA diet group [Hlavackova et al., 2007].

More detailed analysis revealed that the relative content of PKC δ increased most dramatically in mitochondria- and nuclear-cytoskeletal-enriched fractions and to a somewhat smaller extent in the microsomal fraction of chronically hypoxic (IHH) rat myocardium [Neckar et al., 2005]. An increased association of PKC δ predominantly with mitochondria was confirmed in our pilot immunohistochemical study, which documented much higher level of co-localization of this isoform with the marker of OXPHOS complexes in the hypoxic myocardium compared to normoxic tissue; this effect was reversed by administration of a PKC δ-selective inhibitor rottlerin [Hlavackova et al., 2008]. In addition, our experiments revealed that chronic IHH of 7000 m could significantly reduce the content in the particulate fraction of another isoform, PKC ε, which is a key player in various forms of preconditioning [Kolar et al., 2007; Hlavackova et al., 2007]. In contrast to the effect of IHH in adult rats, the exposure of neonatal rabbits to CNH (10% O_2) for 10 days resulted in increased phosphorylation of PKC ε and its redistribution to the particulate fraction [Rafiee et al., 2002]. Interestingly, similar redistribution of this isoform, but not of PKC α, β, γ, δ and ζ, was observed in the right atrial tissue of infant patients undergoing surgery for cyanotic congenital heart defects [Rafiee et al., 2002]. Last but not least, the exposure of pregnant rats to CNH for 7 days led to a decreased content of PKC ε in the myocardium of their offspring at adulthood while contents of PKC α, β_1, β_2, δ and ζ were not affected [Li et al., 2004].

Involvement of PKC in Cardioprotection by Chronic Hypoxia

Taken together, it appears that chronic hypoxia results, at least in some cases, in the upregulation of specific PKC isoforms and/or their subcellular redistribution and activation. However, these effects do not necessarily mean that the enzyme is involved in the cardioprotective mechanism. Several studies, using various protocols of IHH and INH in adult rats or CNH in neonatal rabbits, addressed this question on different *in vivo* and *ex vivo* models of acute oxygen deprivation with the aid of pharmacological tools (Table 2). All these studies in concert succeeded to demonstrate that a general PKC inhibitor (chelerythrine or calphostin C) administered prior to ischemic or anoxic insult completely abolished cardioprotective effects of chronic hypoxia that manifested as an improved postischemic recovery of cardiac contractile function [Rafiee et al., 2002; Ding et al., 2004], reduced myocardial infarct size [Neckar et al. 2005], improved ionic transport and prevention of intracellular Na^+ and Ca^{2+} overload [Ding et al., 2004; Yeung et al. 2007], and attenuation of intracellular acidification [Li et al., 2007]. These results clearly indicate that the activity of PKC during the acute ischemic insult is required for the manifestation of protected phenotype of chronically hypoxic hearts. In line with this conclusion, chelerythrine was able to abolish the infarct size-limiting effect of *in vivo* intermittent hypoxic preconditioning in rats when administered before test ischemia but not before the initial hypoxic stimulus [Beguin et al., 2007], suggesting that PKC is an important mediator while the trigger phase of delayed protection is independent of this enzyme.

Table 1. Summary of reports on effects of chronic hypoxia on various protein kinases in the left ventricular myocardium.

