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Postnatal development of phospholipids and their fatty acid profile in rat heart

František Novák,[1] Eva Tvrzická,[2] Blanka Hamplová,[3] František Kolář[4] and Olga Nováková[3]

[1]*Department of Biochemistry, Faculty of Science, Charles University, Prague, Czech Republic;* [2]*1st Faculty of Medicine, Charles University, Prague, Czech Republic;* [3]*Department of Physiology and Developmental Biology, Faculty of Science, Charles University, Prague, Czech Republic;* [4]*Institute of Physiology, Academy of Sciences of the Czech Republic and Centre for Cardiovascular Research, Prague, Czech Republic*

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Abstract

The aim of this study was to determine the concentration of phospholipids (PL), plasmalogen components of choline (PC) and ethanolamine (PE) phosphoglycerides (PLPC, PLPE) and fatty acid profile of PL and triacylglycerols (TAG) in developing rat left ventricular myocardium between postnatal day (d) 2 and 100. The steepest increase of total PL (TPL) concentration occurs between d2 and d5, followed by a further slower increase between d20 and d40. Similar developmental changes were observed in PC and PE. The PLPE concentration rises by d10, whereas PLPC does not change during the whole period investigated, except for the transient decline on d5. The concentration of diphosphatidylglycerol (DPG) increases by d60; the steepest rise occurs between d20 and d40. Phosphatidylinositol (PI) concentration rises only by d5. The concentration of phosphatidylserine (PS) decreases between d5 and d10 and then it does not change. Sphingomyelin (SM) concentration is maintained till d10, it declines on d20 and does not change thereafter. The proportion of saturated fatty acids (SFA) increases by d5 in PC, PE, PS and TAG, and by d10 in DPG and PI. After d20 the SFA proportion gradually decline in all lipids. Monounsaturated FA (MUFA) proportion decreases in PC, PE, PI and PS from d2 till d10, and in the weaning period it tends to rise again. In contrast, in DPG and TAG the proportion of MUFA declines during the whole postnatal period. N-6 polyunsaturated FA (PUFA) decrease in all PL by d20 and rise again thereafter; in TAG they decline between d2 and d10 and return to the initial level by d100. N-3 PUFA increase in all PL during the suckling period and decline after weaning; in TAG they increase only by d5 and then they decline. This remodeling of myocardial PL and TAG composition during postnatal development may affect membrane properties and contribute to developmental changes in the function of membrane proteins and cell signaling. (Mol Cell Biochem **293:** 23–33, 2006)

Key words: fatty acids, phospholipids, plasmalogens, postnatal development, rat heart

Introduction

Postnatal development is characterized by several critical periods that play a crucial role in the maturation of myocardium from delivery to adulthood. Immediately after birth the maternal supply of high-carbohydrate substrates ceases abruptly and newborns start to be fed with milk, which has a high-fat and low-carbohydrate composition. Successful adaptation of neonates to the nutritional and environmental changes requires an important modification of glucose and fatty acid

Address for offprints: F. Novák, Department of Biochemistry, Faculty of Science UK, Hlavova 8, 128 43 Prague 2, Czech Republic (E-mail: franov@natur.cuni.cz)

metabolism, which is mainly regulated by humoral factors. On the other hand, during the suckling–weaning transition, the milk intake is gradually replaced by solid food diet, which is higher in carbohydrate and lower in fat contents. Rat weanlings begin with the typical rhythm of food intake in 4 weeks of postnatal life when the circadian hormone secretion is initiated. All organs grow rapidly during the first postnatal weeks and regulatory mechanisms for biochemical activities must be reset during this period, which is connected with the high growth rate of the heart. This period is characterized by the preponderance of biosynthesis over degradation of membrane components while both processes are more balanced in the mature myocardium (for review see [1]). Early after birth, adaptation to increased workload stimulates hypertrophic growth of ventricular myocytes and leads to morphological and functional remodeling of myocardial tissue [2].

Membrane structures belong to the main targets of this maturation process including both protein [3] and lipid components [4]. Phospholipids (PL) as the main membrane lipids create the environment for membrane proteins, the function of which depends either on the proportion of individual PL species or on their fatty acid (FA) composition. Despite the importance of PL for membrane function, only a limited number of reports dealing with PL composition in the developing heart have appeared. Rogers [5] compared the PL composition and their FA profile between hearts of newborn and adult rats. Gudmundsdottir and Gudbjarnason [4] performed PL analysis in rat myocardium at several developmental stages. Although the reports mentioned above have shown that substantial alterations in cardiac membrane PL occur during maturation, they dealt with total PL (TPL), choline (PC) and ethanolamine (PE) phophoglycerides only. More detailed analysis of all PL species, which would help to clarify critical transitions in membranes during postnatal development, is still missing. Therefore, in our study we focused on changes in the concentration of individual PL species and their FA profile, the concentration of the plasmalogen component of PC (PLPC) and PE (PLPE) and the FA profile of triacylglycerols (TAG) in membranes of rat left ventricular (LV) myocardium at several developmental stages between postnatal day (d) 2 and 100.

Materials and methods

Animals

Hearts of male Wistar rats of different age were used for analysis. All animals including nursing mothers were fed the same vitamin-enriched, low-fat (3.5% by weight) standard diet (Velaz, Czech Republic). The diet contained, by our analysis (in mol %), 1.5 of 14:0, 19.2 of 16:0, 2.2 of 16:1n-7, 5.5 of 18:0, 25.4 of 18:1n-9, 1.9 of 18:1n-7, 38.7 of 18:2n-6 and 4.2 of 18:3n-3. Rats were sacrificed by cervical dislocation on postnatal days 2, 5, 10, 20, 40, 60, 80 and 100. Hearts were dissected into the right ventricle and the left ventricle including the septum (LV) and weighed. The LV was rinsed in cold saline, frozen and stored in liquid nitrogen. Each group contained six samples; for one sample we used five ventricles on d2, four on d5, 3 on d10, and one on d20–d100. The investigation conforms to 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).

Lipid analyses

Frozen LV myocardium was pulverized and homogenized. PL were extracted in three consecutive steps according to the method of Folch *et al.* [6]. PC, PE, diphosphatidylglycerol (DPG), sphingomyelin (SM), phosphatidylinositol (PI) and phosphatidylserine (PS) were separated by two-dimensional thin-layer chromatography according to the method of Rouser *et al.* [7]. Plasmalogen components of PC (PLPC) and PE (PLPE) were analyzed according to the method of Horrocks *et al.* [8]. PL spots were detected by iodine vapor, scraped out and analyzed for phosphorus [7].

For FA analysis, the PL were separated on plates with Silica Gel H (0.5 mm) according to the method of Rouser *et al.* [7]. Triacylglycerols (TAG) were separated by one-dimensional thin-layer chromatography using the solvent mixture hexane–ether–acetic acid (80:20:3, by vol.). Spots were observed under UV light after staining with 0.005% 2,7-dichlorofluorescein in methanol, scraped out and stored in nitrogen atmosphere at −20 °C until the next day when the methyl esters were prepared. For FA methyl ester preparation, sodium methanolate was added to tubes with silica gel and tubes were then incubated for 60 min at room temperature in the dark. Methyl esters were extracted with hexane; the extracts were evaporated under a stream of nitrogen and stored at −20 °C. FA methyl esters were separated by the gas chromatograph CP 438 A (Chrompack, Middelburg, The Netherlands) using middle polar column CP WAX 52 CB (25 m × 0.25 mm i.d.). The oven temperature was programmed from 145 to 230 °C at 2 °C/min. Hydrogen was used as carrier gas. FAs were identified using a mixture of FA methyl esters [9]. All chemicals were from Sigma unless otherwise indicated.

Statistical analysis

All results are expressed as means ± S.E.M. The statistical significance of differences between groups was determined by one-way ANOVA and subsequent Newman-Keul's test ($p < 0.05$).

Results

PL concentration

Growth of the body as well as of the right and left ventricles between d2 and d100 is documented in Table 1. The concentration of TPL, PC and PE in the LV myocardium is presented in Fig. 1A. The concentration of TPL increases between d2 and d10, followed by a steady-state between d10 and d20. A further increase in TPL follows between d20 and d40. PC concentration does not change between d2 and d20, a moderate increase occurs by d40, followed by a gradual decline by d80. PE concentration rises gradually by d80. The concentration of PLPC and PLPE is shown in Fig. 1B. PLPC maintains a constant level during the whole developmental period, except for the transient decline on d5. The level of PLPE increases by d10 and does not change thereafter.

Figure 1C illustrates developmental changes in the concentration of DPG, PI, PS and SM. The concentration of DPG increases by d60 with the steepest rise between d20 and d40. In comparison with DPG, the changes in concentration of minor PL (PI, PS and SM) are less prominent. The initial rise in the concentration of PI occurs on d5 and then it does not change. The concentration of PS decreases between d5 and d10 and remains constant thereafter. SM concentration declines between d10 and d20 and then it does not change.

Table 2 demonstrates the distribution of individual PL as a percentage of TPL.

Distribution of FA in individual PL and TAG

FA composition of individual PL species and TAG of the developing rat heart is documented in Tables 3–8. The most abundant saturated FA (SFA) in PC, PE and PI (Tables 3–5, respectively) are palmitic (16:0) and stearic (18:0) acids. In PC, the proportion of 16:0 decreases gradually during postnatal development, whereas that of 18:0 increases by d5 and then it keeps constant; on the other hand, it declines from d60 in PE and PI. The most prominent monounsaturated FA (MUFA) are oleic (18:1n-9) and vaccenic (18:1n-7) acids. The proportion of 18:1n-9 decreases from d2 to d20 and rises again in adulthood, whereas 18:1n-7 decreases from d2 to d10 and then it returns to the initial level in PC, PE and PI. The main representatives of n-6 polyunsaturated FA (n-6 PUFA) are linoleic (18:2n-6) and arachidonic (20:4n-6) acids. The proportion of 20:4n-6 in all three PL is 10–20-fold higher than the proportion of 18:2n-6 just after birth. The situation changes after d20 when the proportion of 18:2n-6 starts to rise and that of 20:4n-6 declines. This divergent time course of changes in the proportion of both FA leads to a substantial decline in the 20:4n-6/18:2n-6 ratio during ontogeny. The percentage

Table 1. Weight parameters

Days (*n*)	2 (30)	5 (24)	10 (18)	20 (6)	40 (6)	60 (6)	80 (6)	100 (6)
BW (g)	6.81 ± 0.19	13.33 ± 0.37	24.49 ± 0.70	52.02 ± 4.33	152.2 ± 5.3	252.1 ± 8.0	342.3 ± 8.9	406.3 ± 9.1
LV (mg)	27.22 ± 0.71	47.58 ± 1.02	75.01 ± 1.92	157.2 ± 5.0	351.4 ± 9.4	492.3 ± 7.1	628.8 ± 13.0	740.2 ± 12.1

Values are mean ± S.E.M. *n*, number of animals; BW, body weight; LV, left ventricular weight including the septum.

Table 2. Phospholipid distribution (% of TPL) in left ventricular myocardium of rats during ontogenetic development

Days	2	5	10	20	40	60	80	100
PC	46.22 ± 0.56	45.89 ± 0.40	45.20 ± 1.05	43.49 ± 0.26*#	43.08 ± 0.57*	40.70 ± 0.25*#	39.80 ± 0.28*	39.69 ± 0.37*
PE	32.37 ± 0.68	32.87 ± 0.33	34.12 ± 1.28*	35.26 ± 0.20*	34.80 ± 0.66*	36.33 ± 0.23*	37.53 ± 0.28*	35.48 ± 0.34*#
DPG	7.19 ± 0.62	7.34 ± 0.24	8.32 ± 0.17*#	10.31 ± 0.19*#	13.63 ± 0.49*#	14.32 ± 0.19*	14.70 ± 0.17*	14.66 ± 0.15*
PI	5.41 ± 0.21	5.82 ± 0.09	5.36 ± 0.10	5.02 ± 0.09	3.98 ± 0.22*#	4.14 ± 0.09*	3.94 ± 0.07*	4.24 ± 0.20*
PS	4.63 ± 0.13	4.26 ± 0.17*	3.34 ± 0.16*#	3.43 ± 0.12*	2.35 ± 0.13*#	2.49 ± 0.06*	2.33 ± 0.06*	2.41 ± 0.09*
SM	4.17 ± 0.15	3.82 ± 0.13*	3.68 ± 0.13*	2.48 ± 0.11*#	2.17 ± 0.17*	2.02 ± 0.05*	1.69 ± 0.05*	2.22 ± 0.07*#
PLPC	7.46 ± 0.77	3.16 ± 0.21*	6.74 ± 0.88#	5.53 ± 0.31*	4.62 ± 0.14*	5.93 ± 0.30	5.68 ± 0.31	6.13 ± 0.52
PLPE	33.84 ± 0.99	30.55 ± 1.07	32.42 ± 1.58	28.89 ± 0.70*	25.40 ± 1.21*#	23.94 ± 0.53*	23.97 ± 0.81*	25.10 ± 1.31*

Values are mean ± S.E.M. from six experiments. TPL, total phospholipids; PC, choline phosphoglycerides; PE, ethanolamine phosphoglycerides; DPG, diphosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyeline; PLPC, choline plasmalogen; PLPE, ethanolamine plasmalogen; PLPC and PLPE values represent percentage of corresponding phosphoglycerides. *$p < 0.05$, significant difference compared with d2; #$p < 0.05$, significant difference compared with the previous stage.

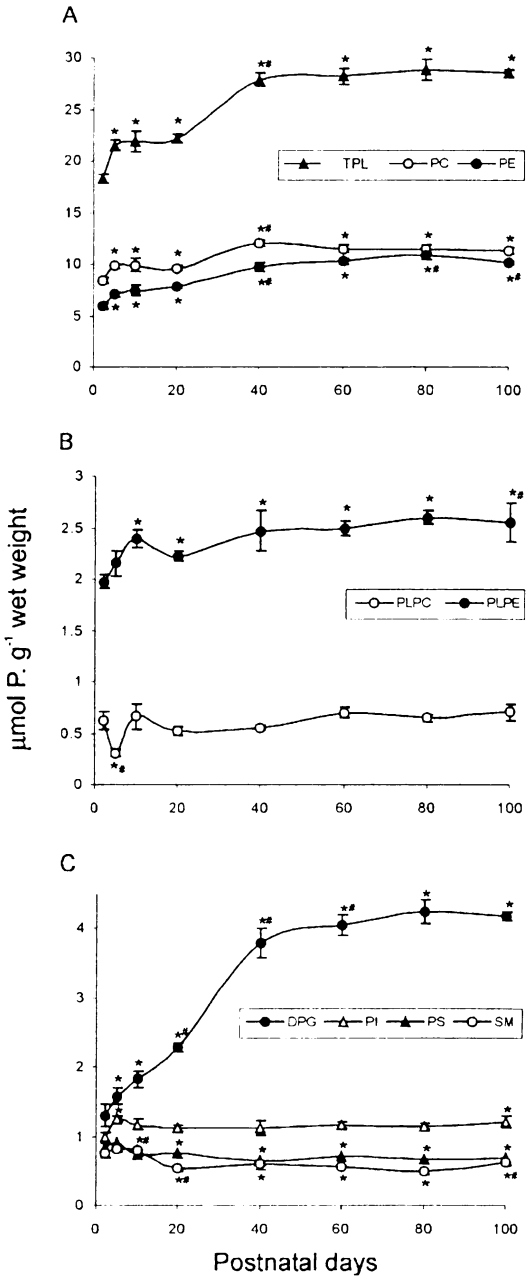

Fig. 1. Concentration of total phospholipids (TPL), individual phospholipid species and plasmalogen components of choline and ethanolamine phosphoglycerides in left ventricular myocardium of rats on postnatal days 2, 5, 10, 20, 40, 60, 80 and 100. PC, choline phosphoglycerides; PE, ethanolamine phosphoglycerides; PLPC, choline plasmalogen; PLPE, ethanolamine plasmalogen; DPG, diphosphatidylglycerol; PI, phosphatidylinositol; PS, phoshatidylserine; SM, sphingomyelin. Values are means ± S.E.M. from six experiments. $^{*}p < 0.05$, significant difference compared with d2; $^{\#}p < 0.05$, significant difference compared with the previous stage.

of 22:4n-6 is roughly comparable to that of 18:2n-6 on d2; it declines gradually till adulthood. Both main n-3 PUFA, docosapentaenoic (22:5n-3) and docosahexaenoic (22:6n-3) acids, rise by d20 and drop afterwards. Therefore, the ratio of n-6/n-3 PUFA is the highest just after birth, than it declines gradually by d20 and increases again during maturation in PC, PE and PI. The value of the unsaturation index (UI) in PC and PE reaches a maximum on d20 and returns to the initial level in adulthood. UI of PI keeps a constant value during the whole developmental period under study.

Table 6 shows the FA composition of DPG. The proportion of SFA and n-3 PUFA is very low in this PL, about 9 and 7 mol % after birth, respectively, and about 2.6 and 2.2 mol % in adulthood, respectively. The percentage of 16:0 and 18:0 remains unchanged between d2 and d20 and it declines thereafter. The prevailing FA in DPG of neonates are MUFA (34 mol %) and n-6 PUFA (49 mol %). During maturation, the relative content of all MUFA and n-6 PUFA declines gradually to very low levels on d100, except for 18:2n-6, the proportion of which increases after weaning up to 88% of total FA in adults. Therefore, the ratio of SFA to unsaturated FA is much lower in DPG in comparison with other PL. The proportion of eicosapentaenoic (20:5n-3) acid in DPG is relatively high after birth as compared with other PL, but it drops to a very low level in adulthood. The percentage of 22:5n-3 and 22:6n-3 rises by d20 and declines thereafter. The ratio of n-6/n-3 keeps constant during the suckling period, but it increases fivefold after the weaning.

Table 7 presents the FA composition of PS. The most abundant FA in this PL is 18:0, which does not change from birth till adulthood. Like in DPG, the proportion of 20:4n-6 is relatively low in PS. On the other hand, the proportion of 22:4n-6 is higher during the whole postnatal period as compared with other PL. The characteristic feature of developmental changes in PS is the gradual increase in the relative content of 22:6n-3 between d2 and d100; in contrast, the decline of 22:6n-3 from d20 occurs in other PL. UI of PS increases from birth till adulthood.

Table 8 demonstrates the FA composition of TAG. The proportion of most FA does not change appreciably during the first 10 days after birth. However, on d100 the proportion of all SFA and nearly of all PUFA is lower, whereas the proportion of all MUFA and 18:2n-6 is higher than after birth. Figure 2 shows the distribution of main FA classes in individual PL and TAG.

Discussion

Developmental changes in myocardial PL concentration

The time course of changes in TPL reveals that their myocardial concentration is generally proportional to ventricular growth. Nevertheless, there are two exceptions, viz. the postnatal periods d2 – d5 and d20 – d40, when the increment of TPL is higher than that of ventricular weight. These periods are considered to be the most critical and stressful periods

Table 3. Fatty acid (FA) composition (mol %) of choline phosphoglycerides in left ventricular myocardium of rats during ontogenetic development

Days	2	5	10	20	60	100
16:0	27.42 ± 2.85	26.93 ± 0.45	25.19 ± 0.74	21.90 ± 0.62*#	17.98 ± 0.65*#	16.91 ± 0.38*
18:0	18.09 ± 0.74	21.79 ± 0.35*	23.74 ± 0.30*	24.30 ± 0.54*	23.55 ± 0.52*	23.55 ± 0.41*
18:1n-9	11.26 ± 0.36	7.08 ± 0.13*	4.86 ± 0.10*#	3.27 ± 0.20*#	5.30 ± 0.47*#	4.99 ± 0.12*
18:1n-7	5.80 ± 0.16	4.64 ± 0.08*	4.24 ± 0.08*	4.88 ± 0.13*	6.89 ± 0.50*#	6.11 ± 0.17#
18:2n-6	3.18 ± 0.15	2.96 ± 0.07	3.84 ± 0.07	6.95 ± 0.32*#	21.36 ± 1.49*#	24.60 ± 0.97*#
20:4n-6	27.15 ± 1.75	27.63 ± 0.51	26.56 ± 0.60	21.80 ± 0.55*#	14.46 ± 1.31*#	15.78 ± 0.89*
22:4n-6	2.27 ± 0.33	1.49 ± 0.04*	0.83 ± 0.04*#	0.44 ± 0.03*#	0.31 ± 0.05*	0.18 ± 0.01*
22:5n-3	1.33 ± 0.22	1.75 ± 0.07	2.70 ± 0.07*#	4.43 ± 0.27*#	2.55 ± 0.42*#	1.49 ± 0.09#
22:6n-3	1.87 ± 0.37	3.29 ± 0.32*	5.79 ± 0.22*#	9.33 ± 0.63*#	2.30 ± 0.61*#	3.88 ± 0.24*#
SFA/UFA	0.85 ± 0.09	0.99 ± 0.02*	0.99 ± 0.03*	0.88 ± 0.03#	0.71 ± 0.01*#	0.68 ± 0.01*
n-6/n-3	10.69 ± 1.30	6.20 ± 0.41*	3.62 ± 0.07*#	2.17 ± 0.13*#	4.60 ± 0.71*#	7.00 ± 0.33*#
20:4/18:2	8.55 ± 0.37	9.35 ± 0.28*	6.92 ± 0.15*#	3.19 ± 0.19*#	0.70 ± 0.12*#	0.67 ± 0.07*
UI	162.4 ± 11.3	166.7 ± 2.6	179.1 ± 3.9	190.1 ± 5.2*#	165.1 ± 8.0#	161.4 ± 2.8

Values are mean ± S.E.M. from six experiments. SFA, sum of saturated FA; UFA, sum of unsaturated FA; UI, unsaturation index is the sum of mol % of individual unsaturated FA multiplied by the number of double bonds. FA reaching at least 1 mol % are shown only. *$p < 0.05$, significant difference compared with d2; #$p < 0.05$, significant difference compared with the previous stage.

Table 4. Fatty acid composition (mol %) of ethanolamine phosphoglycerides in left ventricular myocardium of rats during ontogenetic development

Days	2	5	10	20	60	100
16:0	7.05 ± 1.18	14.67 ± 1.22*	15.94 ± 0.36*	13.40 ± 0.57*	10.10 ± 0.61*#	8.70 ± 0.28*
18:0	23.41 ± 0.30	27.42 ± 0.93*	27.66 ± 0.55*	25.90 ± 0.54*	28.21 ± 0.37*#	25.36 ± 0.39*#
18:1n-9	5.83 ± 0.27	3.77 ± 0.20*	2.78 ± 0.11*#	2.76 ± 0.29*	4.54 ± 0.23*#	4.25 ± 0.13*
18:1n-7	2.56 ± 0.31	1.63 ± 0.16	1.48 ± 0.08*	2.10 ± 0.20	2.43 ± 0.37	3.23 ± 0.14*#
18:2n-6	1.47 ± 0.19	1.21 ± 0.10	1.41 ± 0.04	2.79 ± 0.20	9.71 ± 0.75*#	13.25 ± 0.60*#
20:4n-6	38.14 ± 0.85	32.39 ± 1.11*	26.99 ± 0.41*#	19.50 ± 0.29*#	15.57 ± 0.38*#	18.87 ± 0.37*#
22:4n-6	8.34 ± 0.53	4.14 ± 0.27*	2.37 ± 0.11*#	0.94 ± 0.04*#	0.57 ± 0.05*	0.52 ± 0.03*
22:5n-3	4.00 ± 0.48	3.27 ± 0.21	4.23 ± 0.11#	5.60 ± 0.18*#	4.63 ± 0.29#	3.16 ± 0.15*#
22:6n-3	7.93 ± 0.53	10.26 ± 0.78	15.63 ± 0.46*#	23.70 ± 0.92*#	21.90 ± 1.13*	20.11 ± 0.88*
SFA/UFA	0.44 ± 0.02	0.74 ± 0.05*	0.78 ± 0.02*	0.68 ± 0.02*#	0.63 ± 0.02*	0.52 ± 0.01*#
n-6/n-3	4.11 ± 0.39	2.84 ± 0.16*	1.54 ± 0.04*	0.81 ± 0.03*	0.98 ± 0.07*	1.40 ± 0.08*
20:4/18:2	27.79 ± 3.66	27.58 ± 2.83	19.26 ± 0.67*#	7.31 ± 0.63*#	1.64 ± 0.16*#	1.45 ± 0.06*
UI	268.3 ± 5.9	234.5 ± 8.9	243.9 ± 3.1	261.1 ± 6.2	252.2 ± 7.7	249.0 ± 4.1

Values are mean ± S.E.M. from six experiments. For symbols see Table 3.

of rat postnatal life [10]. The first period, which is specified as a neonatal one, is characterized by an abrupt increase of cardiac workload and concomitant transition from predominantly anaerobic fetal metabolism of carbohydrates to aerobic metabolism of FA. The crucial events of the second period are the nutritional transition from suckling of mother's milk to solid food intake and the initiation of circadian rhythms [1].