Model of chronic hypoxia	Species	Protein kinase	Effect	Reference
IHH, 5000 m, 6 h/day, 6 wk	Adult rats	PKC α, δ, ε	↑ Level in particulate fraction	Ding et al. 2004
IHH, 5500 m, 23 h/day, 2 wk	Adult rats	PKC α, δ, ε, ζ	No effects on levels, subcellular distribution and activity	Morel et al. 2003
IHH, 7000 m, 8 h/day, 5–6 wk	Adult rats	PKC δ PKC ε	↑ Levels in all cellular fractions No effect	Neckar et al. 2005
IHH, 7000 m, 8 h/day, 5-6 wk	Adult rats	PKC δ PKC ε	↑ Level in particulate fraction ↓ Level in particulate fraction	Kolar et al. 2007, Hlavackova et al. 2007
INH, 10% O_2, 23 h/day, 1-3 wk	Adult rats	PKC	No effect on activity or level in homogenate	Rouet-Benzineb et al. 1999
INH, 10 % O_2, 12 h/day, 12 days	Neonatal to senescent rats	PKC α	↑ Phosphorylation in homogenate	Cataldi et al. 2004
CNH, 10.5% O_2, 7 days	Prenatal rats	PKC ε PKC α, β_1, β_2, δ, ζ	↓ Level in homogenate (late effect examined at adulthood) No effect	Li et al. 2004
CNH, 10% O_2, 10 days	Neonatal rabbits	PKC ε p38 MAPK JNK ERK	↑ Phosphorylation and translocation to particulate fraction ↑ Phosphorylation and translocation to particulate fraction ↑ Phosphorylation and translocation to particulate fraction No effect	Rafiee et al. 2002
INH, 12% O_2, 8 h/day, 8 wk	Adult rats	p38 MAPK MAPKK5 ERK5	↑ Phosphorylation in tissue extract ↑ Level in tissue extract ↑ Level in tissue extract	Chen et al. 2007
CNH, 10% O_2, 2 wk INH, 10% O_2, 23 h/day, 2 wk	Adult rat	p38 MAPK JNK1/2 ERK1/2 p38 MAPK JNK1/2 ERK1/2	↑ Phosphorylation and activity in cytosol and nucleus No effect No effect No effect ↑ Activity in cytosol and nucleus, no effect on phosphorylation ↑ Phosphorylation and activity in cytosol and nucleus	Morel et al. 2006
IHH, 7000 m, 8 h/day, 5 wk	Adult rats	p38 MAPK JNK ERK1/2 Akt	↑ Phosphorylation in soluble fraction ↓ Level No effect ↑ Phosphorylation	Strniskova et al. 2006
CNH, 10% O_2, 10 days	Neonatal rabbits	Akt	↓ Phosphorylation in tissue extract	Shi et al. 2002
INH, 12% O_2, 8 h/day, 4-8 wk	Adult rats	Akt	↓ Phosphorylation in tissue extract	Lee et al. 2007

IHH, intermittent hypobaric hypoxia; INH, intermittent normobaric hypoxia; CNH, continuous normobaric hypoxia; PKA, cAMP-dependent protein kinase; CAMKII, Ca^{2+}/calmodulin-dependent protein kinase II; Akt (PKB), protein kinase B; MAPK, mitogen-activated protein kinase; ERK (p42/p44 MAPK), extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase.

Although the role of PKC in cardioprotection by chronic hypoxia appears to be well established, the identity of isoform(s) involved has not been fully elucidated. Individual PKC isoforms regulate distinct cellular functions depending on their subcellular translocation upon activation, which places them in a close proximity to their molecular targets. This process is mediated by binding of each isoform to its selective anchoring protein, receptor for activated C-kinase [Mochly-Rosen, 1995]. Based on changes in their expression and subcellular distribution, mainly two novel PKC isoforms, δ and ε, can be considered as the potential mediators of protection in chronically hypoxic hearts. Activation of PKC ε, the key component of signal transduction of preconditioning, has been proposed to be responsible for the CNH-induced cardioprotection in neonatal rabbits [Rafiee et al., 2002; 2003]. In this experimental model, chelerythrine reversed translocation of PKC ε from the cytosolic to the

particulate fractions and abolished the protection. However, this cannot be taken as a proof of the involvement of this particular isoform because chelerythrine acts as a non-selective inhibitor of all PKC isoforms. On the other hand, our experiments demonstrated that adaptation of adult rats to IHH decreased the myocardial expression of PKC ε, and this effect was independent of interventions (antioxidant treatment, diet enriched with n-6PUFA) that prevented the induction of protected cardiac phenotype [Kolar et al., 2007; Hlavackova et al., 2007], suggesting that this isoform is unlikely to play a crucial role. It is unclear whether these diverse results are due to different models of hypoxia, animal species used or their age.

Table 2. Summary of studies of the involvement of various protein kinases in cardioprotection by chronic hypoxia.