PC and PE are major PL species, accounting for about 79% of TPL in neonatal LV and 75% in the adult one. The time course of changes in the concentration of both major PL during postnatal development is similar to that observed in TPL. In line with our results, Gudmunsdottir and Gudbjarnason [4] found the greatest increase in the amount of PC and PE in rat hearts during the first 3 weeks of postnatal life and between the 2nd and the 6th months in adults. However, the important period from d20 to d40 was not examined in their study.

The concentration of DPG, which is the marker of inner mitochondrial membrane, increases markedly by d40. This increase suggests that membranes of mitochondria are predominant structures accounting for the growth of TPL concentration during the first 5 weeks of life. Quantitative ultrastructural analyses have shown that the area of mitochondrial inner membrane per unit cell volume exceeds that of the

Table 5. Fatty acid composition (mol %) of phosphatidylinositol in left ventricular myocardium of rats during ontogenetic development

Days	2	5	10	20	100
16:0	4.83 ± 0.84	7.22 ± 0.60*	7.09 ± 0.50*	7.53 ± 0.35*	5.36 ± 0.33#
18:0	30.28 ± 5.86	36.58 ± 1.57	38.45 ± 0.53*	39.20 ± 0.69*	34.70 ± 0.57#
18:1n-9	5.77 ± 0.86	4.26 ± 0.38*	2.94 ± 0.28*	3.50 ± 0.43*	6.07 ± 0.37#
18:1n-7	1.98 ± 0.31	1.48 ± 0.19	1.11 ± 0.06*	1.76 ± 0.13	2.47 ± 0.22#
18:2n-6	2.19 ± 0.24	1.38 ± 0.17	1.52 ± 0.17	2.22 ± 0.10	9.50 ± 0.39*#
20:3n-6	1.40 ± 0.22	1.49 ± 0.28	0.97 ± 0.17	0.87 ± 0.05	1.36 ± 0.11
20:4n-6	38.81 ± 1.08	37.30 ± 1.22	37.72 ± 0.85	30.10 ± 1.01*#	28.71 ± 0.72*
22:4n-6	3.10 ± 0.63	2.75 ± 0.49	1.18 ± 0.08*#	0.61 ± 0.04*	0.76 ± 0.42*
22:5n-3	2.02 ± 0.21	2.32 ± 0.31	2.59 ± 0.23	3.94 ± 0.13*#	1.88 ± 0.17#
22:6n-3	3.02 ± 0.63	4.44 ± 0.58*	5.34 ± 0.30*	6.31 ± 0.40*	5.13 ± 0.32*
SFA/UFA	0.60 ± 0.11	0.79 ± 0.03*	0.85 ± 0.02*	0.90 ± 0.02*	0.69 ± 0.02#
n-6/n-3	9.32 ± 1.35	6.27 ± 0.68*	5.12 ± 0.41*	3.24 ± 0.18*#	5.54 ± 0.25*#
20:4/18:2	18.96 ± 2.72	29.23 ± 4.62*	26.18 ± 2.87*	13.86 ± 1.04#	3.07 ± 0.16*#
UI	215.1 ± 2.5	214.8 ± 4.1	212.9 ± 3.0	192.6 ± 3.5*#	197.9 ± 2.9*

Values are mean ± S.E.M. from six experiments. For symbols see Table 3.

Table 6. Fatty acid composition (mol %) of diphosphatidylglycerol in left ventricular myocardium of rats during ontogenetic development

Days	2	5	10	20	60	100
16:0	2.32 ± 0.47	3.94 ± 0.56	2.63 ± 0.30	3.66 ± 0.68	1.90 ± 0.41#	1.02 ± 0.18
16:1n-7	2.17 ± 0.55	2.79 ± 0.14*	1.49 ± 0.09#	0.56 ± 0.07*#	1.49 ± 0.23#	0.69 ± 0.08*#
18:0	3.83 ± 0.77	3.37 ± 0.75	4.57 ± 0.36	3.50 ± 0.71	1.99 ± 0.28	1.37± 0.24*
18:1n-9	21.82 ± 0.70	17.99 ± 0.24*	14.64 ± 0.64*#	5.02 ± 0.56*#	2.16 ± 0.30*#	1.68 ± 0.20*
18:1n-7	12.60 ± 0.57	14.39 ± 0.75*	13.23 ± 0.67	12.55 ± 0.36	2.59 ± 0.21*#	1.91 ± 0.09*
18:2n-6	29.24 ± 0.49	30.23 ± 0.87	29.08 ± 1.19	45.70 ± 1.69*#	86.15 ± 1.48*#	87.90 ± 0.97*
20:3n-6	7.23 ± 0.76	4.87 ± 0.58*	4.87 ± 0.30*	5.49 ± 0.20*	0.86 ± 0.06*#	0.65 ± 0.04*
20:4n-6	10.22 ± 0.74	11.01 ± 0.72	12.96 ± 0.56*#	7.13 ± 0.43*#	1.22 ± 0.17*#	1.02 ± 0.17*
20:5n-3	2.30 ± 0.23	0.09 ± 0.02*	1.11 ± 0.25*#	0.25 ± 0.02*#	0.22 ± 0.03*	0.11 ± 0.02*
22:4n-6	3.29 ± 0.21	1.57 ± 0.12*	1.51 ± 0.11*	0.42 ± 0.04*#	0.11 ± 0.01*#	0.07 ± 0.01*
22:5n-3	1.54 ± 0.36	1.83 ± 0.20	2.54 ± 0.23*#	2.40 ± 0.19*	0.58 ± 0.03*#	0.41 ± 0.05*
22:6n-3	2.80 ± 0.19	6.12 ± 0.71*	11.28 ± 0.81*#	9.69 ± 1.08*	1.53 ± 0.15#	1.63 ± 0.37
SFA/UFA	0.07 ± 0.01	0.09 ± 0.02	0.09 ± 0.01	0.09 ± 0.02	0.04 ± 0.01	0.03 ± 0.01*
n-6/n-3	7.36 ± 0.35	6.06 ± 0.53	3.24 ± 0.13	5.16 ± 0.60	35.52 ± 2.86*#	41.31 ± 5.70*
20:4/18:2	0.35 ± 0.03	0.35 ± 0.02	0.45 ± 0.03*#	0.16 ± 0.01*#	0.01 ± 0.01*#	0.01 ± 0.01*
UI	208.0 ± 4.7	208.0 ± 7.7	247.1 ± 7.4*#	226.3 ± 6.8#	200.5 ± 2.0#	203.2 ± 3.1

Values are mean ± S.E.M. from six experiments. For symbols see Table 3.

sarcoplasmic reticulum 16-fold and that of the sarcolemma about 41-fold in LV myocytes of adult rats [11]. It has been shown that the interval between d1 and d4 was associated with rapid and large accumulation of mitochondria and myofibrils in rabbit LV myocardium. Mitochondria were packed more densely with cristae and the area of mitochondrial inner membrane per unit myofibrillar volume increased progressively throughout the perinatal period [12]. The substantial increase in the concentration of DPG between d2 and d40 in our study reflects the increasing proportion of mitochondrial membranes during this period. DPG is known to be associated with several inner mitochondrial membrane proteins [13], most notably with cytochrome *c* oxidase [14]. In accordance with the rise of DPG in our study, the specific content of cytochrome *c* oxidase increased twofold between birth and d30 in mitochondria isolated from rat LV [15], accompanied by a large increase in activities of other enzymes of aerobic metabolism [16]. The rapid increase in the concentration of PL we observed in neonatal rat hearts as compared with adult ones, is in agreement with several incorporation studies using

Table 7. Fatty acid composition (mol %) of phosphatidylserine in left ventricular myocardium of rats during ontogenetic development

Days	2	5	10	20	100
16:0	5.80 ± 0.43	7.00 ± 0.77	5.16 ± 0.33	5.25 ± 0.61	2.89 ± 0.30*#
18:0	41.90 ± 0.77	42.37 ± 0.88	42.44 ± 0.80	40.70 ± 1.29	39.66 ± 0.98
18:1n-9	7.76 ± 0.58	6.63 ± 0.51	7.38 ± 0.35	7.17 ± 0.26	6.10 ± 0.30
18:1n-7	2.09 ± 0.20	1.84 ± 0.07	1.29 ± 0.07*	1.69 ± 0.24	1.32 ± 0.11*
18:2n-6	1.61 ± 0.13	1.76 ± 0.18	2.13 ± 0.13	2.37 ± 0.15	4.63 ± 0.35*#
20:3n-6	1.99 ± 0.15	2.31 ± 0.32	2.07 ± 0.09	2.17 ± 0.11	2.01 ± 0.25
20:4n-6	12.34 ± 1.27	12.23 ± 1.23	7.54 ± 0.29*#	5.46 ± 0.32*	6.54 ± 0.34*
22:4n-6	8.70 ± 1.41	7.29 ± 0.56	5.14 ± 0.22*#	2.09 ± 0.05*#	2.06 ± 0.14*
22:5n-3	4.47 ± 0.68	4.71 ± 0.24	6.51 ± 0.29*	6.03 ± 0.21	5.00 ± 0.60
22:6n-3	11.24 ± 1.86	12.30 ± 0.83	18.80 ± 0.45*#	21.30 ± 0.86*	24.26 ± 1.30*#
SFA/UFA	0.94 ± 0.04	1.00 ± 0.03	0.93 ± 0.02	0.89 ± 0.03	0.77 ± 0.03*#
n-6/n-3	1.69 ± 0.37	1.38 ± 0.09	0.67 ± 0.03*#	0.51 ± 0.02*	0.60 ± 0.05*
20:4/18:2	7.85 ± 0.98	7.46 ± 1.38	3.57 ± 0.19*#	2.36 ± 0.17*	1.46 ± 0.11*
UI	196.9 ± 4.4	197.1 ± 4.3	217.5 ± 2.7*#	203.4 ± 5.4#	238.4 ± 4.5*#

Values are mean ± S.E.M. from six experiments. For symbols see Table 3.

Table 8. Fatty acid composition (mol %) of triacylglycerols in left ventricular myocardium of rats during ontogenetic development

Days	2	5	10	100
14:0	2.65 ± 0.88	3.71 ± 0.90	3.84 ± 1.58	1.21 ± 0.16#
16:0	30.1 ± 3.14	34.8 ± 2.07	35.3 ± 4.73	25.61 ± 0.65#
18:0	10.3 ± 0.56	10.5 ± 0.38	13.4 ± 2.41	7.73 ± 0.39#
18:1n-9	15.4 ± 0.63	12.3 ± 0.38*	11.7 ± 1.16*	28.44 ± 0.48*#
18:1n-7	4.33 ± 0.17	3.89 ± 0.15	3.81 ± 0.54	5.90 ± 0.17*#
18:2n-6	8.48 ± 0.53	6.20 ± 0.26	6.40 ± 0.94	17.51 ± 0.64*#
20:3n-6	1.92 ± 0.23	1.17 ± 0.08*	0.92 ± 0.14*	0.65 ± 0.09*
20:4n-6	6.95 ± 0.87	5.52 ± 0.44	6.07 ± 2.31	1.53 ± 0.08*#
22:4n-6	4.04 ± 0.48	2.45 ± 0.26*	1.21 ± 0.19*#	0.39 ± 0.09*#
22:5n-3	3.35 ± 0.31	4.45 ± 0.65	3.81 ± 0.52	1.45 ± 0.13*#
22:6n-3	7.15 ± 0.9	10.3 ± 1.4	9.21 ± 1.38	3.03 ± 0.38*#
SFA/UFA	0.80 ± 0.12	1.03 ± 0.14	1.27 ± 0.31	0.54 ± 0.02#
n-6/n-3	2.10 ± 0.09	1.11 ± 0.08	1.13 ± 0.15	3.76 ± 0.24*#
20:4/18:2	0.81 ± 0.05	0.89 ± 0.05	1.08 ± 0.54	0.09 ± 0.01*#
UI	151.5 ± 15.2	153.5 ± 14.8	140.5 ± 18.8	114.8 ± 3.3*

Values are mean ± S.E.M. from six experiments. For symbols see Table 3.

radioactive precursors. Kako *et al.* [17] showed that the synthesis of phosphatidic acid is 1.3-fold higher in mitochondrial and fourfold higher in microsomal fractions from newborn rat hearts than in adults. Phosphatidic acid as a common precursor may reflect the rate of glycerophospholipid synthesis in sarcoplasmic and mitochondrial compartments. While most of the PL are synthesized in the sarcoplasmic reticulum [18], the biosynthesis of DPG occurs in mitochondria [19]. The rate of DPG biosynthesis was fourfold higher in mitochondria isolated from neonatal rat hearts than from adults [20]. The higher DPG biosynthesis in neonatal mitochondria reflects its importance for the incorporation of precursor proteins into mitochondrial membranes during maturation [21].

We observed that the concentration of PI in the LV myocardium reaches its maximum on d5. In line with this finding, Stuhne-Sekalec *et al.* [20] demonstrated a twofold higher rate of PI biosynthesis in the microsomal fraction isolated from 5-day-old rat hearts compared with adults. PI is the most abundant phosphoinositide of the ventricular myocardium and it serves as a precursor for more phosphorylated phosphoinositides, which are involved in signal transduction [22]. In contrast to the increase of PI concentration, we observed

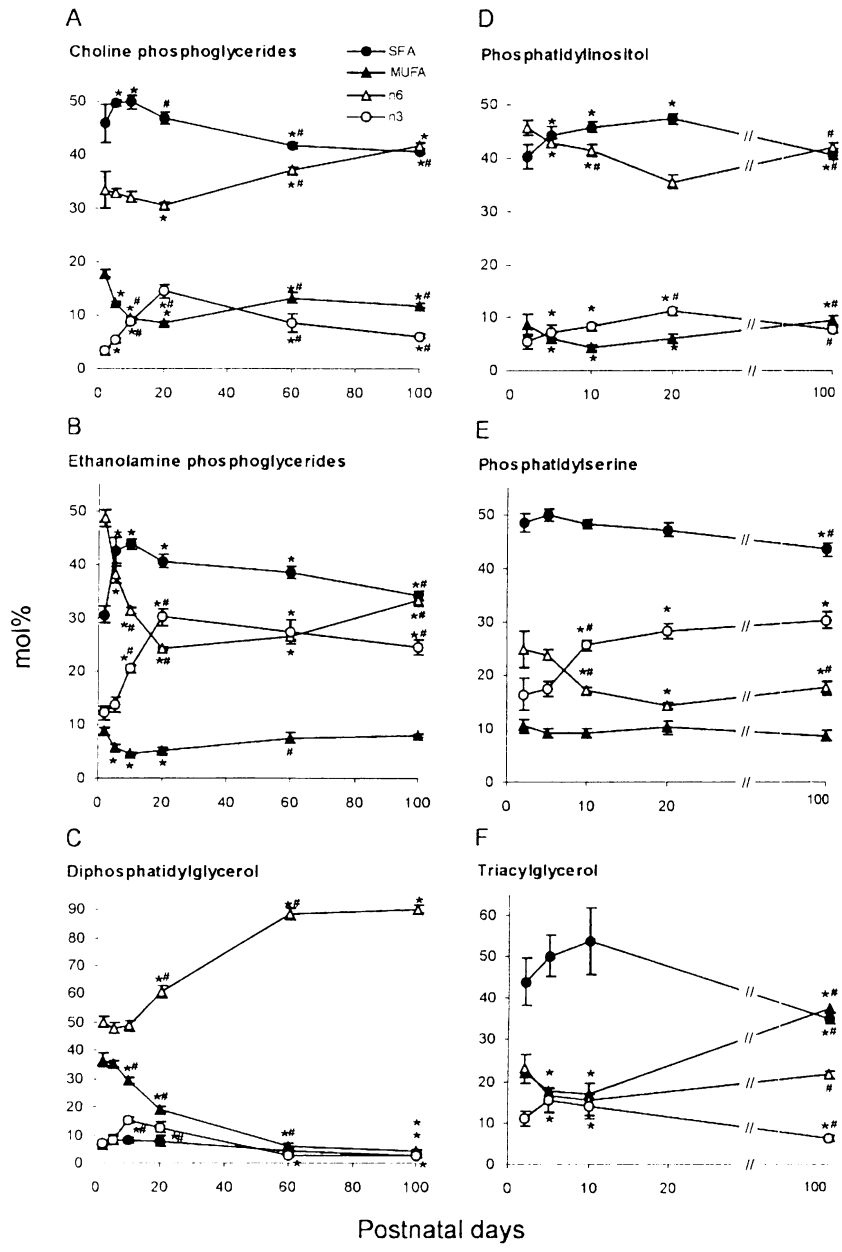

Fig. 2. Composition of main fatty acid (FA) classes in individual phospholipid species in left ventricular myocardium of rats on postnatal days 2, 5, 10, 20, 60 and 100. SFA, sum of saturated FA; MUFA, sum of monounsaturated FA; n-6, n-3, sum of corresponding polyunsaturated FA. Values are means ± S.E.M. from six experiments. $^{*}p < 0.05$, significant difference compared with d2; $^{\#}p < 0.05$, significant difference compared with the previous stage.

recently a significant drop in the concentration of myocardial diacylglycerol on d5 [23]. In view of the fact that diacylglycerol is the final product of PIP_2 breakdown by phospholipase C, our results suggest a possible inhibition of the signaling pathway through phospholipase C in the key period of transition from hyperplastic to hypertrophic growth of ventricular myocytes [2].

In agreement with data published on heart plasmalogens [24, 25], we found an approximately fivefold higher myocardial concentration of PLPE than that of PLPC. Substantial differences between mammalian species with respect to the proportion of PLPC in cardiac tissue were reported [26]. The amount of PLPC is rather high in human, rabbit, dog and guinea pig myocardium (up to 20–40% of PC), whereas in rat, mouse and hamster it comprises 3–8% of PC only (for review see [27]). The dramatic time course of changes in concentrations of both plasmalogens as were observed during the first postnatal days, is interesting with respect to the

transition from hyperplastic to hypertrophic growth [2]. In view of the participation of bioactive lipid molecules in signaling pathways involved in proliferation and differentiation of cardiac cells, the significance of plasmalogens may be inherent in many aspects of these processes. Plasmalogens are important precursors of lipid signaling molecules such as diacylglycerols, modulators of protein kinase C activity [28]. It was shown that plasmalogens contain high amounts of arachidonic acid, the potent signaling molecule and precursor of eicosanoids [29, 30].

Developmental changes in FA composition of myocardial PL and TAG

From the present study, it is evident that the FA composition of individual myocardial PL and TAG undergoes similar developmental changes. The changes concern an increase in the proportion of SFA during the first 10 postnatal days followed by a decline till adulthood, and a decrease in MUFA just after birth with subsequent rise afterwards. As for PUFA, a decrease in the n-6 class and an increase in the n-3 class occur during the suckling period, with an opposite tendency after weaning.

Several factors may play a role in the developmental changes of the FA profile of PL, such as nutrition, metabolic transition, hormonal changes and increasing workload. During the first two postnatal weeks, nutrients are solely supplied from mother's milk and hence the quality and quantity of the milk fat influence substantially the FA profile of heart PL, as indicated by studies with modified composition of FA in mother's diet [31, 32]. On the other hand, in agreement with our data, Gudmundsdottir and Gudbjarnason [4] observed a high amount of PUFA incorporated into myocardial PC and PE of suckling rats. This finding does not correspond to the FA composition of milk (containing predominantly 16:0, 18:1, 18:2n-6 and shorter chain FA). As we used the standard diet of similar FA composition as the authors mentioned above, the high content of PUFA in myocardial PL and TAG in our study suggests that these PUFA were primarily derived from endogenous sources. In view of the limited ability for FA synthesis [33] and desaturation [34] in the myocardium, PUFA in PL and TAG must be derived from plasma TAG and/or nonesterified FA (NEFA) originating from shorter chain precursors by a desaturation-elongation process in the liver [35]. A marked quantitative increase was observed in long-chain FA in TAG of rat liver during the first postnatal week. Total TAG containing predominantly one, two, or even three 20–22 PUFA increased more than 10-fold in the neonatal liver [36, 37]. The unique accumulation of long-chain TAG species during early postnatal development, especially in the liver, indicates that highly unsaturated TAG are probably a quantitatively important pool of PUFA as a direct substrate for the synthesis of membrane PL in different tissues.

Schroedl *et al.* [38] underlined the significance of exogenous NEFA in the membrane biosynthesis of developing heart. They have shown that most of the NEFA incorporated into cultured neonatal cardiomyocytes were directed towards polar rather than neutral lipid stores. These results contradict the classical view that exogenous NEFA are directed intracellularly primarily to the pathways of oxidation or to storage as TAG in the lipid-dependent adult heart [39, 40] or cultured adult cardiac myocytes [41].

18:2n-6 and 20:4n-6, the main n-6 PUFA in heart PL, have quite different starting levels at birth: the relative content of 18:2n-6 is low, whereas that of 20:4n-6 is high. During suckling-to-weaning transition, 18:2n-6 starts to increase probably due to its high content in the solid food. In contrast, 20:4n-6 declines gradually in all PL after weaning. Gudmundsdottir and Gudbjarnason [4] published a similar time course of changes in the proportion of 18:2n-6 and 20:4n-6 in PC and PE. Similar experimental conditions (the same strain of rats and analogous FA composition of the diet) allow the comparison of their and our results. These authors observed a marked increase in 20:4n-6 between d1 and d7 in PC and PE; however, more detailed analysis in our study showed that the maximal proportion of 20:4n-6 is already reached on d2 and then maintained till d10 in all PL. Ghebremeskel *et al.* [42] also did not detect the maximal gain of the 20:4n-6 content, as they analyzed the FA profile of PC and PE in rat liver and heart only at delivery and on d15. The substantial rise of 20:4n-6 in the myocardium of newborn rats may reflect an early response of the heart to stress connected with transition from fetal to neonatal life [43]. This assumption is in line with the results of Decrok *et al.* [44], who showed that, although in the king penguin there is a preponderance of n-3 PUFA in the yolk, two days post-hatching the main PUFA in heart PL is 20:4n-6. In agreement with the high content of 20:4n-6 in myocardial PL, the level of TAG enriched by 20:4n-6 increases significantly throughout the late pregnancy to the first three days of lactation in maternal liver and milk [45] as well as in the liver of rat neonates [46].

The gradual elevation of 22:6n-3 content in all PL, which was observed in our and other studies during the suckling period till weaning [4, 42], is also difficult to explain by nutritional intake only. Its content in mother's milk even decreases during the development of rat [47] and human neonates [48]. The process of hormonal maturation during postnatal development offers another explanation for the increase of 22:6n-3. It is well known that the plasma level of thyroid hormones in the rat increases gradually till the third postnatal week [49], which resembles the time course of 22:6n-3 increase in PL. 22:6n-3 gain in neonatal heart can be even accelerated by the hyperthyroid state [50]. The rise of this FA can be also related

to increasing adrenergic responsiveness in the developing rat heart [51]. Stimulation of the heart by catecholamines led to an increase of 22:6n-3 in myocardial PL of adult rats [52]. A similar remodeling of FA composition was observed in the myocardium in response to different stress conditions, such as exposure of immature [53] or adult rat heart to chronic hypoxia [54] and induction of pressure overload [55]. Gudbjarnason *et al.* [56] described a positive correlation between the heart rate of mammals, ranging from mice to whales, and the 22:6n-3 content in their myocardial PL. Although the mode of action is not quite clear, the fundamental role of 22:6n-3 in membranes of many tissues is evident. This unique PUFA is known to readily incorporate into PL and thus alter significantly the basic properties of membranes, including fluidity, permeability, and function of many proteins [57].

In summary, the increasing concentration of myocardial PL during the early postnatal period is in line with the rapid increase in membrane mass, in particular of mitochondria. Each PL species has a characteristic FA composition, which might be related to its specific role in membrane functions. However, FA composition of all PL undergoes qualitatively similar developmental changes, although their magnitude and detailed time course differ in individual PL. This remodeling of myocardial PL, reflecting altered environmental conditions, may affect membrane properties and contribute to developmental changes in the function of membrane proteins and cell signaling.

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References

1. Girard J, Pascal F, Pégorier JP, Duée PH: Adaptation of glucose and fatty acid metabolism during perinatal period and suckling-weaning transition. Physiol Rev 72: 507–562, 1992
2. Li F, Wang X, Capasso JM, Gerdes AM: Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. J Mol Cell Cardiol 28: 1737–1746, 1996
3. Wibo M, Bravo G, Godfraind T: Postnatal maturation of excitation-contraction coupling in rat ventricle in relation to the subcellular localization and surface density of 1,4-dihydropyridine and ryanodine receptors. Circ Res 68: 662–673, 1991
4. Gudmundsdottir A, Gudbjarnason S: Neonatal changes in fatty acid profile of phospholipids in rat heart muscle. Biochim Biophys Acta 752: 284–290, 1983
5. Rogers CG: Fatty acids and phospholipids of adult and newborn rat hearts and of cultured, beating neonatal rat myocardial cells. Lipids 9: 541–547, 1974
6. Folch J, Lees M, Sloan-Stanley GH: A simple method for the isolation and purification of total lipids from animal tissue. J Biol Chem 226: 497–509, 1957
7. Rouser G, Fleischer S, Yamamoto A: Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids 5: 494–496, 1970
8. Horrocks LA: The alk-1-enyl group content of mammalian myelin phosphoglycerides by quantitative two-dimensional thin-layer chromatography. J Lipid Res 9: 469–472, 1968
9. Tvrzicka E, Vecka M, Stankova B, Zak A: Analysis of fatty acids in plasma lipoproteins by gas chromatography-flame ionisation detection. Quantitative aspects. Anal Chim Acta 465: 337–350, 2002.
10. Ostadal B, Ostadalova I, Dhalla NS: Development of cardiac sensitivity to oxygen deficiency: Comparative and ontogenetic aspects. Physiol Rev 79: 635–659, 1999
11. Page E: Quantitative ultrastructural analysis in cardiac membrane physiology. Am J Physiol 235: C147–C158, 1978
12. Smith HE, Page E: Ultrastructural changes in rabbit heart mitochondria during the perinatal period. J Ultrastruct Res 55: 31–41, 1976
13. Hoch FL: Cardiolipins and biomembrane function. Biochim Biophys Acta 1113: 71–133, 1992
14. Paradies G, Fuggiero FN, Petrosillo G, Quagliariello EI: Peroxidative damage to cardiac mitochondria: cytochrome oxidase and cardiolipin alterations. FEBS Lett 424: 155–158, 1998
15. Skarka L, Bardova K, Brauner P, Flachs P, Jarkovska D, Kopecky J, Ostadal B: Expression of mitochondrial uncoupling protein 3 and adenine nucleotide translocase 1 genes in developing rat heart: putative involvement in control of mitochondrial membrane potential. J Mol Cell Cardiol 35: 321–330, 2003
16. Bass A, Stejskalova M, Stieglerova A, Ostadal B, Samanek M: Ontogenetic development of energy-supplying enzymes in rat and guinea-pig heart. Physiol Res 50: 237–245, 2001
17. Kako KJ, Zaror-Behrens G, Peckett SD: Phosphatidic acid synthesis in the heart. Effect of age and species differences in the mitochondrial and microsomal synthesis. Can J Biochem 55: 308–314, 1977
18. Parkers JG, Thompson W: Phosphatidylethanolamine in liver mitochondria and endoplasmic reticulum: molecular species distribution and turnover. Can J Biochem 53: 698–705, 1975
19. Stanacev NZ, Stuhne-Sekalec L, Brooks KB, Davidson JB: Intermediary metabolism of phospholipids. The biosynthesis of phosphatidylglycerophosphate and phosphatidyl-glycerol in heart mitochondria. Biochim Biophys Acta 176: 650–653, 1969
20. Stuhne-Sekalec L, Wassenaar M, Jackowski G, Stanacev NZ: Comparison of the biosynthesis and composition of polyglycerophosphatides and phosphatidylinositols in mitochondria and microsomes isolated from neonatal and adult rat heart and liver. Membr Biochem 9: 29–45, 1990
21. Skerjans I: Mitochondrial import: Properties of precursor proteins. Cell Biol 68: 9–16, 1990
22. Berridge MJ: Inositol triphosphate and diacylglycerol: two interacting second messengers. Ann Rev Biochem 56: 159–193, 1995
23. Hamplova B, Novakova O, Kolar F, Tvrzicka E, Novak F: Protein kinase C activity and isoform expression during early postnatal development of rat myocardium. Cell Biochem Biophys 43: 105–118, 2005
24. Hack MH, Helmy FM: On the plasmalogenation of myocardial choline glycerophospholipid during maturation of various vertebrates. Comp Biochem Physiol 89B: 111–118, 1988
25. Lee T, Qian Ch, Snyder F: Biosynthesis of choline plasmalogens in neonatal rat myocytes. Arch Biochem Biophys 286: 498–503, 1991
26. Diagne A, Fauvel J, Record M, Chap H, Douste-Blazy L: Studies on ether phospholipids II. Comparative composition of various tissues from human, rat and guinea pig. Biochim Biophys Acta 793: 221–231, 1984

27. Van der Vusse GJ, Glatz JFC, Stam HCG, Reneman RS: Fatty acid homeostasis in the normoxic and ischemic heart. Physiol Rev 72: 881–940, 1992
28. Ford DA, Gross RW: Activation of myocardial protein kinase C by plasmalogenic diglycerides. Am J Physiol 258: C30–C36, 1990
29. Arthur G, Mock T, Zaborniac Ch, Choy PC: The distribution and acyl composition of plasmalogens in guinea pig heart. Lipids 20: 693–698, 1985
30. Gross RW: High plasmalogen and arachidonic acid content of canine myocardial sarcolemma: a fast atom bombardment mass spectroscopic and gas chromatography-mass spectroscopic characterisation. Biochemistry 23: 158–165, 1984
31. Huang YS, Wainwright PE, Rodden PR, Mills DE, Bulman-Fleming B, Horrobin DF: Effect of maternal dietary fats with variable n-3/n-6 ratios on tissue fatty acid composition in suckling mice. Lipids 27: 104–110, 1992
32. Berger A, Gershwin ME, German JB: Effects of various dietary fats on cardiolipin acyl composition during ontogeny of mice. Lipids 27: 605–612, 1992
33. Ghosal J, Whitworth T, Coniglio JG: Biosynthesis of fatty acids from [1-^{14}C] acetate in the perfused rat heart. Biochim Biophys Acta 187: 576–578, 1969
34. Brenner RR: The desaturation step in the animal biosynthesis of polyunsaturated fatty acids. Lipids 6: 567–575, 1971
35. Cunnane SC, Chen ZY: Triacylglycerol: an important pool of essential fatty acids during early postnatal development in rats. Am J Physiol 262: R8–R13, 1992
36. Cunnane SC, Chen ZY: Quantitative changes in long-chain fatty acids during fetal and early postnatal development in rats. Am J Physiol 262: R14–R19, 1992
37. Chen ZY, Cunnane SC: Early postnatal development is characterized by accumulation of highly unsaturated triacylglycerols. Pediatr Res 31: 47–51, 1992
38. Schroedl NA, Hartzell ChR: Preferential distribution of non-esterified fatty acids to phosphatidylcholine in the neonatal mammalian myocardium. Biochem J 224: 651–659, 1984
39. Apstein CS, Gmeiner R, Brachfeld N: Effect of palmitate on hypoxic myocardial metabolism and contractility. Recent Adv Stud Cardiac Struct Metab 1: 136–146, 1972
40. Vasdev CS, Kako KJ: Incorporation of fatty acids into rat heart lipids. In vivo and in vitro studies. J Mol Cell Cardiol 9: 617–631, 1977
41. Gloster J, Achillea M, Harris P: Subcellular distribution of [1-^{14}C]palmitate and [1-^{14}C]oleate incorporated into lipids in the perfused rat heart: a comparison under isothermal and hypothermic conditions. J Mol Cell Cardiol 10: 439–448, 1978
42. Ghebremeskel K, Bitsanis D, Koukkou E, Lovy C, Poston L, Crawford MA: Post-natal modulation of heart and liver phosphoglyceride fatty acids in pups. Ann Nutr Metab 43: 365–373, 1999
43. Gudbjarnason S: Dynamics of n-3 and n-6 fatty acids in phospholipids of heart muscle. J Int Med 225: Supl 1, 117–128, 1989
44. Decrock F, Groscolas R, Speake BK: FA composition of heart and skeletal muscle during embryonic development of the king penguin. Lipids 37: 407–415, 2002
45. Cunnane SC, Armstrong JK: Long chain fatty acid composition of maternal liver lipids during pregnancy and lactation in the rat comparison of triglyceride to phospholipid. J Nutr 120: 338–345, 1990
46. Chen ZY, Cunnane SC: Short-term energy deficit causes net accumulation of linoleoyl-enriched triacylglycerols in rat liver. FEBS Lett 280: 393–396, 1991
47. Guesnet P, Alasnier C, Alessandri JM, Durand G: Modifying the n-3 fatty acid content of the maternal diet to determine the requirements of the fetal and suckling rat. Lipids 32: 527–534, 1997
48. Gibson RA, Kneebone GM: Fatty acid composition of human colostrum and mature breast milk. Am J Clin Nutr 34: 252–257, 1981
49. Vigouroux E: Dynamic study of post-natal thyroid function in the rat. Acta Endocrinol (Copenh) 83: 752–762, 1976
50. Hamplova B, Novakova O, Tvrzicka E, Pelouch V, Novak F: Effect of hypo- and hyperthyroid states on phospholipid composition in developing rat heart. Mol Cell Biochem 252: 295–303, 2003
51. Novotny J, Bourova L, Malkova O, Svoboda P, Kolar F: G proteins, beta-adrenoreceptors and beta-adrenergic responsiveness in immature and adult rat ventricular myocardium: influence of neonatal hypo- and hyperthyroidism. J Mol Cell Cardiol 31: 761–772, 1999
52. Emilsson A, Gudbjarnason S: Reversible alterations in fatty acid profile of glycerophospholipids in rat heart muscle induced by repeated norepinephrine administration. Biochim Biophys Acta 750: 1–6, 1983
53. Novak F, Tvrzicka E, Pelouch V, Jezkova J, Smik D, Novakova O: Phospholipid composition of immature rat myocardium exposed to chronic hypoxia and the effect of normoxic recovery. Collect Czech Chem Commun 69: 674–688, 2004
54. Jezkova J, Novakova O, Kolar F, Tvrzicka E, Neckar J, Novak F: Chronic hypoxia alters fatty acid composition of phospholipids in right and left ventricular myocardium. Mol Cell Biochem 232: 49–56, 2002
55. Novakova O, Pelouch V, Mrnka L, Tvrzicka E, Novak F: Phospholipid composition in mitochondria of pressure overloaded maturating rat heart. J Mol Cell Cardiol 34: A87, 2002
56. Gudbjarnason S, Doell B, Oskardottier G, Hallgrimsson J: Modification of cardiac phospholipids and catecholamine stress tolerance. In: C. deDuve, O. Hayaishi (eds). Tocoferol, Oxygen and Biomembranes. Elsevier, Amsterdam, 1978, pp. 297–310
57. Stillwell W, Wassall SR: Docosahexaenoic acid: membrane properties of a unique fatty acid. Chem Phys Lipids 126: 1–27, 2003

SUPPLEMENT 2

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Effect of hypo- and hyperthyroid states on phospholipid composition in developing rat heart

Blanka Hamplová,[1] Olga Nováková,[1] Eva Tvrzická,[2] Václav Pelouch[3] and František Novák[1]

[1]*Faculty of Science;* [2]*1st Faculty of Medicine;* [3]*2nd Faculty of Medicine and Centre for Experimental Cardiovascular Research, Charles University, Prague, Czech Republic*

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Abstract

The aim of this study was to examine the effect of hypo- and hyperthyroidism on the phospholipid composition in developing rat heart. The hypothyroid state (PTU) was induced by 0.05% 6-n-propyl-2-thiouracil in drinking water given to nursing mothers from the postnatal day 2–21. The hyperthyroidism (T_3) was made by daily injection of 3,3',5-triiodo-L-thyronine (10 μg/100 g body wt) to newborns in the same time period. Age matched intact littermates were taken as euthyroid controls. PTU decreased the concentration of total phospholipids (PL), choline phosphoglycerides (PC), ethanolamine phosphoglycerides (PE) and diphosphatidylglycerol (DPG) and increased the proportion of plasmalogen component of PE (PLPE). T_3 increased the concentration of PL, PC, PE, DPG and decreased PLPE in comparison with euthyroid controls. The ratio of saturated/unsaturated fatty acids (FA) in PE was decreased in PTU and increased in T_3 group. The ratio of n-6/n-3 polyunsaturated FA in PC, PE and phosphatidylinositol (PI) was increased in PTU due to increase of 18:2n-6 and decrease of 22:6n-3 proportion. T_3 decreased this ratio because of decline in 20:4n-6 and rise in 22:6n-3 proportion. Both hypo- and hyperthyroidism decreased the ratio of 20:4n-6/18:2n-6 in the majority of phospholipids. PTU decreased the unsaturation index in PC, PI and phosphatidylserine. It is concluded that thyroid state plays an essential role in the development of membrane phospholipid components in cardiac membranes during the early postnatal period. (Mol Cell Biochem **252**: 295–303, 2003)

Key words: hypothyroidism, hyperthyroidism, phospholipids, fatty acids, rat myocardium

Introduction

Heart development during the early postnatal period is characterised by the increase in magnitude and quality of membrane structures in cardiac cells. The sarcoplasmic reticulum (SR) is not fully developed after birth and the percentage volume of its network doubles in the first 11 postnatal days of the rat [1]. Progress in the biogenesis of mitochondrial membranes coupled with increased energy demand in the developing heart is indicated by the increase in concentration of mitochondrial enzymes [2], cytochromes [3] and diphosphatidylglycerol phospholipid of the inner mitochondrial membrane [4].

Ontogenetic development of cardiac tissue is controlled by thyroid hormones (TH), which appear in plasma of rats at the prenatal day 18 [5] and reach their peak levels in the third postnatal week [6]. It has been well documented that hypothyroidism induced after birth inhibits the postnatal maturation of ventricular tissue whereas hyperthyroidism accelerates this process. The presence of TH is essential for normal postnatal changes in myosin and troponin I isoform profiles [7, 8] and for postnatal maturation of sarcolemmal and SR-mediated Ca^{2+} handling [9–12].

Altered thyroid states affect the concentration of phospholipids and their fatty acid (FA) profiles in adult myocardium. These changes in quality of cell membranes modulate

Address for offprints: F. Novák, Faculty of Science, Charles University in Prague, Department of Biochemistry, Hlavova 2030, 128 43, Prague 2, Czech Republic (E-mail: franov@natur.cuni.cz)

pyruvate transport [13, 14], carnitine-acylcarnitine translocase activity [15], cytochrome c oxidase activity in inner mitochondrial membranes [16] and Ca^{2+} transport function of SR [17].

The only analysis dealing with the effect of hyperthyroidism on phospholipids in developing myocardium was reported by Vasdev *et al.* [18]. Their study showed that triiodothyronine administered during the first 30 days of postnatal life significantly increased the concentration of major phospholipids in rat heart. No complete analysis of FA composition in phospholipids of developing cardiac tissue influenced by altered thyroid states was published until now. Our study provides detailed analyses of the concentration and fatty acid composition of individual classes of cardiac phospholipids in the left ventricular myocardium of eu-, hypo- and hyperthyroid 21-day-old rats.

Materials and methods

Animal model

Newborn male Wistar rats were used in this experiment. Hypothyroidism (PTU) was induced by inclusion of 0.05% 6-n-propyl-2-thiouracyl in drinking water supplied to mothers from the 2nd to 21st day postpartum. Hyperthyroid animals (T_3) were made by daily subcutaneous injection of 3,3',5-triiodo-L-thyronine (Serva, Heidelberg, FRG) at the dose 10 μg per 100 g body weight for the same period. Thyroid hormone was dissolved in the solution containing physiological saline and 0.02 M NaOH. Hypothyroid and euthyroid rats (C) were injected with physiological saline. In each experimental group there were six rats. All animals were fed the same vitamin-enriched, low fat (3.5% by weight) standard diet ST1 (Velaz). The diet contained, by our analyses, 1.5% of 14:0; 19.2% of 16:0; 2.2% of 16:1n-7; 5.5% of 18:0; 25.4% of 18:1n-9; 1.9% of 18:1n-7; 38.7% of 18:2n-6 and 4.2% of 18:3n-3. Rats were killed by cervical dislocation at the postnatal day 21. Hearts were dissected free of atrial tissue, large blood vessels and the right ventricle. The left ventricle including the septum (LV+S) was rinsed in cold saline, weighed, frozen and stored in liquid nitrogen. The investigation conforms to 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).

Lipid analysis

Frozen tissues of LV+S were pulverized and homogenized. Phospholipids (from 100 mg wet tissue) were extracted in three consecutive steps according to the modified method of Folch *et al.* [19]. The first extraction was performed in three portions (0.25 ml each) of the chloroform-methanol mixture (1:3, 2:1 and 2:1) in a chilled mortar. Subsequent extractions were performed in a mixture 2:1 (0.6 ml each). 0.9% NaCl in water (20% volume of extract) was added and after a vigorous shaking the lower lipid layer was dried at 40°C under a stream of nitrogen.

Choline phosphoglycerides (PC), ethanolamine phosphoglycerides (PE), diphosphatidylglycerol (DPG), sphingomyelin (SM), phosphatidylinositol (PI), phosphatidylserine (PS) were separated by two-dimensional thin layer chromatography. Silica Gel H (Merck) as a slurry of 22.5 g in 62 ml water containing 2.5 g of magnon (Merck) was spread in a 0.25 mm layer with a spreader (Desaga) on glass plates (20 × 20 cm). Solvent mixtures were used according to the method of Rouser *et al.* [20]. Plasmalogen component of PC (PLPC) and PE (PLPE) were analysed according to the method of Horrocks *et al.* [21]. Silica Gel G (Merck) in 0.5 mm layer was used for their separation. Phospholipid spots were detected by iodine vapours, scraped out and analysed for phosphorus [20].

For FA analyses, phospholipids were separated on plates with Silica Gel H (0.5 mm) according to the method of Rouser *et al.* [20]. Spots were observed under UV light after staining with 0.005% 2',7'-dichloroflourescein in methanol, scraped out and stored in a nitrogen atmosphere at –20°C until the next day when the methyl esters were prepared. For FA methyl esters preparation, sodium methanolate was added to tubes with silica gel; tubes were then incubated for 60 min at room temperature in the dark; methyl esters were extracted with hexane; the extracts were evaporated under a stream of nitrogen and stored at –20°C. FA methyl esters were separated by the gas chromatograph Chrompack CP 438 A (Chrompack, Middelburg, The Netherlands) using middle polar column CP WAX 52 CB (25 m × 0.25 mm i.d.). The oven temperature was programmed from 145–230°C at 2°C/min. Hydrogen was used as carrier gas. FAs were identified using a mixture of FA methyl esters (Sigma-Aldrich Co., St. Louis, MO, USA).

Statistical analyses

All results are expressed as means ± S.E.M. The statistical significance of differences between groups was determined by one-way ANOVA and subsequent Bonferroni *t*-test ($p < 0.05$).

Results

Weight parameters

Both hypo- and hyperthyroidism led to body growth retardation. PTU and T_3 treatments decreased body weight by 28

and 23%, respectively. PTU reduced absolute weights of both left and right ventricles (by 50 and 55%) and reduced their relative weights as well (by 22 and 29%). T_3 increased absolute weights of left and right ventricles by 15 and 35% and their relative weights by 55 and 80%, respectively. There was no significant difference in percentage of dry tissue in both RV and LV of hypo- and hyperthyroid hearts (Table 1).

Concentration and distribution of phospholipids

PTU significantly decreased the concentration of total cardiac phospholipids by 14% in comparison with euthyroid rats. This decrease was caused by reduced concentration of PC, PE and DPG (by 16, 17 and 14%, respectively). T_3 had the opposite effect: it increased the concentration of total phospholipids by 13% due to higher concentration of PC, PE and DPG (by 10, 12 and 32%, respectively). No changes were observed in the concentration of other phospholipids (PI, PS, SM) in any experimental group (Table 2).

The effect of hypo- and hyperthyroidism on distribution of individual phospholipids in rat heart is shown in Table 3. PTU decreased the proportion of PE and increased PI, PS and SM whereas T_3 caused the rise in proportion of DPG and the reduction of PC.

The proportion of PLPC in PC was nearly 6-fold lower than that of PLPE in PE in euthyroid hearts. Neither hypo- nor hyperthyroidism had any effect on PLPC. On the other hand, PTU increased PLPE proportion by 35% and T_3 reduced it by 23% as compared with euthyroid controls (Fig. 1).

Fatty acid composition in individual phospholipids

The proportions of FA in phospholipids are presented in Tables 4–6. The sums of saturated FA (SFA), monounsaturated FA (MUFA), n-6 and n-3 polyunsaturated FA (PUFA) are expressed as percent of euthyroid control in Fig. 2.

Table 1. Weight parameters of euthyroid, hypothyroid and hyperthyroid 21-day-old rats

Weight	Euthyroid	Hypothyroid	Hyperthyroid
BW (g)	52.0 ± 1.7	33.4 ± 2.3*	38.2 ± 0.6*
HW (mg)	270.1 ± 6.9	131.4 ± 4.2*	319.2 ± 8.5*
LV+S (mg)	211.0 ± 6.0	104.3 ± 4.4*	240.0 ± 12.7*
RV (mg)	59.1 ± 7.3	27.1 ± 3.9*	79.2 ± 10.5*
LV+S/BW (mg/g)	4.04 ± 0.01	3.16 ± 0.11*	6.29 ± 0.32*
RV/BW (mg/g)	1.14 ± 0.06	0.81 ± 0.09*	2.07 ± 0.12*
LV+S (dry substance [%])	20.8 ± 0.3	19.5 ± 0.3	21.9 ± 0.3
RV (dry substance [%])	20.3 ± 0.5	19.0 ± 0.3	22.8 ± 0.5

Values are mean ± S.E.M. from 6 hearts; BW – body weight; LV – left ventricle; S – septum; RV – right ventricle. *$p < 0.05$, significantly different from euthyroid controls.

Regarding choline phosphoglycerides, PTU increased the proportion of palmitic acid (16:0) and simultaneously decreased the proportion of stearic acid (18:0); oleic acid (18:1n-9) increased by 35% and this caused the increase of total MUFA by 12%. PTU as well as T_3 decreased 20:4n-6/18:2n-6 ratio by 32 and 43%, respectively. In both altered thyroid states this decrease was due to the increase of linoleic acid (18:2n-6); in addition, T_3 decreased arachidonic acid (20:4n-6) content. The ratio of n-6/n-3 PUFA was changed in a different way: PTU increased it (by 46%), because of decreased docosapentaenoic acid (22:5n-3) and docosahexaenoic acid (22:6n-3) contents, and T_3 decreased n-6/n-3 ratio by 34%, mainly due to decreased 20:4n-6 content and increased eicosapentaenoic acid (20:5n-3) and 22:6n-3 contents. UI was lower by 12% in PTU and unaltered in T_3 group.

In phosphatidylinositol, the ratio of 20:4n-6/18:2n-6 was reduced only in T_3 group (by 50%), because of the reduction of 20:4n-6 content (by 17%). In PTU group, n-6/n-3 ratio was higher by 55%; this was caused by decreased content of 22:5n-3 and 22:6n-3. In T_3 group, this ratio was lower by

Table 2. Phospholipid concentration in left ventricle and septum of euthyroid, hypothyroid and hyperthyroid 21-day-old rats

Phospholipids	Concentration (μmol P. g^{-1} w.w.)		
	Euthyroid	Hypothyroid	Hyperthyroid
Total phospholipids	22.10 ± 0.27	18.90 ± 0.42*	24.90 ± 0.87*
Choline phosphoglycerides	9.64 ± 0.15	8.07 ± 0.23*	10.56 ± 0.38*
Ethanolamine phosphoglycerides	7.81 ± 0.09	6.45 ± 0.13*	8.78 ± 0.30*
Diphosphatidylglycerol	2.31 ± 0.05	1.99 ± 0.08*	3.05 ± 0.11*
Phosphatidylserine	0.75 ± 0.03	0.72 ± 0.02	0.75 ± 0.03
Phosphatidylinositol	1.10 ± 0.02	1.03 ± 0.03	1.13 ± 0.05
Sphingomyelin	0.53 ± 0.02	0.62 ± 0.04	0.58 ± 0.03
Choline plasmalogen[a]	0.52 ± 0.03	0.50 ± 0.02	0.58 ± 0.08
Ethanolamine plasmalogen[a]	2.22 ± 0.04	2.59 ± 0.07*	1.99 ± 0.09*

Values are mean ± S.E.M. from 6 hearts; w.w. – wet weight; [a]choline and ethanolamine plasmalogens are included in choline and ethanolamine phosphoglycerides, respectively. *$p < 0.05$, significantly different from euthyroid controls.

Table 3. Phospholipid distribution in left ventricle and septum of euthyroid, hypothyroid and hyperthyroid 21-day-old rats

Phospholipids	Distribution (%)		
	Euthyroid	Hypothyroid	Hyperthyroid
Choline phosphoglycerides	43.50 ± 0.26	42.7 ± 0.46	42.40 ± 0.26*
Ethanolamine phosphoglycerides	35.30 ± 0.20	34.2 ± 0.23*	35.20 ± 0.19
Diphosphatidylglycerol	10.30 ± 0.19	10.6 ± 0.36	12.20 ± 0.24*
Phosphatidylserine	3.43 ± 0.12	3.84 ± 0.13*	3.08 ± 0.05
Phosphatidylinositol	5.02 ± 0.09	5.46 ± 0.12*	4.73 ± 0.14
Sphingomyelin	2.48 ± 0.11	3.26 ± 0.20*	2.39 ± 0.07

Values are mean ± S.E.M. from 6 hearts. *$p < 0.05$, significantly different from euthyroid control.