Model of chronic hypoxia	Species	Model of acute O_2 deprivation	Protective effect	Protein kinase	Effect of protein kinase inhibitor on protection	Reference
IHH, 5000 m, 6 h/day, 6 wk	Adult rats	Perfused heart, global I/R Ventricular myocytes, A/R	↑ Recovery of function Prevention of Na^+ and Ca^{2+} overload	PKC	Abolished (chelerythrine) Abolished (chelerythrine)	Ding et al. 2004
IHH, 7000 m, 8 h/day, 5-6 wk	Adult rats	In vivo, regional I/R	↓ Infarct size	PKC PKC δ	Abolished (chelerythrine) Attenuated (rottlerin)	Neckar et al. 2005
IHH, 5000 m, 6 h/day, 6 wk	Adult rats	Ventricular myocytes, A/R	↓ Intracellular acidification	PKC	Abolished (chelerythrine)	Li et al. 2007
INH, 10% O_2, 6 h/day, 1 wk	Adult rats	Ventricular myocytes, A/R	Improved Ca^{2+} handling	PKC PKA CAMKII	Abolished (chelerythrine, calphostin C) Abolished (KT5720) No effect (KN-93)	Yeung et al. 2007
IHH, 5000 m, 6 h/day, 6 wk	Adult rats	Perfused heart, global I/R	↑ Recovery of function	PKA CAMKII	Abolished (H-89) Abolished (KN-93)	Xie et al. 2005
IHH, 7000 m, 8 h/day, 5 wk	Adult rat	In vivo, regional I/R	↓ Infarct size	PI3-K	Attenuated (LY294002)	Ravingerova et al. 2007
CNH, 10% O_2, 10 days	Neonatal rabbits	Perfused heart, global I/R	↑ Recovery of function	PKC p38 MAPK JNK ERK	Abolished (chelerythrine) Abolished (SB203580) Abolished (curcumin) No effect (PD98059)	Rafice et al. 2002

IHH, intermittent hypobaric hypoxia; INH, intermittent normobaric hypoxia; CNH, continuous normobaric hypoxia; I/R, ischemia/reperfusion; A/R, anoxia/reoxygenation; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; CAMKII, Ca^{2+}/calmodulin-dependent protein kinase II; PI3-K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; ERK (p42/p44 MAPK), extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase.

The function of PKC δ in myocardial ischemia/reperfusion injury and protection is a matter of controversy. It seems that PKC δ has the greatest flexibility among all isoforms to affect various cellular functions because the consequences of its activation depend on its localization to various subcellular compartments that is finely controlled by phosphorylation at multiple serine/threonine and tyrosine residues [Steinberg, 2004]. In terms of ischemic tolerance, this isoform can be both protective and detrimental depending on the time of activation as reported in a growing number of articles. Considerable experimental evidence has accumulated indicating that PKC δ aggravates myocardial injury when activated at reperfusion. Convincing data in support of this conclusion were reported mainly by Mochly-Rosen and co-workers [e.g. Inagaki et al., 2003] using the PKC δ-selective inhibitor peptide δV1-1. This detrimental effect of PKC δ seems to be due to stimulation of excess ROS production and activation of proapototic pathways [Murriel et al., 2004]. On the other hand, the same isoform has been shown to play a beneficial role as a mediator of various forms of

cardioprotection including ischemic and pharmacological preconditioning [Hirotani & Sadoshima, 2005] as well as anesthetic preconditioning [Bouwman et al., 2006]. It appears that protective effects of PKC δ are manifested when its activation occurs well before an ischemic insult: according to Inagaki and Mochly-Rosen [2005], about an hour is needed for a salutary effect to predominate. Obviously, this condition is more than satisfied in any setting of chronic hypoxia. Results of our studies implicated involvement of PKC δ in the cardioprotective mechanism induced by IHH, at least in adult rats. Indirect evidence in favor of this conclusion is as the follows:

1) Chronic hypoxia increased PKC δ expression and caused its subcellular redistribution with a predominant localization into mitochondria. This effect was reversed by rottlerin [Hlavackova et al., 2008]. Similarly, the infarct size-limiting effect of IHH was significantly attenuated by rottlerin [Neckar et al. 2005]. However, it is necessary to admit that, in addition to inhibiting PKC δ, several side effects of this drug were reported recently [Soltoff, 2007] that might complicate an interpretation of these results.
2) Chronic treatment of animals during the adaptation to hypoxia with an antioxidant prevented both the cardioprotection and PKC δ upregulation/redistribution [Kolar et al., 2007].
3) Unlike in other diet groups, IHH did not increase myocardial ischemic tolerance in rats supplemented with n-6 PUFA-enriched diet in line with the absence of PKC δ upregulation in this group (Figure 1 top and middle panels) [Hlavackova et al., 2007].
4) As revealed by regression analysis, the relative content of PKC δ in the myocardial particulate fraction exhibited a close negative correlation with myocardial infarct size: the higher isoform content, the smaller infarction (Figure 1 bottom panel) [Hlavackova et al., 2007]. This last point provides probably the most convincing piece of evidence available so far in support of the role of this PKC isoform in cardioprotection by chronic hypoxia although it cannot be taken as a proof of causality. Clearly, more direct evidence is needed, based, for example, on effects of PKC δ-selective inhibitor and activator peptides and use of relevant transgenic models.

Another important yet unresolved question concerns potential downstream molecular targets of PKC that might be responsible for cardioprotective effects of chronic hypoxia. Although several speculative possibilities have been proposed mostly on the basis of analogy with preconditioning, none of them has been proved experimentally so far. At present, therefore, it appears to be premature to discuss this issue here.

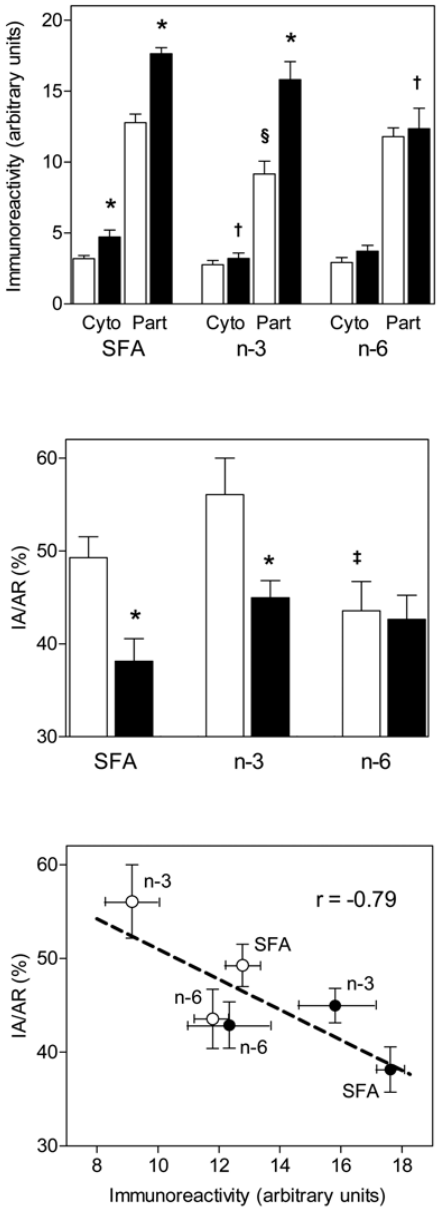

Figure 1. (Top panel) Expression of PKC δ in myocardial cytosolic (Cyto) and particulate (Part) fractions, (Middle panel) myocardial infarct area (IA) normalized to the area at risk (AR), and (Bottom panel) the relationship between the mean values of PKC δ expression in the Part and the mean IA/AR in normoxic (N, open columns or circles) and chronically hypoxic (H, black columns or circles) rats fed diets enriched with different fatty acid (FA) classes. Animals were fed a nonfat diet enriched with 10% lard (saturated FA, SFA), fish oil (n-3 polyunsaturated FA) or corn oil (n-6 polyunsaturated FA) for 10 weeks. After 4 weeks on the diet, each group was divided into two subgroups that were either exposed to intermittent hypobaric hypoxia (7000 m, 8 h/day) or kept at normoxia for an additional 5-6 weeks. Values are mean±S.E.M. *P<0.05 vs. normoxic group, †P<0.05 vs. SFA group, ‡P<0.05 vs. n-3 group, §P<0.05 vs. other diet groups; r indicates correlation coefficient. Redrawn from Hlavackova et al. (*Exp. Biol. Med.* 232: 823-832, 2007) with permission.