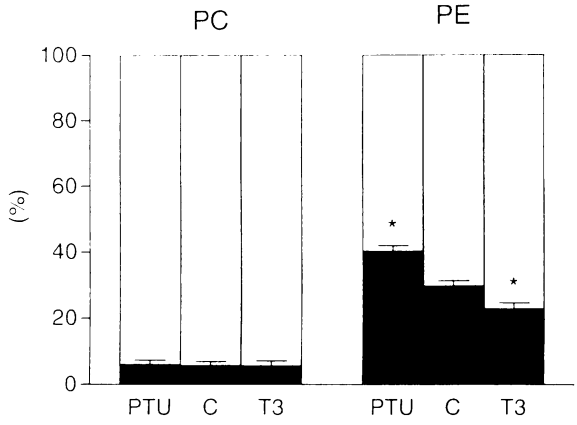

Fig. 1. Plasmalogen proportions of choline and ethanolamine phosphoglycerides. PTU – hypothyroid; C – control; T_3 – hyperthyroid rats; PC – choline phosphoglycerides; PE – ethanolamine phosphoglycerides. Black columns represent % of plasmalogens from corresponding phosphoglycerides. Values are means ± S.E.M. from 6 hearts. *$p < 0.05$, significantly different from euthyroid control.

33% due to decreased content of 20:4n-6 followed by increased 22:6n-3 content. Hypothyroidism decreased UI by 7% in PI.

In ethanolamine phosphoglycerides, PTU decreased 18:0 content, the sum of SFA and SFA/UFA ratio. On the contrary, T_3 decreased the proportion of 16:0 and increased 18:0 FA. SFA sum and SFA/UFA ratio were unaltered. Both altered thyroid states declined 20:4n-6/18:2n-6 ratio: PTU by 50% due to the marked increase of 18:2n-6 (by 62%) and T_3 by 86% due to the decrease of 20:4n-6 (by 36%). In PTU group, higher content of 18:2n-6 and 20:4n-6 caused the rise in n-6/n-3 PUFA ratio. On the contrary, hyperthyroidism decreased n-6/n-3 ratio because of decreased 20:4n-6 and increased 22:6n-3 proportion.

The fatty acid composition in PS was influenced more by the hypothyroid state than the hyperthyroid one. PTU increased the proportion of 16:0 and total SFA. In addition, hypothyroidism decreased the sum of n-3 FA by 18% and the UI by 12%. The effect of T_3 was expressed by decreased contents of 20:4n-6 and 22:5n-3.

The FA composition of DPG was different in comparison with other phospholipids in euthyroid cardiac tissue. The sum of SFA was about 6-fold lower, MUFA about 2-fold higher and the total n-6 FA was about 3-fold higher than in other phospholipids. Less pronounced response to both altered thyroid states was observed in remodelling of fatty acid composition of DPG as compared with other phospholipids. Hypothyroidism decreased the sum of MUFA, the ratio of 20:4n-6/18:2n-6 and increased content of n-6 PUFA. Hyperthyroidism increased the proportion of 18:1n-7 and decreased the proportion of 20:4n-6 in DPG.

Discussion

In our study, both hypo- and hyperthyroidism resulted in the retardation of body growth. During the first three weeks after birth, PTU reduced heart weight whereas in T_3 animals the heart weight increased. The proportional degree of weight

Table 4. Fatty acid composition (mol%) of choline phosphoglycerides and phosphatidylinositol in left ventricle and septum of euthyroid, hypothyroid and hyperthyroid rats

Fatty acids	Choline phosphoglycerides			Phospatidylinositol		
	Euthyroid	Hypothyroid	Hyperthyroid	Euthyroid	Hypothyroid	Hyperthyroid
16:0	21.90 ± 0.62	25.90 ± 0.60*	21.80 ± 0.70	7.53 ± 0.35	7.52 ± 0.45	7.80 ± 0.79
18:0	24.30 ± 0.54	22.40 ± 0.45*	25.90 ± 0.60	39.20 ± 0.69	40.70 ± 1.17	39.50 ± 0.93
18:1n-9	3.27 ± 0.20	4.40 ± 0.10*	3.04 ± 0.09	3.50 ± 0.43	3.94 ± 0.35	3.94 ± 0.53
18:1n-7	4.88 ± 0.13	4.76 ± 0.15	5.32 ± 0.18	1.76 ± 0.13	1.54 ± 0.09	1.89 ± 0.18
18:2n-6	6.95 ± 0.32	9.48 ± 0.27*	8.56 ± 0.57*	2.22 ± 0.10	2.67 ± 0.18	2.93 ± 0.36
20:2n-6	0.46 ± 0.07	0.47 ± 0.05	0.38 ± 0.02	1.45 ± 0.14	1.35 ± 0.13	1.27 ± 0.16
20:3n-6	0.72 ± 0.03	0.67 ± 0.02	0.49 ± 0.03*	0.87 ± 0.05	0.77 ± 0.02	0.78 ± 0.05
20:4n-6	21.80 ± 0.55	20.30 ± 0.25	15.40 ± 0.73*	30.10 ± 1.01	31.70 ± 0.80	25.10 ± 0.83*
20:5n-3	0.52 ± 0.03	0.60 ± 0.03	0.72 ± 0.07*	0.51 ± 0.07	0.55 ± 0.05	0.78 ± 0.20
22:4n-6	0.44 ± 0.03	0.25 ± 0.02*	0.31 ± 0.01*	0.61 ± 0.04	0.42 ± 0.03*	0.47 ± 0.07
22:5n-3	4.43 ± 0.27	2.69 ± 0.15*	3.52 ± 0.19*	3.94 ± 0.13	2.09 ± 0.07*	3.20 ± 0.18*
22:6n-3	9.33 ± 0.63	6.58 ± 0.32*	13.42 ± 0.35*	6.31 ± 0.40	4.23 ± 0.37*	10.01 ± 0.69*
n-6/n-3	2.17 ± 0.13	3.16 ± 0.14*	1.43 ± 0.11*	3.24 ± 0.18	5.03 ± 0.18*	2.16 ± 0.13*
SFA/UFA	0.88 ± 0.03	0.97 ± 0.02	0.94 ± 0.03	0.90 ± 0.02	0.97 ± 0.04	0.93 ± 0.02
20:4/18:2	3.19 ± 0.19	2.16 ± 0.07*	1.82 ± 0.09*	13.86 ± 1.04	12.19 ± 0.84	9.27 ± 1.20*
UI	195.4 ± 5.2	170.3 ± 3.1*	193.3 ± 1.6	200.7 ± 3.7	186.1 ± 4.4*	201.6 ± 3.8

Values are mean ± S.E.M. from 6 hearts; SFA – sum of saturated fatty acids; UFA – sum of unsaturated fatty acids; UI – unsaturation index is sum of mol% of individual unsaturated fatty acids x number of double bonds. *$p < 0.05$, significantly different from euthyroid controls.

Table 5. Fatty acid composition (mol%) of ethanolamine phosphoglycerides and phosphatidylserine in left ventricle and septum from euthyroid, hypothyroid and hyperthyroid rats

Fatty acids	Ethanolamine phosphoglycerides			Phosphatidylserine		
	Euthyroid	Hypothyroid	Hyperthyroid	Euthyroid	Hypothyroid	Hyperthyroid
16:0	13.40 ± 0.57	14.40 ± 0.53	10.20 ± 0.50*	5.25 ± 0.61	8.84 ± 1.28*	4.58 ± 0.75
18:0	25.90 ± 0.54	22.40 ± 1.00*	33.50 ± 0.60*	40.70 ± 1.29	40.70 ± 1.41	43.60 ± 0.90
18:1n-9	2.76 ± 0.29	2.59 ± 0.47	2.44 ± 0.08	7.17 ± 0.26	7.63 ± 0.75	7.51 ± 0.72
18:1n-7	2.10 ± 0.20	1.98 ± 0.20	1.86 ± 0.29	1.69 ± 0.24	2.19 ± 0.20	1.37 ± 0.05
18:2n-6	2.79 ± 0.20	4.51 ± 0.34*	3.20 ± 0.23	2.37 ± 0.15	3.65 ± 0.47*	2.73 ± 0.30
20:2n-6	0.43 ± 0.03	0.42 ± 0.09	0.30 ± 0.02	1.83 ± 0.29	1.58 ± 0.26	1.52 ± 0.18
20:3n-6	0.47 ± 0.03	0.47 ± 0.04	0.29 ± 0.01*	2.17 ± 0.11	1.44 ± 0.05*	1.82 ± 0.07*
20:4n-6	19.50 ± 0.29	21.50 ± 0.70*	12.50 ± 0.67*	5.46 ± 0.32	6.91 ± 1.07	4.40 ± 0.32*
20:5n-3	0.61 ± 0.02	0.99 ± 0.05*	0.75 ± 0.03*	0.60 ± 0.16	0.37 ± 0.13	0.33 ± 0.06
22:4n-6	0.94 ± 0.04	0.74 ± 0.03*	0.64 ± 0.02*	2.09 ± 0.05	1.47 ± 0.09*	1.72 ± 0.09*
22:5n-3	5.60 ± 0.18	4.89 ± 0.19*	3.36 ± 0.17*	6.03 ± 0.21	5.01 ± 0.24*	4.42 ± 0.31*
22:6n-3	23.70 ± 0.92	24.20 ± 1.09	30.30 ± 0.52*	21.30 ± 0.86	17.20 ± 1.04*	23.30 ± 0.78
n-6/n-3	0.81 ± 0.03	0.93 ± 0.04*	0.49 ± 0.02*	0.51 ± 0.02	0.69 ± 0.10	0.44 ± 0.02
SFA/UFA	0.68 ± 0.02	0.57 ± 0.03*	0.79 ± 0.03*	0.89 ± 0.03	1.03 ± 0.03	0.98 ± 0.06
20:4/18:2	7.31 ± 0.63	4.94 ± 0.43*	3.97 ± 0.29*	2.36 ± 0.17	1.88 ± 0.12	1.72 ± 0.21*
UI	268.9 ± 6.2	281.2 ± 8.2	267.8 ± 5.2	218.8 ± 5.3	191.9 ± 5.4*	214.1 ± 6.2

Values are mean ± S.E.M. from 6 hearts; SFA – sum of saturated fatty acids; UFA – sum of unsaturated fatty acids; UI – unsaturation index is sum of mol% of individual unsaturated fatty acids x number of double bonds. *$p < 0.05$, significantly different from euthyroid controls.

restriction in right and left ventricles in PTU and their hypertrophy in T_3 group with right ventricle preponderance was observed, that corresponds to previously published analysis using the same model [9].

Our present study demonstrates changes in phospholipid concentration, a shift in relative distribution of individual phospholipid species and their fatty acid profile in immature hearts under both hypo- and hyperthyroid states. The decreased amount of PL, PC, PE and DPG in hypothyroid hearts suggests that normal maturation of cardiac membranes was delayed compared with euthyroid rats. This is supported by our previous results showing that phospholipid concentration in ventricular tissue of 21-day-old hypothyroid rats approximately corresponds to values observed in ventricles of 2-day-old euthyroid ones [4]. In hypothyroid adult rats, Taylor *et al.* [22] found the reduced DPG content and DPG synthase activity in heart mitochondria.

We observed that the relative proportion of DPG and PC was unchanged and the relative proportion of PE decreased, whereas proportion of PI, PS and SM increased in hypothyroid hearts. Pehowich [23] also observed decreased proportion of PE, increased proportion of PI and PS, but in addition, he found decreased DPG and increased PC proportion in cardiac inner mitochondrial membrane of hypothyroid rats which were treated with propylthiouracyl from the second week after weaning. Similarly, the relative decrease of PE proportion was observed in SR of the skeletal muscle of hypothyroid adult rats [24]. On the other hand, Paradies *et al.* [15] reported the decreased DPG proportion accompanied with increased PC and PE proportion in cardiac mitochondria from hypothyroid adult rats.

Several factors may play a role in slowing down maturation of phospholipid membrane components observed in hypothyroidism. One of them may be the absence of the stimulating effect of TH on expression and activity of enzymes involved in phospholipid metabolism [25]. Nutrition deficit can be another reason for reduced gain in cardiac

Table 6. Fatty acid composition (mol%) of diphosphatidylglycerol in left ventricle and septum from euthyroid, hypothyroid and hyperthyroid rats

Fatty acids	Diphosphatidylglycerol		
	Euthyroid	Hypothyroid	Hyperthyroid
16:0	3.66 ± 0.68	2.26 ± 0.51	2.36 ± 0.37
18:0	3.50 ± 0.71	2.16 ± 0.34	2.73 ± 0.56
18:1n-9	5.02 ± 0.56	5.69 ± 0.59	4.93 ± 0.89
18:1n-7	12.55 ± 0.36	6.66 ± 0.35*	15.00 ± 0.75*
18:2n-6	45.70 ± 1.69	57.70 ± 1.57*	48.30 ± 2.53
20:2n-6	1.79 ± 0.16	1.86 ± 0.15	1.71 ± 0.12
20:3n-6	5.49 ± 0.20	4.66 ± 0.28*	3.13 ± 0.15*
20:4n-6	7.13 ± 0.43	5.74 ± 0.41*	4.93 ± 0.30*
20:5n-3	0.25 ± 0.02	0.36 ± 0.12	0.25 ± 0.06
22:4n-6	0.42 ± 0.04	0.38 ± 0.05	0.32 ± 0.04
22:5n-3	2.40 ± 0.19	1.66 ± 0.13*	1.98 ± 0.18
22:6n-3	9.69 ± 1.08	8.29 ± 0.93	12.50 ± 1.46
n-6/n-3	5.16 ± 0.60	6.76 ± 0.58	4.25 ± 0.71
SFA/UFA	0.09 ± 0.02	0.06 ± 0.01	0.06 ± 0.01
20:4/18:2	0.16 ± 0.01	0.10 ± 0.01*	0.10 ± 0.01*
UI	233.1 ± 6.8	233.1 ± 6.1	238.9 ± 6.4

Values are mean ± S.E.M. from 6 hearts; SFA – sum of saturated fatty acids; UFA – sum of unsaturated fatty acids; UI – unsaturation index is sum of mol% of individual unsaturated fatty acids x number of double bonds. *$p < 0.05$, significantly different from euthyroid controls.

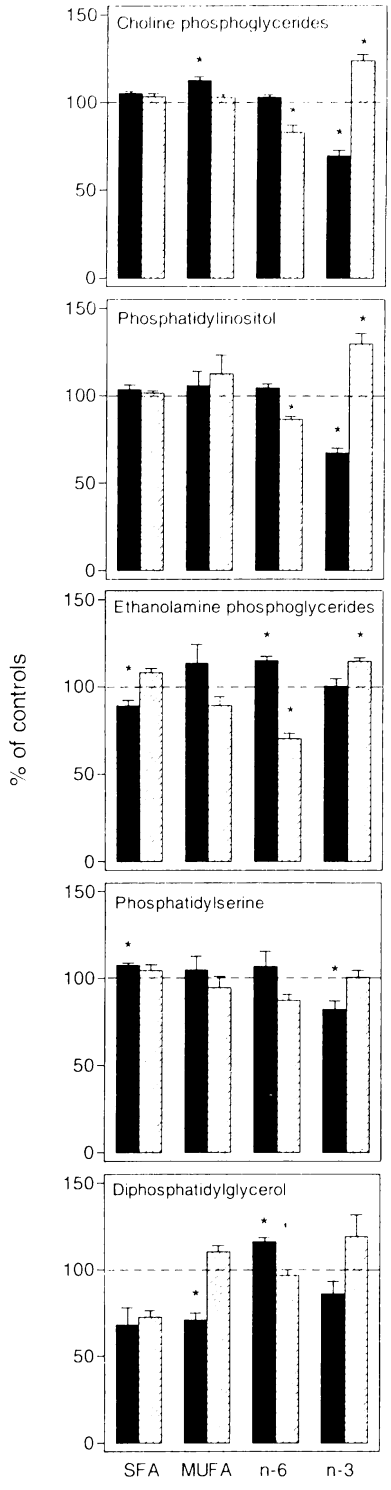

Fig. 2. Composition of main fatty acid classes in individual phospholipid species in left ventricle and septum of hypothyroid and hyperthyroid 21-day-old rats, expressed as percent of euthyroid control values (dotted lines). Values are means ± S.E.M. from 6 hearts; SFA – sum of saturated fatty acids; MUFA – sum of monounsaturated fatty acids; n-6, n-3, sum of corresponding polyunsaturated fatty acids; black columns, hypothyroid rats; stripped columns, hyperthyroid rats. *$p < 0.05$, significantly different from euthyroid controls.

phospholipid content. Because hypothyroid mothers can be hypolactating [26], it is quite likely that neonates from hypothyroid mothers did not receive an adequate amount of important substrates from milk resulting in a decreased phospholipid content [27]. The cardiac hypofunction with low heart rate, low velocity of contraction and low output caused by hypothyroidism may be the additional cause of restricted membrane phospholipid maturation. A similar decrease of phospholipid content as we observed in hypothyroid myocardium was found in transplanted atrophying hypofunctional myocardium of euthyroid rats [18].

The increased concentration of PL, PC, PE and DPG in hyperthyroid heart tissue was consistent with accelerated maturation of ventricular myocytes as was evidenced by proliferation of SR, system of T-tubules and mitochondrial membranes [28]. According to our results and those of Vasdev *et al.* [18], the ratio of dry to wet weight of hyperthyroid and control hearts was similar. Therefore the increased synthesis of phospholipids in hyperthyroid heart tissue must have exceeded accelerated protein synthesis characteristic of this type of hypertrophy [8] resulting in the disproportionate increase of phospholipids per gram of muscle mass. In support of this conclusion previous investigators reported that the number and size of mitochondria as well as number of cristae increased in cardiac muscle of chronically hyperthyroid animals [29]. Cao *et al.* [30] reported the increase of DPG content accompanied with enhanced activity of enzymes involved in DPG metabolic pathway even after a short period of hyperthyroidism in adult rats. The increased concentration of PL and PC was also found in SR from hyperthyroid adult rats [17].

The prominent observation of this study was the fact that while the concentration of all major phospholipids increased in hyperthyroid hearts, the only increased relative proportion was that of DPG that was compensated by decreased proportion of PC. The principle subcellular location of DPG in mammalian tissues is the inner mitochondrial membrane and the critical role for DPG in the process of energy generation via the respiratory electron transport chain has been well documented [31]. Page *et al.* [1] observed that the contribution of myofibrils and mitochondria to myocardial cell volume increased proportionately to each other and to cell volume as a whole during postnatal growth of ventricles of euthyroid rats. In this respect, the normal growth differs from that influenced by T_3 treatment. In hyperthyroid rats the mitochondrial contribution to cell volume increases disproportionately while the volume of myofibrils remains constant at its normal value [32]. In adult rats, Paradies *et al.* [14] found the increased proportion of DPG and in addition, the increased proportion of PI and PS and decreased proportion of PC and PE after five-day T_3 treatment.

We have found changed plasmalogen proportion of PE at both altered thyroid states. Hypothyroidism increased PLPE

proportion in PE, whereas hyperthyroidism reduced it in comparison with controls. Because plasmalogens are predominant phospholipids of cardiac sarcolemma and their content is relatively low in mitochondria [33], the observed differences in plasmalogen proportion between different thyroid states may result from changes in mitochondrial to extramitochondrial membrane proportion.

We analysed the FA composition of cardiac phospholipids from rats that were treated with drugs in the period when important nutritional, endocrine and functional changes are taking place. During first 3 postnatal weeks, changes of FA composition are connected with the relative decrease of 16:0, 18:1n-9 and 20:4n-6 and the increase of 18:0, 18:2n-6 and 22:6n-3 in cardiac PC, PE [34, 35] and DPG [36] in euthyroid rats. In our study, both altered thyroid states caused marked changes in FA composition of individual phospholipids in comparison with euthyroid control.

Hypothyroidism maintained similar phospholipid FA composition in ventricles of 21-day-old rats as in euthyroid rats just after birth [34–36]; except for 18:2n-6, the content of which was higher in all phospholipids compared with the euthyroid group. In a similar way, the content of 18:2n-6 rose in cardiac mitochondria of hypothyroid adult rats [37]. A possible explanation for this observation is the inhibition of Δ-6 desaturase activity under hypothyroid state, which is illustrated by decreased 20:4n-6/18:2n-6 ratio. The diminished Δ-6 desaturase activity, the rate limiting step in conversion of 18:2n-6 to 20:4n-6, was observed in liver of hypothyroid adult rats [38].

The remodelling of FA composition after PTU treatment led to decrease of UI in PC, PI and PS. This decrease was caused by a redistribution of fatty acids in favour of less unsaturated fatty acids and due to marked decrease in proportion of 22:6n-3 in comparison with controls. Pehowich [13, 23] observed the similar decrease of 22:6n-3 content accompanied with reduced ratio of n-6/n-3 PUFA in mitochondria of hypothyroid rats. Interestingly, this effect of hypothyroidism was diminished with n-3 PUFA enriched diet. Decreased UI was also observed in cardiac mitochondria from hypothyroid adult rats [37] and in sarcolemma from hypothyroid adult rabbits [39].

Changes in FA composition observed in T_3 treated hearts were mainly represented by decreased n-6/n-3 PUFA ratio in PC, PE and PI, which was caused by the decrease in 20:4n-6 and increase in 22:6n-3 content. Enhanced conversion of 20:4n-6 to prostaglandin E_2 by the microsomal prostaglandin synthetase may contribute to lower 20:4n-6 level in phospholipids of hyperthyroid hearts [40]. The modification of the balance between n-6 and n-3 FA may have significant biological implications because eicosanoids derived from these two PUFA series exert different biological activities [41]. As was mentioned above, the proportion of 22:6n-3 gradually increases in phospholipids of euthyroid hearts during early postnatal period. Therefore enhanced level of 22:6n-3 in hyperthyroid hearts may be caused by accelerated remodelling process of phospholipids in which the stimulating effect of TH on enzymes of deacylation-reacylation cycle may play a role [25]. Among others, the increase of 22:6n-3 in heart phospholipids may be related to increased heart rate in T_3 rats. A positive correlation between 22:6n-3 and heart rate of various animal species was already reported [42]. Because hypothyroidism decreases and hyperthyroidism increases alpha- and beta-adrenergic stimulation in developing rat heart [43–45], the changes in accumulation of 22:6n-3 in heart phospholipids may result from the effect of thyroid state on catecholamine sensitivity of immature rat heart. It was well documented that the stimulation of heart by increased doses of catecholamines led to enhanced accumulation of n-3 PUFA in heart phospholipids of adult rats [46, 47]. Peifer [48] reported increased proportion of 22:6n-3 and decreased ratio of n-6/n-3 PUFA in total heart lipids from chronically hyperthyroid adult rats. The similar remodelling of lipid composition occurred in heart membranes in response to different stress conditions like exposure to chronic hypoxia [49], pressure-overload [50] and heat acclimation [51]. The altered phospholipid FA composition, particularly increased 22:6n-3 and decreased n-6/n-3 PUFA ratio, is a common feature of the response to these stressors. The membrane enrichment with 22:6n-3 has been suggested to protect membrane proteins by providing increased membrane stability [52]. It was shown that 22:6n-3 can specifically influence electrophysiological properties of cardiac membranes by altered function of ion channels [53] and level of their gene expression [54].

We observed that the outcome of hyperthyroidism on FA remodelling in DPG differed in rats treated immediately after birth from those treated in the adulthood. While there was the absence of any alteration in the relative FA content in heart DPG of hyperthyroid adult rats [55, 56], the significant redistribution of unsaturated FA in DPG was found in young rats of our study. The FA composition of DPG is important for proper function of key mitochondrial enzymes as was evidenced in the study where different diets, which altered FA profile of heart DPG, modified the activity of cytochrome c oxidase [57].

In summary, our data demonstrate that thyroid hormones are essential for the maturation of phospholipid component in rat myocardial membranes during early postnatal development. We have also shown that thyroid control over FA composition of myocardial phospholipids is active from early postnatal period. Number of reports confirmed that the alteration of thyroid state induced in neonatal period affects the expression and activity of membrane proteins involved in the signal transduction [43–45], excitation-contraction coupling [10, 11] and energy metabolism [58]. In addition, the changes in phospholipid environment modulate function of cardiac

membrane proteins as was well documented in studies where phospholipid composition was specifically altered [13, 23]. We suppose that phospholipid remodelling at least partly contributes to the modification of function of integral and membrane-associated proteins under altered thyroid states.

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References

1. Page E, Earley J, Power B: Normal growth of ultrastructures in rat left ventricular myocardial cells. Circ Res 35(suppl 6): 1974
2. Baldwin KM, Campbell PJ, Hooker AM, Lewis RE: Effects of thyroid deficiency and sympathectomy on cardiac enzymes. Am J Physiol 236: C30–C34, 1979
3. Hallman M, Maenpaa P, Hassinen I: Levels of cytochromes in heart, liver, kidney and brain in the developing rat. Experientia 28: 1408–1410, 1972
4. Novák F, Chvojkova S, Jarkovská D, Kolar F, Tvrzická E, Nováková O: Ultrastructure, phospholipid composition and fatty acid profile of hearts after aortic constriction in newborn rats. J Mol Cell Cardiol 29: A132, 1997
5. Pic P, Bouquin JP: Thyrotropic hormone and thyroidal function initiation in fetal rat. J Dev Physiol 7: 207–214, 1985
6. Vigouroux E: Dynamic study of post-natal thyroid function in the rat. Acta Endocrinol (Copenh) 83: 752–762, 1976
7. Dieckman LJ, Solaro RJ: Effect of thyroid status on thin-filament Ca regulation and expression of troponin I in perinatal and adult rat hearts. Circ Res 67: 344–351, 1990
8. Cernohorsky J, Pelouch V, Kolar F, Milerova M, Ostadal B: Protein profiling of developing rat myocardium is altered by theroid hormone. Physiol Res 47: 9P, 1998
9. Kolar F, Seppet EK, Vetter R, Vetter R, Prochazka J, Grunermel J, Zilmer K, Ostadal B: Thyroid control of contractile function and calcium handling in neonatal rat heart. Pflügers Arch 421: 26–31, 1992
10. Wibo M, Feron O, Zheng L, Maleki M, Kolar F, Godfraind T: Thyroid status and postnatal changes in subsarcolemmal distribution and isoform expression of rat cardiac dihydropyridine receptors. Cardiovasc Res 37: 151–159, 1998
11. Cernohorsky J, Kolar F, Pelouch V, Korecky B, Vetter R: Thyroid control of sarcolemmal Na^+/Ca^{++} exchanger and SR Ca^{++}-ATPase in developing rat heart. Am J Physiol 275: H264–H273, 1998
12. Wibo M, Kolar F, Zheng L, Godfraind T: Influence of thyroid status on postnatal maturation of calcium channels, beta-adrenoceptors and cation transport ATPases in rat ventricular tissue. J Mol Cell Cardiol 27: 1731–1743, 1995
13. Pehowich DJ: Hypothyroid state and membrane fatty acid composition influence cardiac mitochondrial pyruvate oxidation. Biochim Biophys Acta 1235: 231–238, 1995
14. Paradies G, Ruggiero FM: Effect of hyperthyroidism on the transport of pyruvate in rat-heart mitochondria. Biochim Biophys Acta 935: 79–86, 1988
15. Paradies G, Ruggiero FM, Petrosillo G, Quagliariello E: Alterations in carnitine-acylcarnitine translocase activity and in phospholipid composition in heart mitochondria from hypothyroid rats. Biochim Biophys Acta 1362: 193–200, 1997
16. Paradies G, Petrosillo G, Ruggiero FM: Cardiolipin-dependent decrease of cytochrome c oxidase activity in heart mitochondria from hypothyroid rats. Biochim Biophys Acta 1319: 5–8, 1997
17. Limas CJ: Calcium transport ATPase of cardiac sarcoplasmic reticulum in experimental hyperthyroidism. Am J Physiol 235: H745–H752, 1978
18. Vasdev SC, Korecky B, Rastogi RB, Singhal RL, Kako KJ: Myocardial lipid metabolism in cardiac hyper- and hypo-function. Studies on triiodothyronine-treated and transplanted rat hearts. Can J Physiol Pharmacol 55: 1311–1319, 1977
19. Folch J, Lees M, Sloan-Stanley GH: A simple method for the isolation and purification of total lipids from animal tissue. J Biol Chem 226: 497–509, 1957
20. Rouser G, Fleischer S, Yamamoto A: Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids 5: 494–496, 1970
21. Horrocks LA: The alk-1-enyl group content of mammalian myelin phosphoglycerides by quantitative two-dimensional thin-layer chromatography. J Lipid Res 9: 469–472, 1968
22. Taylor WA, Xu FY, Ma BJ, Mutter TC, Dolinsky VW, Hatch GM: Expression of monolysocardiolipin acyltransferase activity is regulated in concert with the level of cardiolipin and cardiolipin biosynthesis in the mammalian heart. BMC Biochemistry 3: 9, 2002
23. Pehowich DJ: Hypothyroid state and membrane fatty acid composition influence cardiac mitochondrial pyruvate oxidation. Biochim Biophys Acta 1235: 231–238, 1995
24. Simonides WS, van Hardeveld C: Effects of hypothyroidism on the distribution and fatty acyl composition of phospholipids in sarcoplasmic reticulum of fast skeletal muscle of the rat. Biochim Biophys Acta 924: 204–209, 1987
25. Hoch FL: Lipids and thyroid hormones. Prog Lipid Res 27: 199–270, 1988
26. Babicky A, Novakova V: Influence of thyroxine and propylthiouracil administration on the intake of maternal milk in sucklings of the laboratory rat. Physiol Bohemoslov 34: 193–199, 1985
27. Steven HZ, Douglas C, Nancy FS: Choline, phosphatidylcholine and sphingomyelin in human and bovine milk and infant formulas. J Nutr 116: 50–58, 1986
28. Jarkovska D, Kolar F, Prochazka J, Ostadal B: Structural maturation of the newborn rat myocardium: The influence of thyroid hormones. Funct Develop Morph 4: 167–168, 1994
29. Page E, McCallister LP: Quantitative electron microscopic description of heart muscle cells. Application to normal, hypertrophied and thyroxin-stimulated hearts. Am J Cardiol 31: 172–181, 1973
30. Cao SG, Cheng P, Angel A, Hatch GM: Thyroxine stimulates phosphatidylglycerolphosphate synthase activity in rat heart mitochondria. Biochim Biophys Acta 1256: 241–244, 1995
31. Hoch FL: Cardiolipins and biomembrane function. Biochim Biophys Acta 1113: 71–133, 1992
32. McCallister LP, Page E: Effects of thyroxin on ultrastructure of rat myocardial cells: A stereological study. J Ultrastruct Res 42: 136–155, 1973
33. Post JA, Verkleij AJ, Roelofsen B, Op de Kamp JA: Plasmalogen content and distribution in the sarcolemma of cultured neonatal rat myocytes. FEBS Lett 240: 78–82, 1988
34. Gudmundsdottir A, Gudbjarnason S: Neonatal changes in fatty acid profile of phospholipids in rat heart muscle. Biochim Biophys Acta 752: 284–290, 1983
35. Ghebremeskel K, Bitsanis D, Koukkou E, Koukkou E, Lowy C, Poston L, Crawford MA: Post-natal modulation of heart and liver phosphoglyceride fatty acids in pups. Ann Nutr Metab 43: 365–373, 1999

36. Berger A, Gershwin ME, German JB: Effects of various dietary fats on cardiolipin acyl composition during ontogeny of mice. Lipids 27: 605–612, 1992
37. Hoch FL: Thyroid control over biomembranes. VII. Heart muscle mitochondria from $_{L}$-Triiodothyronine-injected rats. J Mol Cell Cardiol 14: 81–90, 1982
38. Faas FH, Carter WJ: Fatty acid desaturation and microsomal lipid fatty acid composition in experimental hypothyroidism. Biochem J 207: 29–35, 1982
39. Szymanska G, Pikula S, Zborowski J: Effect of hyper- and hypothyroidism on phospholipid fatty acid composition and phospholipases activity in sarcolemma of rabbit cardiac muscle. Biochim Biophys Acta 1083: 265–270, 1991
40. Gudbjarnason S: Prostaglandins and polyunsaturated fatty acids in heart muscle. J Mol Cell Cardiol 7: 443–449, 1975
41. Weber PC, Leaf A: Cardiovascular effects of ω3 fatty acids. Atherosclerosis risk factor modification by ω3 fatty acids. World Rev Nutr Diet 66: 218–232, 1991
42. Gudbjarnason S, Hallgrímson J, Doell B, Steingrímsdóttir U, Emilsson A, Sigmundson S: In: P. Padieu, J.P. Didier (eds). International Symposium for Heart Research – European Section, 1975, pp 73–82
43. Noguchi A, Whitsett JA: Ontogeny of alpha 1-adrenergic receptors in the rat myocardium: Effects of hypothyroidism. Eur J Pharmacol 86: 43–50, 1982
44. Whitsett JA, Pollinger J, Matz S: beta-Adrenergic receptors and catecholamine sensitive adenylate cyclase in developing rat ventricular myocardium: Effect of thyroid status. Pediatr Res 16: 463–469, 1982
45. Novotny J, Bourova L, Malkova O, Svoboda P, Kolar F: G proteins, beta-adrenoreceptors and beta-adrenergic responsiveness in immature and adult rat ventricular myocardium: Influence of neonatal hypo- and hyperthyroidism. J Mol Cell Cardiol 31: 761–772, 1999
46. Emilsson A, Gudbjarnason S: Reversible alterations in fatty acid profile of glycerophospholipids in rat heart muscle induced by repeated norepinephrine administration. Biochim Biophys Acta 750: 1–6, 1983
47. Benediktsdottir VE, Gudbjarnason S: Reversible alterations in fatty acid composition of heart muscle membrane phospholipids induced by epinephrine in rats fed different fats. J Lipid Res 29: 765–772, 1988
48. Peifer JJ: Disproportionately higher levels of myocardial docosahexaenoate and elevated levels of plasma and liver arachidonate in hyperthyroid rats. J Lipid Res 9: 193–199, 1968
49. Ježková J, Nováková O, Kolář F, Tvrzická E, Neckář J, Novák F: Chronic hypoxia alters fatty acid composition of phospholipids in right and left ventricular myocardium. Mol Cell Biochem 232: 49–56, 2002
50. Reibel DK, O'Rourke B, Foster KA, Hutchinson H, Uboh CE, Kent RL: Altered phospholipid metabolism in pressure-overload hypertrophied hearts. Am J Physiol 250: H1–H6, 1986
51. Shmeeda H, Kaspler P, Shleyer RJ, Yer J, Honen R, Horowitz M, Barenholz Y: Heat acclimation in rats: Modulation via lipid polyunsaturation. Am J Physiol Reg Integrative Comp Physiol 283: R389–R399, 2002
52. Mitchel DC, Litman BJ: Modulation of receptor signalling by phospholipid acyl chain composition. In: D.I. Mostovofsky, S. Yehuda, N. Salem (eds). Fatty Acids. Physiological and Behavioral Functions. Humana, Totowa, NJ, 2001, pp 1–18
53. Goel DP, Maddaford TG, Pierce GN: Effects of ω-3 polyunsaturated fatty acids on cardiac sarcolemmal Na^+/H^+ exchange. Am J Physiol Heart Circ Physiol 283: H1688–H1694, 2002
54. Kang JX, Li Y, Leaf A: Regulation of sodium channel gene expression by class I antiarrhytmic drugs and n-3 polyunsaturated fatty acids in cultured neonatal rat cardiac myocytes. Proc Natl Acad Sci USA 94: 2724–2728, 1997
55. Paradies G, Ruggiero FM, Petrosillo G, Quagliariello E: Enhanced cytochrome oxidase activity and modification of lipids in heart mitochondria from hyperthyroid rats. Biochim Biophys Acta 1225: 165–170, 1994
56. Mutter T, Dolinsky VW, Ma BJ, Taylor A, Hatch GM: Thyroxine regulation of monolysocardiolipin acyltransferase activity in rat heart. Biochem J 346:403–405, 2000
57. Yamaoka-Koseki S, Urade R, Kito M: Cardiolipins from rats fed different diets affect bovine heart cytochrome C oxidase activity. J Nutr 121: 956–958, 1991
58. Stevens RJ, Nishio ML, Hood DA: Effect of hypothyroidism on the expression of cytochrome c and cytochrome c oxidase in heart and muscle during development. Mol Cell Biochem 143:119–127, 1995

SUPPLEMENT 3

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SHORT COMMUNICATION

Phospholipid Composition of Myocardium in Children with Normoxemic and Hypoxemic Congenital Heart Diseases

B. HAMPLOVÁ[1], V. PELOUCH[2], O. NOVÁKOVÁ[1], J. ŠKOVRÁNEK[3], B. HUČÍN[3], F. NOVÁK[4]

[1]Department of Animal Physiology and Developmental Biology, [4]Department of Biochemistry, Faculty of Science, [2]Second Faculty of Medicine, Charles University, [3]Cardiocenter of Faculty Hospital Motol, Prague, Czech Republic

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Summary

Samples of myocardial tissue were obtained during cardiac surgery from children operated for different types of normoxemic and hypoxemic congenital heart diseases. The phospholipid composition was analyzed by thin layer chromatography. The concentration of total phospholipids (PL), phosphatidylcholine and phosphatidylethanolamine (PE) was found lower in atrial tissue of both normoxemic and hypoxemic groups in comparison with the ventricles. When comparing the difference between hypoxemic and normoxemic defects, hypoxemia was found to increase the concentration of total PL, PE and phosphatidylserine in ventricles and total PL and PE in the atria. The increased level of particular phospholipid species may represent adaptive mechanisms to hypoxemia in children with congenital heart diseases.

Key words

Phospholipids • Human myocardium • Congenital heart disease • Ventricle • Atrium

Introduction

Congenital heart diseases are caused by abnormalities developed in the first six to eight weeks of fetal life. The incidence of heart malformations is about eight per thousand live births. These congenital cardiac defects may be grouped according to both the status of blood flow to the lungs and the presence and type of cardiac shunts. Right to left sided shunt mixes unoxygenated and oxygenated blood; oxygen blood saturation is thus significantly reduced and leads to hypoxemic (cyanotic) disease. On the other hand, left to right shunt is characteristic for normoxemic (acyanotic) disease. Recent medical progress in pediatric cardiac surgery allows successful repairing of almost all congenital heart defects. Nevertheless, only limited data are available on biochemical remodeling of both atrial and ventricular parts of diseased myocardium. We demonstrated that protein profiles of atrial musculature are totally different as compared with ventricular ones; the concentration of contractile proteins was higher in ventricle, while the concentration of extracellular matrix

E-mail: physres@biomed.cas.cz Fax +420 241 062 164
http://www.biomed.cas.cz/physiolres

proteins was higher in atrial musculature. Hypoxemia did not affect this protein profile in either cardiac part (Pelouch *et al.* 1993, 1995a, 1995b, 1997). There was no atrio-ventricular difference in the concentration of metabolic proteins but higher activity of carbohydrate and lipid aerobic metabolism enzymes was observed in ventricles of both normoxemic and hypoxemic patients (Bass *et al.* 1988, Pelouch *et al.* 1993). All metabolic differences mentioned above depended neither on the type of congenital heart disease nor on the reference values used (wet weight, metabolic or non-collagenous proteins) (Bass *et al.* 1988). The remodeling of human cardiac phospholipids associated with aging and coronary heart disease was observed (Gudbjarnason 1989, Skuladottir *et al.* 1988). However, there are no available data dealing with phosholipid composition and their remodeling in human myocardium with congenital heart diseases. Therefore, the aim of this work was to determine phospholipid composition of atrial and ventricular musculature and to compare the effect of chronic normoxemic and hypoxemic defects on membrane phospholipid remodeling in children myocardium.

Table 1. Characterization of patients with congenital heart diseases

	normoxemic		hypoxemic	
	ventricle	atrium	ventricle	atrium
Age	10.0 ± 2.2	8.5 ± 2.5	4.8 ± 2.0	4.3 ± 1.2
pO_2 (%)	94.7 ± 1.2	95.0 ± 0.9	76.7 ± 2.0 *	78.1 ± 1.6 *

Values are mean ± S.E.M. *p<0.05, significant difference vs. normoxemic tissue.

Table 2. Phospholipid concentration (μmol P. g^{-1} w.w.) in myocardium from children with congenital heart diseases

Phospholipids	normoxemic		hypoxemic	
	ventricle	atrium	ventricle	atrium
PC	5.74 ± 0.39	3.07 ± 0.50 #	7.27 ± 0.91	4.44 ± 0.52 #
LPC	0.87 ± 0.20	n.d.	0.50 ± 0.16	0.58 ± 0.06
PE	3.96 ± 0.49	1.84 ± 0.40 #	5.80 ± 0.56 *	3.65 ± 0.48 # *
LPE	0.39 ± 0.10	0.27 ± 0.05	0.55 ± 0.10	0.47 ± 0.08
DPG	1.37 ± 0.14	0.78 ± 0.14	1.77 ± 0.30	1.28 ± 0.31
SM	1.04 ± 0.12	0.79 ± 0.13	0.94 ± 0.07	0.86 ± 0.09
PI	0.96 ± 0.10	0.52 ± 0.08	0.95 ± 0.20	0.73 ± 0.10
PS	0.38 ± 0.02	0.36 ± 0.03	0.53 ± 0.04 *	0.41 ± 0.04 #
Total PL	14.71 ± 0.81	8.01 ± 0.97 #	18.46 ± 1.42 *	12.42 ± 1.37 # *

Values are means ± S.E.M. PL (phospholipids), PC (choline phosphoglycerides), LPC (lysophosphatidylcholine), PE (ethanolamine phosphoglycerides), LPE (lysophosphatidylethanolamine), DPG (diphosphatidylglycerol), SM (sphingomyelin), PI (phosphatidylinositol), PS (phosphatidylserine). # p<0.05, significant difference atrium vs. ventricle; * p<0.05, significant difference hypoxemic vs. normoxemic tissue.

The study was performed on 23 cardiac tissue samples obtained during surgery of children (1 - 18 years) with congenital heart disease. The samples were taken from right atria (n=6) and right ventricles (n=6) of patients with normoxemic defects (ventricular or atrial septal defect), and also from right atria (n=5) and right ventricles (n=6) of patients with hypoxemic defects (tetralogy of Fallot). For further characterization of patients see Table 1. The samples of cardiac tissue were rapidly weighed, frozen to –80 °C and stored at this temperature until the phospholipid analysis. The frozen tissue of both cardiac parts was pulverized and homogenized. Lipids were extracted according to the method of Folch *et al.* (1959) and evaporated under nitrogen. Phosphatidylcholine (PC), phosphatidyl-ethanolamine (PE), diphosphatidylglycerol (DPG),

sphingomyelin (SM), phosphatidylinositol (PI) and phosphatidylserine (PS) were separated by two-dimensional thin layer chromatography and the spots of individual phospholipids were analyzed for phosphorus (Rouser *et al.* 1970). Experimental values are given as means ± S.E.M. Differences were evaluated by one-way ANOVA and considered significant for $p<0.05$.

Table 1 confirms that the oxygen blood saturation was significantly lower in hypoxemic children than in normoxemic ones. As for the patients' age, we did not observe any significant difference either between atrial and ventricular defects or between normoxemic and hypoxemic ones. Table 2 shows that the concentration of total phospholipids (PL), PC and PE was significantly lower in atrial samples of both normoxemic (by 42 %, 47 % and 54 %, respectively) and hypoxemic groups (by 33 %, 39 % and 37 %, respectively) in comparison with the ventricles. Besides, we observed decreased concentration of PS (by 23 %) in hypoxemic atria in comparison with hypoxemic ventricles. Hypoxemia has a tendency to elevate the concentration of both total PL and individual phospholipid species. However, this increase was significant only in total PL (by 25 %), PE (by 46 %) and PS (by 39 %) in ventricular tissue and in total PL (by 55 %) and PE (by 98 %) in atrial samples. The relative proportion of individual phospholipids was unaltered with exception of the higher proportion of PS in normoxemic atria in comparison with normoxemic ventricles (by 83 %) and lower proportion of PS in hypoxemic atria in comparison with normoxemic ones (Table 3).

Table 3. Phospholipid distribution (%) in myocardium from children with congenital heart diseases

Phospholipids	normoxemic		hypoxemic	
	ventricle	atrium	ventricle	atrium
PC	39.00 ± 1.20	37.50 ± 2.50	38.80 ± 1.90	35.70 ± 0.90
LPC	6.18 ± 1.44	n.d.	3.93 ± 1.31	4.97 ± 0.82
PE	26.50 ± 2.20	26.10 ± 3.10	31.50 ± 1.40	29.10 ± 1.30
LPE	2.75 ± 0.76	3.87 ± 1.09	3.16 ± 0.58	3.89 ± 0.60
DPG	9.16 ± 0.56	9.84 ± 0.98	9.34 ± 0.89	9.82 ± 1.55
SM	7.22 ± 1.00	11.31 ± 2.63	5.23 ± 0.49	7.25 ± 1.16
PI	6.50 ± 0.44	6.45 ± 0.35	5.11 ± 0.50	5.90 ± 0.42
PS	2.69 ± 0.35	4.92 ± 0.75 #	2.93 ± 0.31	3.41 ± 0.33 *

For symbols see Table 2.

Our results demonstrate that the concentration of total PL is significantly higher in ventricles as compared with atria. This is due to the higher concentration of major phospholipids PC and PE, which reflects the higher content of intracellular membranes in ventricles. As the ratio of major phospholipids to mitochondrial DPG is similar in ventricles and atria, the higher total phospholipid concentration observed in ventricles is supposed to reflect the higher content of mitochondrial membranes. Moreover, higher activities of aerobic enzymes were found in ventricles in comparison with atria (Bass *et al.* 1988, Pelouch *et al.* 1988). The concentration of PL that we found in children heart was higher in comparison with values reported in adult human heart (Skuladottir *et al.* 1988). Although no data are available on developmental changes in phospholipid concentration in human myocardium, it is known that during ontogenesis of rat myocardium phospholipid concentration gradually rises from the birth to the adulthood (Nováková *et al.* 1995). We did not observe any ontogenetic difference in our set of patients. The relative distribution of individual phospholipids determined in our study is similar to those found in other studies (Sebedio *et al.* 1982, Rocquelin *et al.* 1989). However, on contrary to these, we found the significant amount of lysophosphatidylcholine and lysophosphatidyl-ethanolamine in nearly all heart tissues under study. Because cardiac tissue of healthy children was not analyzed, we cannot claim that the presence of lysophospholipids was a consequence of heart disease; lysophospholipids might also originate from the process of tissue sampling during the surgery. We observed

higher concentration of aminophospholipids (PE, PS) in both atrial and ventricular tissues from children with hypoxemic defects as compared with normoxemic ones. We do not have a precise explanation for this phenomenon, but it is known that the redistribution of these phospholipid species in cardiac plasma membrane precedes the apoptotic cell death (Maulik *et al.* 1998). It is tempting to speculate that the changes in phospholipid composition observed in hypoxemic heart tissues may reflect either an adaptive response of the heart to the lower oxygen blood saturation or a sign of tissue damage.

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References

BASS A, ŠAMÁNEK M, OŠŤÁDAL B, HUČÍN B, STEJSKALOVÁ M, PELOUCH V: Differences between atrial and ventricular energy supplying enzymes in children. *J Appl Cardiol* **3**: 397-405, 1988.

FOLCH J, LEES M, SLOAN-STANLEY GH: A simple method for the isolation and purification of total lipids from animal tissue. *J Biol Chem* **226**: 497-509, 1957.

GUDBJARNASON S: Dynamics of n-3 and n-6 fatty acids in phospholipids of heart muscle. *J Intern Med* **225**: 117-128, 1989.

MAULIK N, KAGAN VE, TYURIN VA, DAS DK: Redistribution of phosphatidylethanolamine and phosphatidylserine precedes reperfusion-induced apoptosis. *Am J Physiol* **274**: H242-H248, 1998.

NOVÁKOVÁ O, MRNKA L, PELOUCH V, KALOUS M, NOVÁK F: Altered phospholipid composition and oxidative capacity in pressure-overloaded rat myocardium. *J Mol Cell Cardiol* **27**: A85, 1995.

PELOUCH V, MILEROVÁ M, OŠŤÁDAL B, ŠAMÁNEK M, HUČÍN B: Protein profiling of human atrial and ventricular musculature: the effect of normoxaemia and hypoxaemia in congenital heart diseases. *Physiol Res* **42**: 235-242, 1993.

PELOUCH V, MILEROVÁ M, OŠŤÁDAL B, ŠAMÁNEK M, HUČÍN B: Collagenous and noncollagenous proteins of human atrial and ventricular musculature: the effect of normoxaemia and hypoxaemia in congenital heart disease. *J Muscle Res Cell Motil* **16**: 171-172, 1995a.

PELOUCH V, MILEROVÁ M, OŠŤÁDAL B, HUČÍN B, ŠAMÁNEK M: Differences between atrial and ventricular protein profiling in children with congenital heart disease. *Mol Cell Biochem* **147**: 43-49, 1995b.

PELOUCH V, PEXIEDER T, MILEROVÁ M, OŠŤÁDAL B, ŠAMÁNEK M: Protein remodeling of malformed human and mouse heart. In: *The Developing Heart.* B OŠŤÁDAL, M NAGANO, N TAKEDA, NS DHALLA (eds), Raven Press, New York, 1997, pp 357-372.

ROCQUELIN G, GUENOT L, ASTORG PO, DAVID M: Phospholipid content and fatty acid composition of human heart. *Lipids* **24**: 775-780, 1989.

ROUSER G, FLEISCHER S, YAMAMOTO A: Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* **5**: 494-496, 1970.

SEBEDIO JL, FARQUHARSON TE, ACKMAN RG: Improved methods for the isolation and study of the C-18, C-20 and C-22 monoethylenic fatty acid isomers of biological samples-HG adducts, HPLC, $AgNO_3$-TLC/FID and ozonolysis. *Lipids* **17**: 469-475, 1982.