Phosphatidylinositol 3-Kinase/Akt

Data from several laboratories have implicated activation of the Akt (PKB) family of pro-survival kinases in various cardioprotective phenomena. The Akt family of serine-threonine kinases consists of three isoforms, but the majority of total Akt protein in the myocardium is formed by Akt1 and Akt2 [Yellon & Downey, 2003]. Akt is the main downstream target of phosphatidylinositol 3-kinase (PI3-K); activation of PI3-K by a number of stimuli activates Akt *via* PDK-1. The PI3-K/Akt as well as the ERK1/2 pathways are often referred as reperfusion injury salvage kinase (RISK) pathway, because their protective signaling is essential during the postischemic reperfusion phase [Hausenloy & Yellon, 2004].

In contrast to the involvement of PI3-K/Akt in preconditioning and postconditioning, the role of this pathway in long-lasting cardiac protection by chronic hypoxia is unknown. Only few studies attempted to determine the activation of Akt in chronically hypoxic myocardium, however yielded diverse results (Table 1). Increased phosphorylation at serine^{473} without a change in total protein level of Akt was observed in adult rats adapted to severe IHH (7000 m) [Strniskova et al., 2006] while two other studies detected markedly decreased expression and phosphorylation of Akt in rats adapted to INH (12% O_2) [Lee et al., 2007] and in neonatal rabbits maintained at CNH (10% O_2) [Shi et al., 2002]. To our knowledge, only one study examined this pathway using pharmacological tools: administration of a selective inhibitor of PI3-K (LY294002) before an ischemic insult attenuated the infarct size-limiting effect of IHH in rats, suggesting that the activation of PI3-K/Akt cascade may play a role in this cardioprotective mechanism [Ravingerova et al., 2007]. Interestingly, this pathway has been shown to play a role also in protective signaling induced in heart-derived H9c2 cells by their exposure to mild (13% O_2) hypoxia for 24 hours [Crawford et al., 2003]. However, it should be mentioned that chronic activation of Akt might also have negative effects as it is involved in the progression of heart failure [Naga Prasad et al., 2000]. Needless to say that more experiments are needed to resolve this issue.

Mitogen-Activated Protein Kinases

Mitogen-activated protein kinases are a large family of serine-threonine kinases that are activated by various stimuli and mediate diverse cellular responses including cardiac protection against ischemia/reperfusion injury. Although numerous reports demonstrated the involvement of all major MAPK pathways in the mechanism of ischemic preconditioning and related protective phenomena, their role in myocardial injury or survival is still a matter of debate [reviewed by Ravingerova et al., 2003]. Concerning cardioprotection by chronic hypoxia, only few studies dealt with MAPK, and the results were rather inconsistent and controversial (Table 1). Thus, phosphorylation and activity of ERK1 (p44 MAPK) and ERK2 (p42 MAPK) were increased in the myocardial cytosolic and nuclear fractions of adult rats exposed to INH (10%) for two weeks [Morel at al., 2006] while no effect was observed in rats [Morel et al., 2006] and neonatal rabbits [Rafiee et al., 2002] exposed to CNH of the same degree and duration. Similarly, IHH of 7000 m for 5 weeks did not affect myocardial ERK1/2 level in rats [Strniskova et al., 2006] and no activation of ERK1/2 was detected in chronically hypoxic infant human hearts [Rafiee et al., 2002]. The observation that ERK1/2 inhibitor PD98059 failed to abolish the increase in the postischemic recovery of cardiac contractile

function in chronically hypoxic neonatal rabbits suggests that this pathway does not play a role in the protective mechanism [Rafiee et al., 2002].

On the other hand, there seems to be a consensus that chronic hypoxia activates p38 MAPK. The increased activity, level of phosphorylation and/or subcellular translocation was found in hearts of rats and rabbits adapted to various protocols of CNH, INH or IHH [Rafiee et al., 2002; Morel et al., 2006; Strniskova et al., 2006; Chen et al., 2007] as well as in chronically hypoxic infant human myocardium [Rafiee et al., 2002]. Interestingly, CNH-induced phosphorylation and translocation of p38 MAPK to the particulate fraction was absent in the hypoxic rats, which were exposed daily to normoxia for one hour [Morel et al., 2006], suggesting that periodic reoxygenation may blunt the hypoxic activation of this kinase. However, the fact that others demonstrated positive effects of intermittent hypoxia on p38 MAPK phosphorylation does not support this view (Table 1). It has been proposed that p38 MAPK is involved in the cardioprotective mechanism of chronic hypoxia because its inhibitor SB203580 abolished the beneficial effect on postischemic contractile dysfunction in rabbit hearts [Rafiee et al., 2002]. Moreover, this compound reduced CNH-induced increase in transcript levels of inducible heat shock protein 70 (Hsp70i) and reversed subcellular redistribution of Hsp70i protein, which is assumed to play a role in mediating ischemic resistance [Rafiee et al., 2003].