SKULADOTTIR G, BENEDIKTSDOTTIR E, HARDARSON T, HALLGRIMSSON J, ODDSSON G, SIGFUSSON N, GUDBJARNASON S: Arachidonic acid level of non-esterified fatty acid and phospholipids in serum and heart muscle of patients with fatal myocardial infarction. *Acta Med Scand* **223**: 233-238, 1988.

Reprint requests

Dr. F. Novák, Department of Biochemistry, Faculty of Science, Charles University, Hlavova 2030, 128 43 Prague 2, Czech Republic, Fax: +420 2 2195 2331, e-mail: franov@natur.cuni.cz

SUPPLEMENT 4

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ORIGINAL ARTICLE

Protein Kinase C Activity and Isoform Expression During Early Postnatal Development of Rat Myocardium

B. Hamplova,[1,2] O. Novakova,[2] E. Tvrzicka,[3] F. Kolar,[4] and F. Novak,[1]*

[1]Department of Biochemistry Faculty of Science, Charles University in Prague, Hlavova 2030, 128 43 Prague 2, Czech Republic; [2]Department of Animal Physiology, Faculty of Science, Charles University in Prague, Vinicna 7, 128 44 Prague 2, Czech Republic; [3]1st Faculty of Medicine, Charles University in Prague, U nemocnice 2, 128 08 Prague 2, Czech Republic; and [4]Institute of Physiology, Academy of Sciences of the Czech Republic and Centre for Cardiovascular Research, Videnska 1083, Prague,[4] Czech Republic

Abstract

Total protein kinase C (PKC) activity, its isoform expression, and concentration and fatty acid (FA) composition of diacylglycerol (DAG) were determined in the left ventricular myocardium of the rat during early postnatal development (d 2, 3, 5, 7, and 10). PKC activity measured by the incorporation of ^{32}P into histone IIIS decreased between d 2 and 10 in the homogenate as well as in cytosolic, membrane (100,000*g*), and nuclear-cytoskeletal-myofilament fractions (1000*g*). Likewise, the expression of PKC isoforms (α, δ, and ε) determined by immunoblotting generally declined during the period analyzed, although with a variable pattern. In the membrane and nuclear cytoskeletal myofilament fractions, PKCδ and PKCε expression decreased markedly by d 3, returning to or close to the d 2 level immediately on d 5. PKCα expression in the membrane fraction remained almost unchanged by d 7, declining thereafter. PKCδ and PKCε were associated predominantly with particulate fractions, whereas PKCα was more abundant in the cytosolic fraction. DAG concentration exhibited a significant decline by d 5, consistent with the decrease in maximal PKC activity. The unsaturation index of FA in DAG tended to decrease on d 3 owing to the lowered proportion of all polyunsaturated FA of n-6 and n-3 series. These results demonstrate that the developmental decrease in PKC activity and expression in the rat myocardium is not linear and that subcellular localization of the enzyme exhibits isoform-specific day-by-day changes during the early postnatal period. These changes are compatible with the view that PKC signaling may be involved in the control of a rapid switch of myocardial growth pattern during the first week of life.

Index Entries: Protein kinase C; diacylglycerol; fatty acids; postnatal development; rat heart.

* Author to whom all correspondence and reprint requests should be addressed. E-mail: franov@natur. cuni.cz

INTRODUCTION

Protein kinase C (PKC) is a family of Ser-Thr kinases that plays a key role in the regulation of numerous biological processes. In cardiac tissue, PKC appears to modulate ion transport, contractility, cell proliferation and differentiation, hypertrophy, and apoptosis *(1–5)*. The PKC family can be divided into at least three groups according to the structure and requirement of activators. Members of the classic group (α, $\beta_{1/2}$, and γ) are Ca^{2+} dependent and require diacylglycerol (DAG) and phosphatidylserine (PS) for their activation; novel PKC isoforms (δ, ε, η, and θ) are Ca^{2+} independent and are activated by DAG and PS; atypical isoforms (ζ and ι/λ) are Ca^{2+} and DAG independent but require PS as a cofactor. DAG, which increases the specificity of the enzyme for PS and also increases its affinity for Ca^{2+} *(6)*, originates in the plasma membrane during agonist-induced hydrolysis of inositol phospholipids by phospholipase C and also from phosphatidylcholine (PC) hydrolyzed by phospholipase D (PLD) at a relatively later phase of cellular responses *(5)*. The DAG produced by the PLD pathway appears to activate selectively novel PKC isoforms *(7)*. The function of individual isoforms of PKC can be modulated by factors such as multiple lipid mediators (DAG, PS, PC, lysoPC, phosphatidic acid, sphingomyelin, phosphatidylglycerol, unsaturated fatty acids [FAs]; *[8–13]*) and by phosphorylation *(14)*. The specificity of each isoform action on activation is ensured by its translocation to a proximity of the target substrate that is achieved by the isoform anchoring at its specific binding protein called receptor for activated C kinase *(15–17)*.

In the rat heart, different PKC isoforms were immunochemically detected, depending on the age of animals and on the use of either the whole cardiac extracts or isolated cells. In neonatal rat myocardium, PKCα, PKCβ, PKCδ, PKCε and PKCζ were detected. In neonatal cultured myocytes, certain discrepancies exist regarding the presence of PKCβ; however, PKCα, PKCδ, PKCε, and PKCζ were found in all studies. The situation in adult myocardium is more controversial *(18)*.

The majority of developmental studies dealing with changes in myocardial PKC activity and isoform expression were not sufficiently detailed with respect to dramatic day-by-day structural and functional changes that occur in the heart early after birth *(19)*. For example, one of the prominent features of the neonatal heart is a transition from hyperplastic to hypertrophic growth of myocytes. In the rat, this transition appears to occur sharply after postnatal d 3 *(20)*. Because PKC is an important regulator of cell proliferation and hypertrophy, the aim of our study was to determine detailed developmental changes in total PKC activity and the expression of the prevalent isoforms α, δ, and ε in the left ventricular myocardium of the rat on d 2, 3, 5, 7, and 10. Furthermore, the abundance of the classic and novel PKC isoform activator DAG and its FA composition were analyzed during this period.

MATERIALS AND METHODS

Animal Model

Newborn male Wistar rats were obtained from the animal care facility of the Institute of Physiology in Prague, Czech Republic. Animals were sacrificed on d 2, 3, 5, 7, and 10, and their hearts were dissected free of atrial tissue, large blood vessels, and the right ventricle. The left ventricle including the septum (LV + S) was rinsed in cold saline (5°C), weighed, frozen in liquid nitrogen, and stored at –80°C until use. This study conforms to 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).

Chemicals

Chemical reagents including PKC isoform-specific primary antisera and competing immunizing peptides were obtained from Sigma-Aldrich. The secondary swine anti-rabbit IgG antibody labeled with horseradish

peroxidase (HRP) was from Sevapharma. Radioactive γ[^{32}P]ATP was from ICN.

Tissue Fractionation

One specimen for fractionation contained ventricles from four (d 10) to eight (d 2) animals. The tissue was minced using an Ultra-Turrax (twice, 30 s each) and then homogenized (Potter-Elvehjem homogenizer) in 10 vol of ice-cold homogenization buffer composed of 12.5 mmol/L of Tris-HCl (pH 7.4), 250 mmol/L of sucrose, 2.5 mmol/L of EGTA, 1 mmol/L of EDTA, 100 mmol/L of NaF, 5 mmol/L of dithiothreitol (DTT), 0.3 mmol/L of phenylmethylsulfonyl fluoride, 0.2 mmol/L of leupeptin, and 0.02 mmol/L of aprotinin. The homogenate was fractionated according to a method described previously *(21)* with slight modifications. Briefly, the homogenate was centrifuged at 100*g* for 20 min to remove cellular debris and unbroken cells. The supernatant was centrifuged at 1000*g* for 10 min to produce a nuclear-cytoskeletal-myofilament-enriched fraction (denoted as nuclear fraction hereafter for simplicity) followed by centrifugation at 100,000*g* for 60 min. The 100,000*g* pellet contained the membrane-enriched fraction and the supernatant was the cytosolic fraction. The homogenate and the pellets of nuclear and membrane fractions were suspended in homogenization buffer containing 1% Triton X-100, held on ice for 60 min, and centrifuged at 100,000*g* for a further 60 min. The resulting detergent-treated supernatants were used for activity and immunoblotting analyses. Triton X-100 was also added to the cytosolic fraction to give a final concentration of 1%. Protein content was determined according to Lowry as modified by Peterson *(22)*.

Activity Assay of Total PKC

PKC activity was determined as a difference in incorporation of ^{32}P from γ[^{32}P]ATP into histone IIIS in the presence and absence of PS, 1-octanoyl-2-acetyl-glycerol (OAG), and Ca^{2+}. The required amount of PS and OAG dissolved in chloroform was dried in a stream of nitrogen and solubilized in 0.3% Triton X-100 by vortexing and incubating at 30°C for 5 min. The reaction mixture (0.1 mL) contained 20 mmol/L of Tris-HCl (pH 7.45), 10 mmol/L of $MgCl_2$, 1 mmol/L of DTT, 1 mmol/L of $CaCl_2$, 0.1 mmol/L of vanadate, 21 μg of PS, 5.1 μg of OAG, and protein (2.5 μg of homogenate, 3 μg of cytosolic, 2 μg of nuclear, or 1 μg of membrane fractions). The reaction was started by adding 10 μL of 1 m*M* γ[^{32}P]ATP (50–100 cpm/pmol) and terminated after 5 min at 30°C by adding 1 mL of ice-cold 25% trichloroacetic acid (TCA). The resulting precipitates were filtered through presoaked nitrocellulose filters (Pragopor, Pragochema) and washed three times with 5% TCA. Radioactivity was quantified by Cerenkov counting.

Immunoblot Analyses of PKC Isoforms

Samples were electrophoresed on 8% bisacrylamide polyacrylamide gel. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out at 20 m*A*/gel for 90 min on a Mini-Protean II apparatus (Bio-Rad, Hercules, CA). After electrophoresis, the resolved proteins were transferred to nitrocellulose membranes (Amersham). The membranes were incubated in 5% dry low-fat milk in Tris-buffered saline with Tween-20 (TTBS) for 60 min at room temperature in order to block nonspecific binding. After washing in TTBS buffer (three times quickly and three times for 5 min each), the membranes were probed with PKC isoform-specific primary rabbit antisera (1:8000 in TTBS) for 90 min at room temperature. The membranes were washed again and incubated with secondary swine antirabbit IgG antibody labeled with HRP (1:4000 in TTBS) for 60 min at room temperature. Before enhanced chemiluminescence (ECL), the nitrocellulose membranes were washed as just described and stored in TTBS for at least 2 h. For ECL, we prepared ECL substrates A (Luminol solution) and B (H_2O_2 solution); they were mixed 1:1 and poured onto the immunoblot. The specific signal was documented on autoradiography film (Amersham). Scanning (Epson Perfection

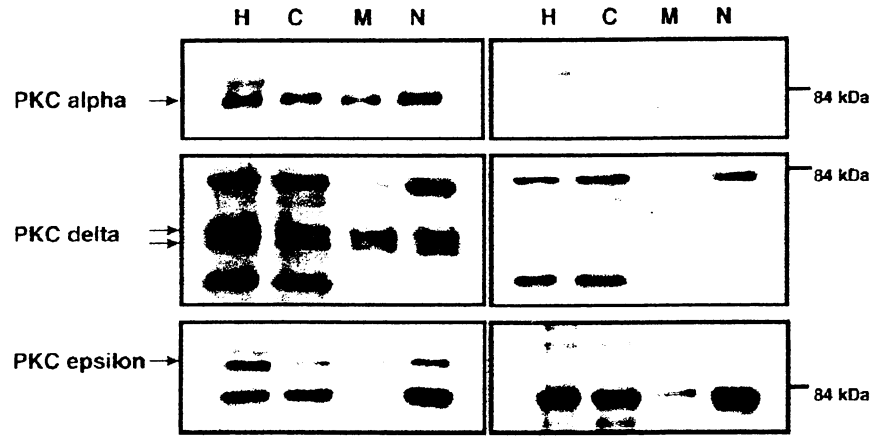

Fig. 1. PKC expression in extracts of homogenate (H) and of cytosolic (C), membrane (M), and nuclear-cytoskeletal-myofilament (N) fractions of left ventricular myocardium of 2-d-old rats. Total amounts of protein loaded were as follows: H (4 μg), C (3 μg), M (3 μg), N (5 μg) (PKCα); H (9 μg), C (12 μg), M (3 μg), N (4 μg) (PKCδ); H (10 μg), C (12 μg), M (5 μg), N (7 μg) (PKCε). Immunoblots on the left show results obtained in the absence of the appropriate competing peptide antigen; blots on the right show results in the presence of the antigen (50 μg/10 mL). The polyclonal antisera were used at a dilution of 1:8000; bound antibody was detected by the ECL method (for details *see* Materials and Methods). The numbers on the right indicate the positions of the prestained molecular mass standards in kilodaltons.

1240U scanner) and ImageQuant software were used for quantification of the relative abundance of individual PKC isoforms. To ensure the specificity of the immunoreactive proteins, Western blots were done in the presence and absence of competing immunizing peptides (*see* Fig. 1). The PKCδ isoform was identified as a doublet of proteins. Both bands were recognized by the antibody and blocked by the appropriate immunizing peptide. Both proteins were taken for the quantification of PKCδ isoform expression. Similarly, Ogita et al. *(23)* immunodetected PKCδ as a doublet of proteins (78 and 76 kDa) in a preparation from rat brain; the 78-kDa protein was identified as a phosphorylated form of the 76-kDa protein by means of protein phosphatase 2A treatment.

Extraction of Lipids

The samples were pulverized and homogenized. Lipids (from approx 150 mg of wet tissue) were extracted in three consecutive steps according to the modified method of Folch et al. *(24)*. The first extraction was performed in three portions (0.5 mL each) of a chloroform-methanol mixture (1:3, 2:1, and 2:1 [v/v]) in a chilled mortar. Subsequent extractions were performed in a 2:1 mixture (0.75 mL each). Saline (20% volume of extract) was added, and after vigorous shaking the lower lipid layer was separated and dried at 40°C in a stream of nitrogen.

Determination of DAG Concentration

The dried extract was solubilized in a chloroform-methanol mixture (2:1). Aliquots (from 8 mg of wet tissue) were applied to high performance thin-layer chromatography (HPTLC) plates (aluminum sheets, 20 × 20 cm; Merck), which were dried at 110°C for 75 min. After cooling, the plates were impregnated with 0.25 *M* boric acid in ethanol-H_2O (1:1) to inhibit acyl migration from the second to the third position in DAG—and subsequently dried at 80°C for 20 min. They were developed using a solvent system of hexane–ether–acetic acid (50:50:1

Table 1
Weight Parameters of Rats During Early Postnatal Development[a]

	Age (d)				
Weight parameter	2 (n = 24)	3 (n = 21)	5 (n = 14)	7 (n = 12)	10 (n = 14)
BW (g)	7.31 ± 0.19	8.73 ± 0.21	13.11 ± 0.30	15.57 ± 0.33	20.83 ± 0.50
LV + S (mg)	25.73 ± 0.80	28.59 ± 1.18	42.02 ± 1.29	49.22 ± 1.49	57.52 ± 1.51
RV (mg)	9.41 ± 0.51	10.28 ± 0.63	15.84 ± 0.83	16.41 ± 0.36	20.14 ± 1.03
LV + S/BW (mg/g)	3.52 ± 0.07	3.27 ± 0.09	3.22 ± 0.08	3.18 ± 0.12	2.77 ± 0.06
RV/BW (mg/g)	1.28 ± 0.05	1.18 ± 0.06	1.20 ± 0.05	1.05 ± 0.03	0.97 ± 0.04

[a] BW, body weight; LV, left ventricle; S, septum; RV, right ventricle; n, number of animals. Values are the means ± SEM

[v/v/v]), air-dried for 90 min, stained with Coomassie Brilliant Blue G (0.03% in 20% methanol in 100 mmol/L of NaCl) for 30 min, and destained for 5 min (twice in 20% methanol in 100 mmol/L of NaCl). Stained spots were quantified using a scanner (Epson Perfection 1240U) and the software ImageQuant. The concentration of DAG was interpolated from the standard curve established from known quantities of 1,2-dioleoylglycerol, which were spotted on the same plates as the samples.

Analysis of FA Composition of DAG

Lipids (from approx 130 mg of wet tissue) were separated using silica gel plates (20 × 20 cm, 0.5 mm; Kiesegel 60 F254) and a solvent system of hexane–ether–acetic acid (70:30:1 [v/v/v]). Areas corresponding to DAG were identified after staining with 0.005% 2,7-dichlorofluorescein in methanol, scraped out, and stored under nitrogen atmosphere at –20°C until the next day, when methyl esters were prepared. For preparation of fatty acid methyl esters (FAMEs), sodium methanolate was added to tubes with silica gel; the tubes were then incubated for 60 min at room temperature in the dark. Methyl esters were extracted with hexane, and the extracts were evaporated in a stream of nitrogen and stored at 0°C. FAMEs were separated by a gas chromatograph Chrompack CP 438 A using a middle polar column CP WAX 52 CB (25 m × 0.25 mm id). Oven temperature was programmed from 145 to 230°C at 2°C/min. Hydrogen was used as the carrier gas. FAs were identified using a mixture of FAMEs.

Statistical Analyses

All results are expressed as means ± SEM. The statistical significance of differences between groups was determined by one-way analysis of variance and subsequent Student-Newman-Keuls test ($p < 0.05$). Linear regression analysis was used to test the relationship between total PKC activity and individual PKC isoform expression in the homogenate.

RESULTS

Weight Parameters

Body and heart growth of rats between d 2 and 10 is documented in Table 1. In line with previous studies, relative weights of both ventricles decreased during the investigated postnatal period.

Total PKC Activity

The absolute values of specific PKC activity, determined as the amount of radioactive ^{32}P incorporated into histone IIIS, were in the range of nanomoles per minute per milligram of tissue. Figure 2 shows the specific activity of Ca^{2+}-, DAG-, and PS-dependent components of PKC in extracts of homogenate and cytosolic, membrane, and nuclear fractions

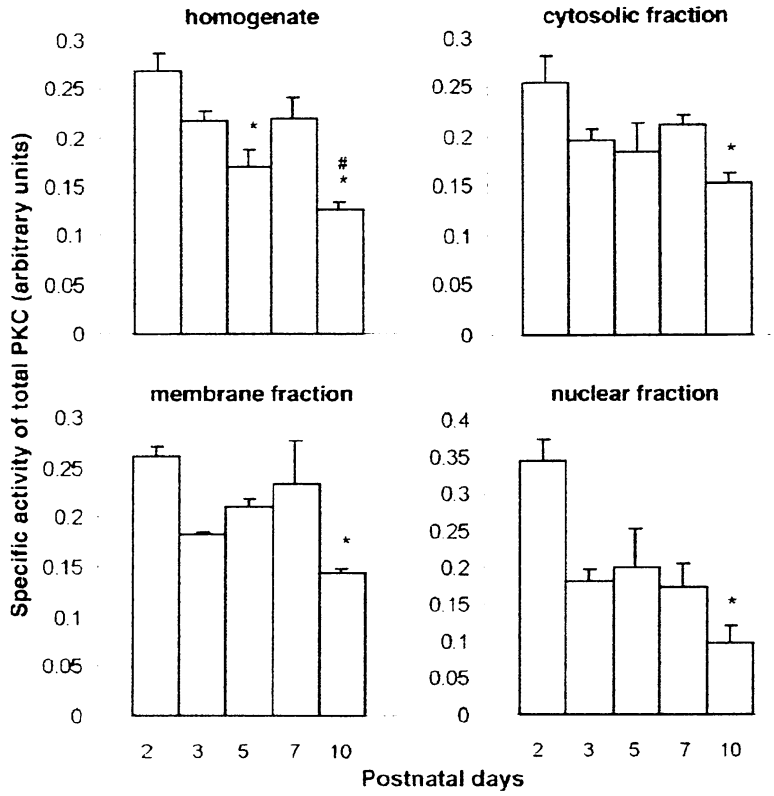

Fig. 2. Developmental changes in total PKC activity in extracts of homogenate and of cytosolic, membrane, and nuclear-cytoskeletal-myofilament fractions of rat myocardium during first 10 postnatal days. PKC activity was determined as the amount of radioactive ^{32}P incorporated into histone IIIS (for details *see* Materials and Methods). Values are expressed as arbitrary units (the sum of specific radioactivity measured on postnatal d 2, 3, 5, 7, and 10 is equal to 1). Data are the means ± SEM from three fractionations in each age group. *$p < 0.05$, significant difference vs d 2; #$p < 0.05$, significant difference vs previous age group.

from rat myocardium between d 2 and 10, expressed as arbitrary units (the sum of specific radioactivity measured on d 2, 3, 5, 7, and 10 is equal to 1). The specific activity significantly declined from d 2 to 10 by 55, 42, 48, and 70%, in the homogenate and in cytosolic, membrane, and nuclear fractions, respectively, although mild transient increases occurred between d 3 and 5–7.

Expression of PKC Isoforms

The expression pattern of PKC isoforms in the homogenate between d 2 and 10 (Fig. 3) essentially reflected the changes in total PKC activity. Regression analysis revealed significant ($p < 0.01$) linear relationships between individual values of total PKC activity and immunoreactivities of all three isoforms in the homogenate during the investigated postnatal period (correlation coefficients reached 0.644, 0.813, and 0.758 for PKCα, PKCδ, and PKCε, respectively).

The time course of changes in the homogenate and in nuclear and cytosolic fractions was essentially similar for all isoforms analyzed, despite obvious quantitative differences (Fig. 3). In the homogenate, the expres-

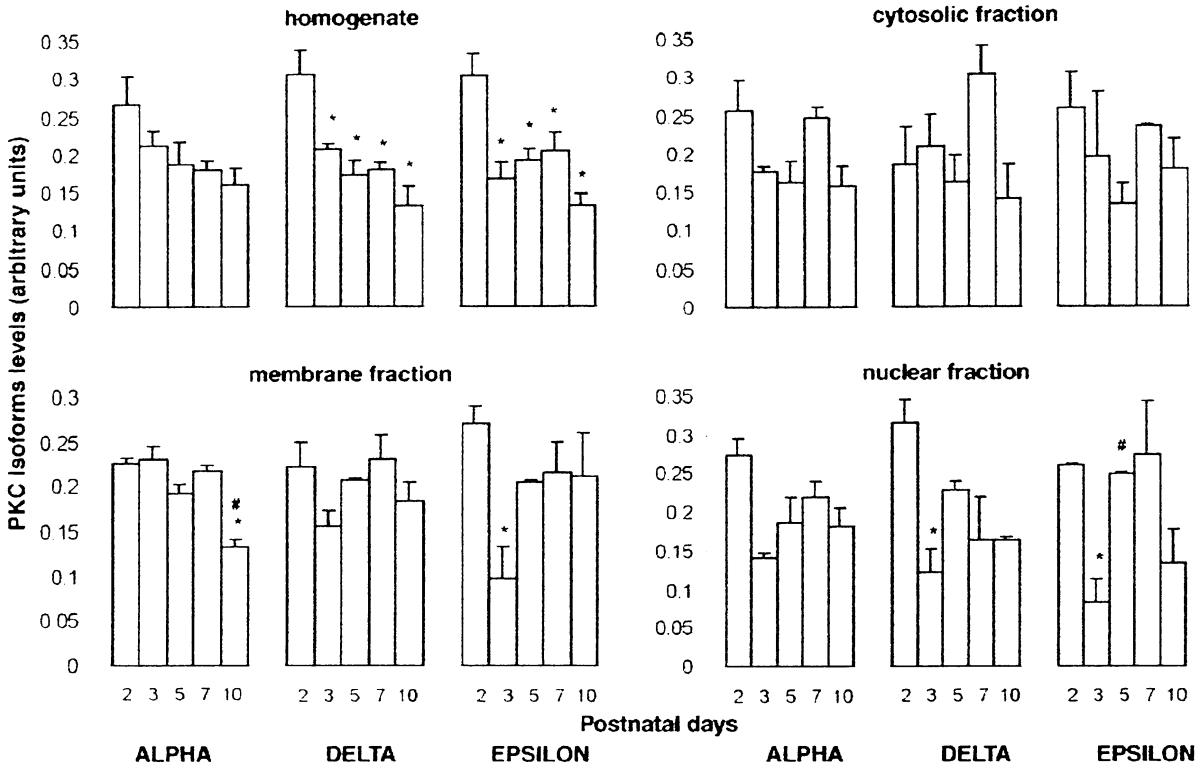

Fig. 3. Developmental changes in expression of PKC isoforms in extracts of homogenate, and of cytosolic, membrane, and nuclear-cytoskeletal-myofilament fractions of rat myocardium during first 10 postnatal days. After electrophoresis and immunoblotting, signals of PKCα, PKCδ, and PKCε were quantified as described in Materials and Methods. Values are expressed as arbitrary units (the sum of densitometry volumes measured on postnatal d 2, 3, 5, 7, and 10 is equal to 1). Data are the means ± SEM from three fractionations in each age group. *$p < 0.05$, significant difference vs d 2; #$p < 0.05$, significant difference vs previous age group.

sion of PKCδ and PKCε dropped sharply between d 2 and 3; it did not significantly change by d 7 and then decreased again on d 10. In the cytosol, we observed a tendency of all PKC isoforms to increase transiently on d 7. In the membrane fraction, the abundance of PKCδ and PKCε decreased transiently on d 3, whereas PKCα remained stable by d 7 and declined significantly only on d 10. The abundance of PKC isoforms in the nuclear fraction decreased transiently more than twofold on d 3; it increased again on d 5, reaching the d-2 level in the case of PKCε.

Subcellular Distribution of PKC Isoforms

To examine whether the distribution of PKC isoforms changed among nuclear, membrane, and cytosolic fractions during the first 10 postnatal days, the amounts of PKCα, PKCδ, and PKCε were expressed as membrane-to-cytosol and nuclear-to-cytosol ratios (Fig. 4). The membrane-to-cytosol PKCα ratio was about one and did not change appreciably during the period analyzed. The nuclear-to-cytosol ratio was about 0.5 on d 2 tending to increase transiently on d 3 and d 5. Changes in PKCδ and

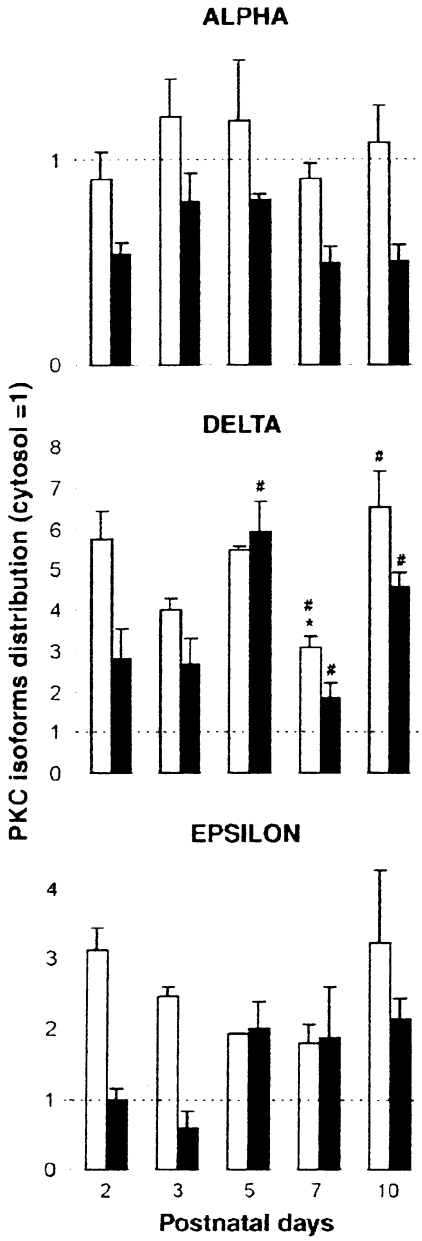

Fig. 4. Changes in subcellular distribution of PKC in rat myocardium during first 10 postnatal days. Values are expressed as membrane-to-cytosol ratio (□) and nuclear-to-cytosol ratio (■) of PKC isoform abundance. Data are the means ± SEM from three fractionations in each age group. *$p < 0.05$, significant difference vs d 2; #$p < 0.05$, significant difference vs previous age group.

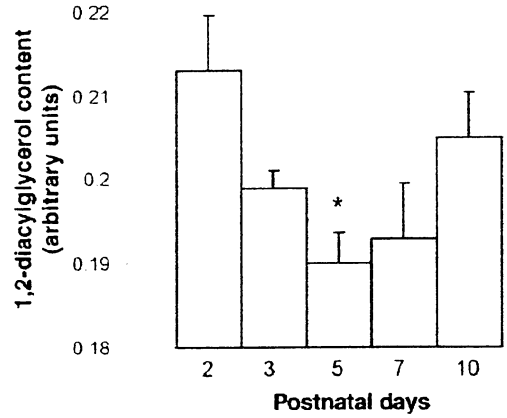

Fig. 5. Developmental changes in DAG concentration in homogenate of rat myocardium during first 10 postnatal days. The amount of DAG was quantified by densitometry from HPTLC plates stained with Coomassie Brilliant Blue (for details, *see* Materials and Methods). Values are expressed as arbitrary units (the sum of concentrations measured on postnatal d 2, 3, 5, 7, and 10 is equal to 1). Data are the means ± SEM from four pooled specimens. *$p < 0.05$, significant difference vs d 2.

PKCε distribution between particulate and cytosolic fractions were more variable compared with those of PKCα. The abundance of PKCδ was about sixfold higher in the membrane fraction in comparison with the cytosol on d 2, 5, and 10, but the ratio dropped to 4 and 3 on d 3 and 7, respectively. The nuclear-to-cytosol ratio of PKCδ was about 3 on d 2 and 3; then it doubled on d 5, dropped markedly to 2 on d 7, and increased again on d 10. PKCε abundance was threefold higher in the membrane fraction compared with the cytosol on d 2, and then it tended to decrease gradually until d 7 and again to increase to the initial level by d 10. The nuclear-to-cytosol ratio of this isoform was equal to 1 on d 2 and even lower on d 3. Then it increased to about 2 on d 5 and kept constant until d 10; these changes did not reach statistical significance.

Table 2
FA Composition of DAGs in Rat Heart During First 10 Postnatal Days[a]

	Age (d)				
FA	2	3	5	7	10
14:0	0.90 ± 0.44	0.88 ± 0.21	0.75 ± 0.14	1.09 ± 0.24	1.23 ± 0.28
16:0	25.43 ± 2.11	26.22 ± 0.89	25.53 ± 1.24	27.72 ± 2.30	28.72 ± 2.34
16:1n-9	2.81 ± 1.45	2.09 ± 0.13	1.79 ± 0.21	1.95 ± 0.33	1.69 ± 0.16
16:1n-7	0.47 ± 0.02	1.12 ± 0.58	0.93 ± 0.31	1.40 ± 0.66	0.78 ± 0.13
18:0	27.72 ± 2.47	30.63 ± 1.45	33.61 ± 1.22	27.42 ± 3.56	27.44 ± 0.40
18:1n-9	12.91 ± 1.64	14.67 ± 5.22	13.51 ± 4.67	16.24 ± 6.81	14.56 ± 5.11
18:1n-7	3.33 ± 0.51	3.33 ± 0.28	3.67 ± 0.30	3.86 ± 0.48	3.80 ± 0.32
18:2n-6	4.25 ± 0.53	3.75 ± 0.30	4.21 ± 1.12	4.55 ± 0.49	5.67 ± 0.76
20:1n-9	0.68 ± 0.02	0.66 ± 0.06	0.84 ± 0.09	0.80 ± 0.18	0.72 ± 0.09
20:4n-6	11.03 ± 2.33	10.42 ± 3.14	8.57 ± 3.50	7.74 ± 2.19	8.33 ± 2.49
22:4n-6	1.28 ± 0.31	0.63 ± 0.15	0.58 ± 0.08	0.57 ± 0.28	0.54 ± 0.30
22:5n-3	1.77 ± 0.50	0.98 ± 0.21	1.04 ± 0.15	1.17 ± 0.47	1.02 ± 0.12
22:6n-3	5.02 ± 0.86	2.63 ± 0.51	2.85 ± 0.37	3.38 ± 1.14	3.12 ± 0.70
SFA	54.64 ± 0.31	58.44 ± 1.11	60.74 ± 1.53	57.0 ± 5.5	58.14 ± 2.89
UFA	45.42 ± 0.27	41.56 ± 1.11	39.32 ± 1.51	43.02 ± 5.51	41.89 ± 2.90
MUFA	20.11 ± 1.88	21.89 ± 4.71	20.67 ± 4.64	24.24 ± 6.50	21.62 ± 5.04
PUFA	25.19 ± 1.91	19.67 ± 3.78	18.56 ± 3.61	18.89 ± 3.92	20.33 ± 3.30
n-6	17.93 ± 1.82	15.56 ± 3.22	14.24 ± 3.33	13.70 ± 2.44	15.54 ± 2.32
n-3	7.36 ± 1.32	4.14 ± 0.66	4.42 ± 0.47	5.21 ± 1.57	4.84 ± 0.96
SFA/UFA	1.20 ± 0.01	1.40 ± 0.06	1.55 ± 0.10	1.40 ± 0.32	1.41 ± 0.17
n-6/n-3	2.60 ± 0.52	3.72 ± 0.44	3.22 ± 0.71	2.85 ± 0.48	3.24 ± 0.21
20:4/18:2	2.75 ± 0.72	2.90 ± 0.96	2.60 ± 1.59	1.80 ± 0.60	1.66 ± 0.73
UI	123.48 ± 6.04	98.78 ± 12.04	92.53 ± 11.56	97.64 ± 16.03	97.81 ± 12.45

[a] SFA, sum of saturated FA; UFA, sum of unsaturated FA; MUFA, sum of monounsaturated FA; PUFA, sum of PUFA; UI, unsaturation index is the sum of molar percent of individual unsaturated FA multiplied by the number of double bonds. Only FAs reaching at least 0.5 mol% are shown. Values are the means ± SEM from three pooled specimens.

Concentration of DAG and Its FA Composition

The development of myocardial DAG abundance during the first 10 d of postnatal life exhibited a statistically significant decrease between d 2 and 5, followed by a tendency to increase by d 10 (Fig. 5). Analysis of FA composition demonstrated that the most abundant FAs in DAG were saturated FA, palmitic acid (16:0), and stearic acid (18:0), accounting for about 55 mol% on d 2 and tending to increase thereafter. The sum of monounsaturated FA was maintained at about 20 mol% throughout the investigated period. The proportion of total polyunsaturated FA (PUFA) tended to decrease on d 3 (owing to decreased proportions of 20:4n-6, 22:4n-6, 22:5n-3, and 22:6n-3), and then it maintained a level close to 20 mol% until d 10. Consequently, the unsaturation index declined by 20–25% after d 2; however, none of the developmental changes in FA composition of DAG reached statistical significance (Table 2).

DISCUSSION

We determined the levels of total PKC activity in the left ventricular myocardium of the rat during the early postnatal period, which is characterized by rapid cardiac growth and differentiation. A major finding is that, in general, the PKC activity declined from d 2 to 10 in the homogenate and subcellular fractions, but this decline was not linear: a tendency to a transient increase was observed in all specimens between d 3 and 5–7. Significant correlations between the activity and immunoreactivities of all three PKC isoforms in the homogenate suggest that the developmental changes in total activity cannot be attributed to only one predominating isoform. Although McGill and Brooks *(25)* observed similar changes in total PKC activity between d 5 and 11, the transient increase was delayed by 2 d in their study. This difference might reflect the unequal degree of maturation of the animals. The absolute values of PKC activity were about 1 nmol/(min·mg) of protein in both studies despite the fact that McGill and Brooks *(25)* used specific peptide ε, the substrate for all DAG-dependent isoforms, whereas we employed histone IIIS, which is the suitable substrate only for classic PKC and PKCε *(26)*.

The expression of PKC isoforms in developing hearts has previously been studied in several animal species, particularly in the rat. We analyzed developmental changes in the expression of classic PKCα and novel isoforms PKCδ and PKCε in the homogenate and in cytosolic, membrane, and nuclear fractions of the rat myocardium during the first 10 postnatal days. The expression of all these isoforms declined from d 2 until 10, but the most prominent decrease was observed already between d 2 and 3. Then a continuous or a transient increase occurred between d 3 and 5–7, in particular of PKCδ and PKCε, in membrane and nuclear fractions. This suggests that these isoforms play a specific role during this narrow period of cardiac development. Other studies on rats reported a continual decline in the expression of myocardial PKC isoforms after birth *(18,27)*; however, the investigators did not follow the postnatal development in such detail as we did. In the mouse heart, the expression of PKCα, PKCδ, and PKCε decreased from the embryonic period until adulthood, but between postnatal d 2 and 9, which corresponds to the developmental period analyzed in the present study, the expression either did not change or it increased continually *(28)*. Thus, species differences in PKC expression appear to exist during early postnatal life, probably reflecting a different time course of maturation.

A variety of factors might potentially contribute to the changes in expression and activity of PKC isoforms in the rat myocardium during the first few postnatal days, but this issue remains unresolved. They include humoral regulations as the levels of a number of hormones increase dramatically just after birth *(29–31)*. For example, an increasing thyroid surge has been shown to specifically repress PKCα and PKCδ in neonatal hearts *(32)*. However, the transient upregulation of PKC expression between d 3 and 5–7 is unlikely to be explained by thyroid hormone, the level of which continually increases during this period *(31)*. The influence of the sympathetic system on the developmental changes in PKC was excluded in a study by Rybin and Steinberg *(27)*.

The differences in the isoforms cofactor requirement, substrate specificity, tissue and cellular distribution, and subcellular localization suggest that each of the different PKC isoforms plays a specific and distinct regulatory role in cellular signal transduction. Novel PKC isoforms appear to be selectively activated by DAG produced from the PLD pathway, which does not induce Ca^{2+} transient *(7)*. On the other hand, the Ca^{2+} signal that accompanies phosphoinositide hydrolysis may be required for full activation of conventional PKC isoforms *(9)*. It appears likely that the isoform-selective phosphorylation of downstream targets should be the basis of isoform-selective function in vivo. However, in vitro studies did not indicate a large degree of substrate specificity *(33)*, because K_m and V_{max} for different

substrates did not differ more than threefold. For this reason, focus has been turned to subcellular localization to explain the selective phosphorylation in vivo. The compartmentation of PKC isoforms close to the localization of specific substrates appears to determine their function. Inactive PKC isoforms were thought to be present mainly in the cytosol, whereas their activators should be present in membranes. However, immunocytochemical studies revealed that inactive isozymes are localized mostly to subcellular structures too, and on activation translocate to new distinct sites. For example, PKCδ and PKCε were reported to be localized mainly to the nucleus in unstimulated rat myocytes and translocated to the myofibrillar, cytoskeletal, and cross-striated structures after activation by norepinephrine and/or phorbol 12-myristate 13-acetate *(16)*. This fact implies that the reason we were unable to detect the translocation of activated PKCδ and PKCε in our study was that we analyzed the mixture of nuclear, cytoskeletal, and myofibrillar structures.

The level of DAG and membrane phospholipid composition, their fatty acid chain length, and their level of unsaturation all are postulated to modulate PKC activity *(10,34,35)*. For example, alterations in the ratio of bilayer-forming lipids to non-bilayer-forming lipids in the membrane can modulate PKC translocation on and off the membrane. DAG is a non-bilayer-forming lipid, and its elevated content in the membrane tends to promote PKC translocation to the membrane, which is accompanied by PKC activation *(36)*. We found a significant transient drop in myocardial DAG concentration between d 2 and 5, which corresponds to the decrease in total PKC activity and expression of PKC isoforms in the homogenate. Regarding the translocation of individual PKC isoforms determined by immunoblotting, only PKCε associated with the membrane fraction followed the drop in DAG level: it tended to translocate from membranes to the cytosol by d 5 and 7 and return again by d 10. On the contrary, the content of PKCε associated with nuclear-cytoskeletal-myofilament structures tended to increase when the level of cellular DAG decreased. Localization of PKCα and PKCδ did not correspond to the changes in tissue DAG level.

Madani et al. *(34)* showed that individual PKC isoforms varied in response to different DAG species containing n-6 or n-3 PUFA in an in vitro study. This phenomenon might contribute to the complex regulation of the total PKC activity and explain some of the diverse results. In addition, it was shown that DAG containing long-chain saturated FA was far less effective in PKC activation in comparison with PUFA *(35)*. However, it is still not clear which DAG species are convenient for the activation of specific PKC isoforms in vivo. We analyzed the FA composition of myocardial DAG, but it is difficult to identify the origin of DAG according to its FA profile. Comparison of FA composition of DAG with that of PC, phosphatidylinositol (PI), and triacylglycerol (TAG) in rat myocardium during the same developmental period suggests that DAG did not originate from either of them exclusively (unpublished observation). It seems more likely that the DAG analyzed was derived from a mixture of those lipids. Moreover, it should be taken into account that the composition of polyphosphoinositides, the direct source of DAG, differs from that of PI. According to Lamers et al. *(37)*, in the myocardium there is a higher proportion of saturated FA in polyphosphoinositides than in PI. The proportion of 16:0 that we determined in DAG indicates that the precursor of DAG could be PC or TAG, but the proportion of 18:0 in DAG is higher than in these lipids. This finding suggests that highly saturated polyphosphoinositides are the important source of DAG in the rat heart during early postnatal development. Concerning n-6 PUFA, the proportion of 18:2n-6 refers to both DAG precursors: $PI(4,5)P_2$ and PC. On the other hand, the proportion of 20:4n-6 (about 10 mol%) differs completely from polyphosphoinositides and PC (<20 mol%) as well as from TAG (about 5 mol%). The proportion of n-6 PUFA in DAG corresponds to that in phosphoinositides *(37)*.

Our study cannot explain the complex role of PKC signaling in the regulation of cellular processes associated with early postnatal cardiac development. Nevertheless, the rapid day-by-day changes in PKC activity, expression, and localization correspond to the time course of development of various structural and functional characteristics of the neonatal heart. For example, changes in contractility of the rat heart exhibit a biphasic pattern, reaching a minimum value in the middle of the first postnatal week *(38)*. We could speculate that this response is owing to a transient decrease in PKC-dependent phosphorylation of proteins involved in the control of cardiac contraction. PKC downregulation on d 3 may also be implicated in the rapid cessation of cardiomyocyte proliferation after birth, because this enzyme, particularly its PKCε isoform has been proposed to contribute to the increase in myocyte number during normal cardiac growth *(39)*. Moreover, the activation of both PKCδ and PKCε leads to myocardial hypertrophy *(40)*. We observed a more than twofold increase in the content of PKCδ and PKCε associated with nuclear-cytoskeletal-myofilament structures between d 3 and 5, within the narrow developmental period, which is characterized by the switch from hyperplastic to hypertrophic growth of cardiomyocytes *(20)*. Thus, our results are compatible with the view that mainly these two novel PKC isoforms could be involved in the regulation of this key developmental process, although further studies are necessary to elucidate their precise role.

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REFERENCES

1. Nishizuka, Y. (1986) Studies and perspectives of protein kinase C. *Science* **233,** 305–312.
2. Puceat, M. and Vassort, G. (1996) Signalling by protein kinase C isoforms in the heart. *Mol. Cell. Biochem.* **157,** 65–72.
3. Malhotra, A., Kang, B. P., Opawumi, D., Belizaire, W., and Meggs, L. G. (2001) Molecular biology of protein kinase C signaling in cardiac myocytes. *Mol. Cell. Biochem.* **225,** 97–107.
4. Sabri, A. and Steinberg, S. F. (2003) Protein kinase C isoform-selective signals that lead to cardiac hypertrophy and the progression of heart failure. *Mol. Cell. Biochem.* **251,** 97–101.
5. Nishizuka, Y. (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258,** 607–614.
6. Newton, A. C. (1995) Protein kinase C: structure, function, and regulation. *J. Biol. Chem.* **270,** 28,495–28,498.
7. Ha, K. S. and Exton, J. H. (1993) Differential translocation of protein kinase C isozymes by thrombin and platelet-derived growth factor: a possible function for phosphatidylcholine-derived diacylglycerol. *J. Biol. Chem.* **268,** 10,534–10,539.
8. Khan, W. A., Blobe, G. C., and Hannun, Y. A. (1995) Arachidonic acid and free fatty acids as second messengers and the role of protein kinase C. *Cell. Signal.* **7,** 171–184.
9. Nishizuka, Y. (1995) Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.* **9,** 484–496.
10. Murray, N. R. and Fields, A. P. (1998) Phosphatidylglycerol is a physiologic activator of nuclear protein kinase C. *J. Biol. Chem.* **273,** 11,514–11,520.
11. Lee, J. Y., Hannun, Y. A., and Obeid, L. M. (1996) Ceramide inactivates cellular protein kinase C alpha. *J. Biol. Chem.* **271,** 13,169–13,174.
12. Dhalla, N. S., Xu, Y. J., Sheu, S. S., Tappia, P. S., and Panagia, V. (1997) Phosphatidic acid: a potential signal transducer for cardiac hypertrophy. *J. Mol. Cell. Cardiol.* **29,** 2865–2871.
13. Khan, W. A., Blobe, G. C., and Hannun, Y. A. (1992) Activation of protein kinase C by oleic acid. Determination and analysis of inhibition by detergent micelles and physiologic membranes: requirement for free oleate. *J. Biol. Chem.* **267,** 3605–3612.
14. Parekh, D. B., Ziegler, W., and Parker, P. J. (2000) Multiple pathways control protein kinase C phosphorylation. *EMBO J.* **19,** 496–503.
15. Newton, A. C. (1996) Protein kinase C: ports of anchor in the cell. *Curr. Biol.* **6,** 806–809.
16. Disatnik, M. H., Buraggi, G., and Mochly-Rosen, D. (1994) Localization of protein kinase

C isozymes in cardiac myocytes. *Exp. Cell Res.* **210,** 287–297.
17. Mochly-Rosen, D. and Gordon, A. S. (1998) Anchoring proteins for protein kinase C: a means for isozyme selectivity. *FASEB J.* **12,** 35–42.
18. Clerk, A., Bogoyevitch, M. A., Fuller, S. J., Lazou, A., Parker, P. J., and Sugden, P. H. (1995) Expression of protein kinase C isoforms during cardiac ventricular development. *Am. J. Physiol. Heart Circ. Physiol.* **269,** H1087–H1097.
19. Ostadal, B., Ostadalova, I., and Dhalla, N. S. (1999) Development of cardiac sensitivity to oxygen deficiency: comparative and ontogenetic aspects. *Physiol. Rev.* **79,** 635–659.
20. Li, F., Wang, X., Capasso, J. M., and Gerdes, A. M. (1996) Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. *J. Mol. Cell. Cardiol.* **28,** 1737–1746.
21. Gu, X. and Bishop, S. P. (1994) Increased protein kinase C and isozyme redistribution in pressure-overload cardiac hypertrophy in the rat. *Circ. Res.* **75,** 926–931.
22. Peterson, G. L. (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83,** 346–356.
23. Ogita, K., Miyamoto, S., Yamaguchi, K., et al. (1992) Isolation and characterization of delta-subspecies of protein kinase C from rat brain. *Proc. Natl. Acad. Sci. USA* **89,** 1592–1596.
24. Folch, J., Lees, M., and Stanley, G. H. (1957) A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem.* **226,** 497–509.
25. McGill, C. J. and Brooks, G. (1997) Expression and regulation of 80K/MARCKS, a major substrate of protein kinase C, in the developing rat heart. *Cardiovasc. Res.* **34,** 368–376.
26. Herget, T., Oehrlein, S. A., Pappin, D. J., Rozengurt, E., and Parker, P. J. (1995) The myristoylated alanine-rich C-kinase substrate (MARCKS) is sequentially phosphorylated by conventional, novel and atypical isotypes of protein kinase C. *Eur. J. Biochem.* **233,** 448–457.
27. Rybin, V. O. and Steinberg, S. F. (1994) Protein kinase C isoform expression and regulation in the developing rat heart. *Circ. Res.* **74,** 299–309.
28. Schreiber, K. L., Paquet, L., Allen, B. G., and Rindt, H. (2001) Protein kinase C isoform expression and activity in the mouse heart. *Am. J. Physiol. Heart Circ. Physiol.* **281,** H2062–H2071.
29. Hunt, R. A., Ciuffo, G. M., Saavedra, J. M., and Tucker, D. C. (1995) Quantification and localisation of angiotensin II receptors and angiotensin converting enzyme in the developing rat heart. *Cardiovasc. Res.* **29,** 834–840.
30. Artman, M. (1992) Developmental changes in myocardial contractile responses to inotropic agents. *Cardiovasc. Res.* **26,** 3–13.
31. Vigouroux, E. (1976) Dynamic study of postnatal thyroid function in the rat. *Acta Endocrinol. (Copenh.)* **83,** 752–762.
32. Rybin, V. and Steinberg, S. F. (1996) Thyroid hormone represses protein kinase C isoform expression and activity in rat cardiac myocytes. *Circ. Res.* **79,** 388–398.
33. Kazanietz, M. G., Areces, L. B., Bahador, A., et al. (1993) Characterization of ligand and substrate specificity for the calcium-dependent and calcium-independent protein kinase C isozymes. *Mol. Pharmacol.* **44,** 298–307.
34. Madani, S., Hichami, A., Legrand, A., Belleville, J., and Khan, N. A. (2001) Implication of acyl chain of diacylglycerols in activation of different isoforms of protein kinase C. *FASEB J.* **15,** 2595–2601.
35. Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U., and Nishizuka, Y. (1980) Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. *J. Biol. Chem.* **255,** 2273–2276.
36. Xu, F. Y., Fandrich, R. R., Nemer, M., Kardami, E., and Hatch, G. M. (1999) The subcellular distribution of protein kinase C-alpha, -epsilon, and -zeta isoforms during cardiac cell differentiation. *Arch. Biochem. Biophys.* **367,** 17–25.
37. Lamers, J. M., Dekkers, D. H., Mesaeli, N., Panagia, V., and van Heugten, H. A. (1993) Myocardial phosphoinositides do not share the same fatty acid profile. *Biochem. Biophys. Res. Commun.* **191,** 487–494.
38. Ostadalova, I., Kolar, F., Ostadal, B., Rohlicek, V., Rohlicek, J., and Prochazka, J. (1993) Early postnatal development of contractile performance and responsiveness to Ca^{2+}, verapamil and ryanodine in the isolated rat heart. *J. Mol. Cell. Cardiol.* **25,** 733–740.
39. Mochly-Rosen, D., Wu, G., Hahn, H., et al. (2000) Cardiotrophic effects of protein kinase C ε: analysis by in vivo modulation of PKCε translocation. *Circ. Res.* **86,** 1173–1179.
40. Chen, L., Hahn, H., Wu, G., et al. (2001) Opposing cardioprotective actions and parallel hypertrophic effects of δPKC and εPKC. *PNAS* **98,** 11,114–11,119.