The third MAPK subgroup, JNK, seems to exhibit rather inconsistent responses to chronic hypoxia. While IHH in rats resulted in a decrease in its myocardial level [Strniskova et al., 2006], CNH in neonatal rabbits led to increased phosphorylation and translocation of JNK to the particulate fraction [Rafiee et al., 2002]. In contrast, INH in rats stimulated the activity of JNK in cytosolic and nuclear fractions but CNH of the same degree and duration had no effect [Morel et al., 2006]. Due to different species and models of hypoxia, these limited data do not allow a convincing conclusion. Nevertheless, the potential involvement of JNK cascade in protective mechanism of chronic hypoxia is supported by the observation that its inhibitor curcumin abolished both the improvement of postichemic recovery of contractility and the subcellular redistribution of Hsp70i in chronically hypoxic infant rabbit hearts [Rafiee et al., 2002; 2003].

OTHER PROTEIN KINASE PATHWAYS

Yet other protein kinase pathways may contribute to cardioprotective effects of chronic hypoxia. Xie et al. [2005] demonstrated that H-89, an inhibitor of cAMP-dependent protein kinase (PKA), abolished the improved postischemic recovery of cardiac function (rate of relaxation) induced by IHH (5000 m) in rats. Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) inhibitor KN-93 exhibited similar effect as H-89. Both drugs also inhibited the IHH-induced phosphorylation of phospholamban, thereby preventing its beneficial effect on depression of the sarcoplasmic reticulum Ca^{2+}-ATPase activity caused by ischemia/reperfusion. In addition, PKA inhibition with KT5720 but not CaMKII inhibition with KN-93 abolished the increased function of ryanodine-sensitive Ca^{2+}-release channels in anoxic/reoxygenated cardiomyocytes isolated from the hearts of INH (10% O_2) rats [Yeung et al., 2007]. These results implicate activation of PKA and CaMKII pathways in improved

myocardial Ca^{2+} handling, which may contribute to the salutary effects of chronic hypoxia against acute ischemia/reperfusion injury.

A few additional protein kinase pathways, which may play a role in survival signaling in myocardial ischemia/reperfusion, had not been investigated in chronically hypoxic hearts. For example, cGMP-dependent protein kinase (PKG) pathway may act as an important pro-survival cascade as recently appreciated [reviewed by Burley et al., 2007]. Baker et al. [1999] proposed that protection of neonatal rabbit hearts by CNH might involve activation of PKG *via* increased production of NO, which activates soluble guanylyl cyclase causing cGMP accumulation. However, they did not analyze PKG directly and provided no experimental evidence indicating its role in this model of protection. Last but not least, AMP-activated protein kinase (AMPK) can be considered in association with cardioprotection by chronic hypoxia as it is activated by a number of stimuli including hypoxia and oxidative stress [Hardie et al., 2003]. Although AMPK pathway may contribute to protection in various settings of ischemia/reperfusion, its role remains controversial [Dyck & Lopaschuk, 2006]. Its potential involvement in the mechanism of improved ischemic tolerance of chronically hypoxic hearts has so far been neglected.

CONCLUSION

Taken together, the available data suggest that several protein kinase pathways may participate in the complex cardioprotective signaling in various models of chronic intermittent or continuous hypoxia. At present, the involvement of PKC seems to be the only well established although some controversy remains over which of its isoforms is of crucial importance. There is limited evidence also in favor of a contribution of other protein kinases, such as p38 MAPK, JNK, PI3-K/Akt cascade, PKA and CaMKII. Significance of these pathways, their regulation, mutual interactions, and molecular targets responsible for protection of chronically hypoxic hearts remain to be elucidated.

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