SUPPLEMENT 5

EFFECT OF PRESSURE OVERLOAD ON PROTEIN KINASE C IN RAT MYOCARDIUM DURING EARLY POSTNATAL DEVELOPMENT

B. Hamplová[1,2], O. Nováková[2], F. Kolář[3], F. Novák[1]

[1]Department of Biochemistry, [2]Department of Physiology and Developmental Biology, Faculty of Science, [3]Institute of Physiology, Academy of Sciences of the Czech Republic and Centre for Cardiovascular Research, Prague, Czech Republic.

Address for offprints:

F. Novák,

Faculty of Science, Charles University in Prague,

Department of Biochemistry, Hlavova 2030, 128 43, Prague 2, Czech Republic

Fax: +420 2 2195 2331

E-mail: franov@natur.cuni.cz

Abstract

Protein kinase C (PKC) appears to play a significant role in the signal transduction of cardiac growth and development. The aim of this study was to determine changes in the total PKC activity and the expression of PKC isoforms α, δ and ε in the rat heart that was affected by the pressure overload imposed on the postnatal day (d) 2. Three groups of Wistar rats were employed for the experiment: intact rats, pressure-overloaded rats submitted to the abdominal aortic constriction (AC) and sham-operated (SH) rats used as age-matched controls for AC rats. Animals were sacrificed on d2, 3, 5 and 10. The total PKC activity was measured by the incorporation of ^{32}P into histone IIIS and the expression of PKC by immunoblotting in the cytosolic, membrane-enriched (10^5 x g) (denoted as membrane fraction hereafter) and nuclear-cytoskeletal-myofilament-enriched (10^3 x g) (denoted as nuclear fraction hereafter) fractions. We noticed a strong effect of SH on the total activity and the expression of PKCδ and PKCε in all cellular fraction as compared to the intact rats. AC targeted the same PKC isoforms, in particular in the membrane fraction of the rat ventricular homogenate. The rapid increase in the amount of PKCδ and PKCε was observed on d3, a minor increase on d5 and a decrease on d10 in the membrane fraction of ventricular homogenate from the AC rats compared to the SH ones. It is concluded that rather PKCδ and PKCε, and not PKCα, are involved in the regulation of the accelerated cardiac growth induced by the pressure overload imposed in the very early postnatal period.

Key words: protein kinase C, pressure overload, postnatal development, rat myocardium

Introduction

A developing heart has to overcome an increased workload, which is associated with the rapid growth of left ventricle during postnatal ontogeny (*1*). Just after birth, the myocardium grows by adding new cells (hyperplasia), but rat cardiomyocytes lose their capability of dividing soon, and the further growth of the heart is due to the increase of cell volume (hypertrophy) (*2*). It is well known that protein kinase C (PKC) is an important regulator of the cell proliferation and cardiac hypertrophy (*3-5*). The Gq/PLC/PKC signaling pathway was proved to be activated to produce the hypertrophy in the pressure-overloaded heart (*6*).

PKC consists of a family of at least 11 isozymes, which are divided into three groups. The members of a classical group (α, $\beta_{1/2}$ and γ) are Ca^{2+} dependent and need 1,2-diacylglycerol (DAG) and phosphatidylserine (PS) for their activation; novel PKC isoforms (δ, ε, η and θ) are Ca^{2+} independent and are activated by DAG and PS; atypical isoforms (ζ and ι/λ) are Ca^{2+} and DAG independent but require PS as a cofactor. PKC isozymes are expressed differentially in various organs and tissues (*7*). PKCα, PKCδ, PKCε and PKCζ were mostly identified in a neonatal rat heart and all of them diminished markedly until adulthood (*8-10*). We have recently described dramatic developmental changes in the expression of PKCα, PKCδ and PKCε in the homogenate and the cellular fractions of rat heart during the first 10 postnatal days. A more then 2-fold increase occurred in the content of PKCδ and PKCε associated with nuclear-cytoskeletal-myofilament structures between d 3 and 5 (*10*). It is worth noting that this narrow developmental period is connected with the transition from the hyperplastic to the hypertrophic growth (*2*).

In the present study we were interested how an additional growth-promoting stimulus can influence the developmental changes in the total activity and the expression of PKCα, PKCδ and PKCε during the first ten postnatal days. We used the unique experimental model of the neonatal pressure-overloaded heart that allows observing a response of the myocardium that is still in the proliferative phase of the cardiomyocyte growth (*11*).

Materials and Methods

Animal model

Newborn male Wistar rats were obtained from the animal care facility of the Institute of Physiology, Prague, Czech Republic. Under a light ether anesthesia, a pressure overload was induced by the constriction of an abdominal aorta (AC) in 2-d-old rats. Sham-operated (SH) littermates were used as the age-matched controls; their aorta was exposed, but not constricted (*11*). Intact littermates were used also. The animals were killed on d2, 3, 5 and 10 and their hearts were dissected free of an atrial tissue, large blood vessels and a right ventricle. A left ventricle including a septum (LV+S) was rinsed in cold saline (5 °C), weighed, frozen in liquid nitrogen and stored at –80 °C until use. This study conforms to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institute of Health (National Institutes of Health publication No. 85-23, revised 1996).

Material

Chemical reagents including the PKC isoform-specific primary antisera and competing immunizing peptides were obtained from Sigma-Aldrich. The secondary swine anti-rabbit IgG antibody labelled with a horseradish peroxidase was from Sevapharma. Radioactive $\gamma[^{32}P]$ATP was obtained from ICN Biomedicals.

Tissue fractionation

A specimen for a fractionation contained ventricles from 4 (d10) to 14 (d2) animals. The tissue was minced using Turrax (30 s, twice) and then homogenized (Potter Evehjem) in 10 volumes of ice-cold homogenization buffer composed of (in mmol/l): 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, 0.02 aprotinin. The homogenate was fractionated according to the method described previously (*12*), with slight modifications. Briefly, the homogenate was centrifuged at 100 × g for 20 min to remove cellular debris and unbroken cells. A supernatant was centrifuged at 1000 × g for 10 min to produce a nuclear-cytoskeletal-myofilament-enriched fraction (denoted as *nuclear fraction* in the text for simplicity) followed by centrifugation at 100,000 × g for 60 min. The

100,000 × g pellet contained a membrane-enriched fraction and the supernatant was a cytosolic fraction. The homogenate and the pellets of nuclearl and membrane fractions were suspended in the homogenization buffer containing 1 % Triton X-100, held on ice for 60 min and centrifuged at 100,000 × g for further 60 min. The resulting detergent-treated supernatants were used for activity and immunoblotting analyses. Triton X-100 was also added to the cytosolic fraction to give the final concentration of 1 %. Protein content was determined according to Lowry, as modified by Peterson (*13*).

Activity assay of total PKC

The PKC activity was determined as a difference in the incorporation of ^{32}P from $\gamma[^{32}P]ATP$ into a histone IIIS in the presence and the absence of PS, 1-octanoyl-2-acetyl-glycerol (OAG) and Ca^{2+}. The required amount of PS and OAG dissolved in chloroform was dried in the stream of nitrogen and solubilized in 0.3 % Triton X-100 by vortexing and incubation at 30 °C for 5 min. A reaction mixture (0.1 ml) contained (in mmol/l): 20 Tris-HCl (pH 7.45), 10 $MgCl_2$, 1 dithiothreitol, 1 $CaCl_2$, 0.1 vanadate, 21 μg PS, 5.1 μg OAG and protein (in μg: 2.5 homogenate, 3 cytosolic, 2 nuclear, 1 membrane fractions). The reaction was started by the addition of 10 μl of 1 mM $\gamma[^{32}P]ATP$ (50-100 cpm/pmol) and terminated after 5 min at 30 °C by the addition of 1 ml ice-cold 25 % trichloroacetic acid. The resulting precipitates were filtered through pre-soaked nitrocellulose filters (Pragopor, Pragochema) and washed three times with 5 % trichloroacetic acid. The radioactivity was quantified using the Cerenkov decay.

Immunoblot analyses of PKC isoforms

Samples were electrophoresed on the 8 % bis-acrylamide polyacrylamide gel. SDS-PAGE was carried out at 20 mA/gel for 90 min on a Mini-Protean II apparatus (Bio-Rad). After the electrophoresis, resolved proteins were transferred to a nitrocellulose membrane (Amersham International). The membranes were incubated in 5 % dry low-fat milk in Tris-buffered saline with Tween 20 (TTBS) for 60 min at room temperature in order to block a nonspecific binding. After washing in TTBS buffer (quickly 3-times, 5 min, 3-times) they were probed with the PKC isoform-specific primary rabbit antisera (1:8000 in TTBS) for 90 min at room temperature. The membranes were washed again and

incubated with the secondary swine anti-rabbit IgG antibody labelled with the horseradish peroxidase (1:4000 in TTBS) for 60 min at room temperature. Before an enhanced chemiluminescence (ECL), the nitrocelluloses were washed as described above and stored in TTBS for at least 2 hours. For ECL, we prepared ECL substrates A (Luminol solution) and B (H_2O_2 solution); they were mixed 1:1 and poured on the immunoblot. A specific signal was documented on an autoradiography film (Amersham Int). A scanning (Epson Perfection 1240U) and ImageQuant software were used for the quantification of a relative abundance of the individual PKC isoforms. To ensure the specificity of the immunoreactive proteins, Western blots were done in the presence and the absence of competing immunizing peptides (see Fig.1 in (*10*)). The PKC δ isoform was identified as the doublet of proteins. Both proteins were recognized using the antibody mentioned above, blocked by the appropriate immunizing peptide and taken for the quantification in our study. Similarly, Ogita *et al.* (*14*) immunodetected PKC δ as the doublet of proteins (78 and 76 kDa) in the preparation from rat brain; the 78 kDa protein was identified as the phosphorylated form of the 76 kDa protein by means of the protein phosphatase 2A treatment.

Statistical analyses

All results are expressed as means ± S.E.M. The statistical significance of differences was determined by one-way ANOVA and subsequent Newman-Keul's test ($p<0.05$).

Results

Weight parameters

A body weight was not significantly altered in any group of the animals. SH increased the weight of LV+S and RV on d5 (by 17% and 29%, respectively) as compared with the intact rats. AC evoked a cardiac enlargement 3 and 7 days after the surgery (by 17 % and 22 %, respectively) as compared with the SH rats (Table 1).

Total PKC activity

The first graph in Fig. 1 shows changes in the total PKC activity in the cytosolic, membrane and nuclear fraction of the intact ventricular homogenate during early

postnatal development. The effect of SH and AC on the total activity within the period of the study is presented in the second and the third graph, respectively. The PKC activity declines gradually from d2 till d10 in the cytosolic fraction of the intact rats. SH induced a decrease in the activity (by 21%) as early as the first day after the surgery. This effect weakened steadily and an increase in the activity (by 15%) was observed on d10 in the SH group compared to the intact controls. AC caused opposite changes in the activity: a rise on d3 (by 10%) and a fall on d10 (by 13%) as compared with the SH rats. In the membrane fraction, the total PKC activity also declines by d10 but with a transient increase on d5 in the intact rats. SH decreased the PKC activity on d3 and d10 (both by 37%) and increased it on d5 (by 23%) in comparison with the intact controls. AC lowered additionally the decrease in the activity on d10 (by 43%) in the membrane fraction as compared with the SH rats. Similarly as in the other cellular fractions, a gradual decline in the PKC activity from d2 till d10 occurs in the nuclear fraction of the intact myocardium. As for the SH effect, a 2-fold increase on d3 and an increase (by 28%) on d5 were observed in the SH rats as compared with the intact ones. The pressure overload did not significantly influence the total PKC activity, but tended to increase it on d10 in the nuclear fraction.

PKC isoforms expression

Developmental changes in the expression of PKC isoforms and the effect of SH and AC are presented in Fig. 2, 3, 4.

The PKCα expression declines gradually from d2 till d10 in the cytosolic fraction of the intact myocardium. The PKCα expression does not change until d5 and decreases on d10 in the membrane fraction, and it drops deeply on d3 and remains unchanged further in the nuclear fraction. Neither SH nor AC influenced the expression of PKCα in any cellular fraction.

The PKCδ expression does not change markedly in the cytosolic fraction of the intact animals. SH tended to increase the PKCδ expression on d5 as compared with the intact animals. The pressure overload tended to elevate the PKCδ expression by d10 in the cytosol as compared with the SH group. The expression of PKCδ drops on d3 and essentially keeps the level after in the membrane fraction during the normal postnatal

development. SH deepened the drop on d3 (by 29%) compared to the intact rats, on the other hand, AC evoked a significant increase (by 93%) on d3 compared to the SH rats. In the nuclear fraction, the abundance of PKCδ falls on d3, elevates on d5 and declines again by d10 in the intact rats. In the group of the SH animals, we observed a decrease of PKCδ level on d5 and d10 (by 53% and 26%, respectively) as compared with the intact controls. After AC a tendency to an additional decrease in the PKC δ expression on d5 and d10 was observed in comparison with the SH rats.

The expression of PKCε declines gradually until d5 and than tends to increase slightly in the cytosolic fraction of the intact rats. SH tended to diminish this decline on d5. The pressure overload did not affect the expression in the cytosolic fraction. During the normal postnatal development, the PKCε expression drops on d3, elevates on d5 and does not change after in the membrane fraction. SH had no effect on the expression, but AC induced a significant increase in the PKCε expression (by 27%) on d5 and a decrease (by 52%) on d10 in the membrane fraction. The time course of developmental changes in the PKCε expression in the nuclear fraction of the intact rats was essentially the same as in PKCδ, however, the effects of SH and AC were dissimilar. We observed nearly a 2-fold elevation in the PKCε expression on d3 and a trend toward a fall in the amount of the enzyme associated with the nuclear fraction on d5 and d10 in the SH group. In contrast, the pressure overload decreased the expression of PKCε on d3 (by 15%) as compared with the SH controls.

Discussion

The aim of this study was to estimate an effect of the pressure overload on the pattern of developmental changes in the total PKC activity and the expression of PKCα, PKCδ and PKCε in the rat heart during the first ten postnatal days in comparison with the SH controls. We observed the strong effect of the operation itself on the cardiac PKC isoforms. The total PKC activity and the PKCδ expression were even more affected by SH than by AC. There are two possible explanations of this observation. Firstly, it could be an effect of anesthetics on the cardiac PKC. Zaugg et al. analyzed the influence of volatile (*15*) and intravenous (*16*) anesthetics on the mitochondrial K_{ATP} channel activity

in the isolated cardiac myocytes. They demonstrated that the particular anesthetics mimic the cardiac preconditioning by priming the activation of the mitochondrial K_{ATP} channel, and that multiple PKC-coupled signaling pathways mediate this protection. Secondly, the marked effect of SH on the PKC isoforms in the rat heart could be connected with a systemic antiinflammatory response to a stress and an injury. The systemic response prevents an inflammation in uninvolved tissues by neutralizing inflammation-induced molecules (such as cytokines, proteases, and oxidants) that enter the bloodstream immediately after the injury (*17*). Tumor necrosis factor-α (TNF-α) belongs among the earliest and the most potent mediators related to the injury and the inflammatory response (*18*) and it can induce the activation of PKCδ and PKCε, as was shown in the human intestinal epithelial cell line (*19*). These findings support our results, as we have observed nearly the 2-fold increase in PKC ε and the total activity, and the trend to the increase in PKC δ expression on d3 in the nuclear fraction of the SH rats as compared with the intact ones. The relevant comparison with the other studies concerning the changes in the PKC isoforms after SH are lacking as they do not show the effect of the operation itself.

Although there are several studies investigating the role of the PKC family in the regulation of the cardiac hypertrophy induced by the aorta constriction (*12; 20; 21*), none of them examines the cardiomegaly induced before d5, i.e. in the proliferative stage of the cardiomyocyte growth (*2*). It was shown that the myocardium responds differently to the pressure overload depending on the time of the intervention. When the aorta constriction is performed just after birth, the cellular hyperplasia contributes significantly to the increase of the heart mass aside from the cardiomyocyte enlargement (*22*) and the hypertrophied hearts of this unique experimental model do not show the fibrosis characteristic of adult pressure-overloaded models (*23*).

Different PKC isoforms have been found upregulated or activated in pressure-overloaded heart in an age-dependent manner (*12; 20; 21*). In this study, we have found the transient translocation of PKCδ and PKCε to the particulate fractions as early as on d3. This rapid translocation is consistent with the shift of PKC to membranes observed immediately after ANG II receptor activation (*24*), and ANG II has been reported to play a role in cardiac hypertrophy due to pressure overload (*25; 26*). The amount of both PKCδ and PKCε in the microsomal membranes decreased with the comparable trend

from d3 till d10. This is in line with the observation that these novel isoforms play parallel roles in the regulation of the cardiac hypertrophy (*27*). Since essential roles of PKCδ and PKCε have been evidenced in the normal cardiac growth as well (*28; 29*), there is probably functional synergism between normal cardiac growth and pathological growth during early postnatal period (*30*). Gu and Bishop (*12*) found activated PKCε and upregulated PKCβ two weeks after the aortic banding. The function of PKCβ in cardiac hypertrophy is controversial. Although PKCβ overexpression in the myocardium led to cardiac hypertrophy and heart failure in an age-dependent manner (*31*), PKCβ-knockout mice demonstrated that this isoform was not necessary for the development of cardiac hypertrophy (*32*). In all accounts, increased PKCβ expression was found in failed human hearts (*33*), which strongly supports its important role in the development of pathological hypertrophy. As for the role of PKCε, studies on transgenic mice have demonstrated that PKCε activation in pathological cardiac hypertrophy is more compensatory than the pathological event (*29; 34; 35*). In adult pressure-overloaded myocardium, PKCα and PKCδ were markedly upregulated, the latter one even as early as the first day after aortic banding (*20; 21*). These results provide indirect evidence that PKCδ might be involved in the induction of compensatory hypertrophy in the adult heart after aorta constriction, whereas both PKCα and PKCδ might be responsible for the transition to heart failure. In agreement with this, recent studies with transgenic mice revealed that PKCα is more important regulator of myocardial contractility (*36*) than cardiac hypertrophy as former analyses proposed (*37*), and the function of PKCδ depends on the strength of hypetrophic stimuli (*28*). A modest increase in PKCδ expression was shown to result in physiological hypertrophy, but on the other hand, high chronic expression of PKCδ evoked cardiomyocyte necrosis and contractile dysfunction (*27; 28*). In line with this, a moderate level in G protein activation induced stable cardiac hypertrophy, whereas, high level induced cardiomyocyte apoptosis (*38*). The latter observations clearly demonstrate that only the extent of G protein/PLC/PKC pathway stimulation is critical for the transition from compensate hypertrophy to heart failure. Heidkamp et al. (*39*) showed that just PKCδ and PKCε act as a switch between cardiomyocyte hypertrophy and apoptosis, and PKCε selectively activates the MAPK cascade implicated in growth response and cell survival, whereas, PKCδ preferentially activates stress-activated protein

kinase cascades implicated in detrimental changes of the heart. Thus, it seems that the fate of cardiomyocytes depends mostly on the balance of nPKC isoforms to downstream signaling cascades.

Apparently, the PKC regulation of the cardiac hypertrophy might depend on multiple factors, including the age, the experimental model, the nature and the strength of pathological stimuli, and the severity of hypertrophy. It should be stressed that the PKC isoform signaling depends all the more on the timing of on a hypertrophic stimuli when it occurs in the period just after birth when each of given PKC isoform follows the specific day-by-day changes (*10*). In this case, slightest the time difference might result in a big diversity in the PKC isoforms activation in the developing myocardium. Furthermore, the PKC acting can be largely influenced by the changes in the membrane phospholipids. They serve as the source of DAG and other lipid second messengers, and additionally can modify the PKC function by the modification of membrane properties (*40*). In our previous study, we showed that the concentration and the fatty acid profile of the membrane phospholipids change dramatically during the early postnatal development (*41*), and moreover, their composition is highly sensitive to the nutritional and humoral changes. Hence, an altered diet or a thyroid state of rats might take an effect on the expression and/or the activity of PKC isoforms in the heart. All these facts together with the impossibility to guarantee the same degree of the aorta constriction in the 2-day-old rats make more difficult to obtain consistent results especially in so dynamic process like the PKC signaling.

Our results suggest that PKCδ and PKCε are involved in the regulation of the development of the adaptive hypertrophy of the developing myocardium after the constriction of abdominal aorta on d2.

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References

1. Smolich, J. J. (1995) Ultrastructural and functional features of the developing mammalian heart: a brief overview, *Reprod. Fertil. Dev. 7(3),* 451-61.

2. Clubb, F. J. J. and Bishop, S. P. (1984) Formation of binucleated myocardial cells in the neonatal rat. An index for growth hypertrophy, *Lab. Invest. 50*, 571-577.

3. Bogoyevitch, M. A. and Sugden, P. H. (1996) The role of protein kinases in adaptational growth of the heart, *Int. J. Biochem. Cell. Biol. 28*, 1-12.

4. Sabri, A. and Steinberg, S. F. (2003) Protein kinase C isoform-selective signals that lead to cardiac hypertrophy and the progression of heart failure, *Mol. Cell. Biochem. 251*, 97-101.

5. Buchner, K. (2000) The role of protein kinase C in the regulation of cell growth and in signalling to the cell nucleus, *J. Cancer. Res. Clin. Oncol. 126*, 1-11.

6. Wettschureck, N., Rutten, H., Zywietz, A., Gehring, D., Wilkie, T. M., Chen, J., Chien, K. R., and Offermanns, S. (2001) Absence of pressure overload induced myocardial hypertrophy after conditional inactivation of Galphaq/Galpha11 in cardiomyocytes, *Nat. Med. 7*, 1236-1240.

7. Wetsel, W. C., Khan, W. A., Merchenthaler, I., Rivera, H., Halpern, A. E., Phung, H. M., Negro-Vilar, A., and Hannun, Y. A. (1992) Tissue and cellular distribution of the extended family of protein kinase C isoenzymes, *J. Cell Biol. 117*, 121-133.

8. Clerk, A., Bogoyevitch, M. A., Fuller, S. J., Lazou, A., Parker, P. J., and Sugden, P. H. (1995) Expression of protein kinase C isoforms during cardiac ventricular development, *Am. J. Physiol. 269*, H1087-H1097.

9. Rybin, V. O. and Steinberg, S. F. (1994) Protein kinase C isoform expression and regulation in the developing rat heart, *Circ. Res. 74*, 299-309.

10. Hamplova, B., Novakova, O., Tvrzicka, E., Kolar, F., and Novak, F. (2005) Protein kinase C activity and isoform expression during early postnatal development of rat myocardium, *Cell. Biochem. Biophys. 43*, 105-117.

11. Kolar, F., Papousek, F., Pelouch, V., Ostadal, B., and Rakusan, K. (1998) Pressure overload induced in newborn rats: effects on left ventricular growth, morphology, and function, *Pediatr. Res. 43*, 521-526.

12. Gu, X. and Bishop, S. P. (1994) Increased protein kinase C and isozyme redistribution in pressure-overload cardiac hypertrophy in the rat, *Circ. Res. 75*, 926-931.

13. Peterson, G. L. (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable, *Anal. Biochem. 83*, 346-356.

14. Ogita, K., Miyamoto, S., Yamaguchi, K., Koide, H., Fujisawa, N., Kikkawa, U., Sahara, S., Fukami, Y., and Nishizuka, Y. (1992) Isolation and characterization of delta-subspecies of protein kinase C from rat brain, *Proc. Natl. Acad. Sci. U. S. A 89*, 1592-1596.

15. Zaugg, M., Lucchinetti, E., Spahn, D. R., Pasch, T., and Schaub, M. C. (2002) Volatile anesthetics mimic cardiac preconditioning by priming the activation of mitochondrial K(ATP) channels via multiple signaling pathways, *Anesthesiology 97*, 4-14.

16. Zaugg, M., Lucchinetti, E., Spahn, D. R., Pasch, T., Garcia, C., and Schaub, M. C. (2002) Differential effects of anesthetics on mitochondrial K(ATP) channel activity and cardiomyocyte protection, *Anesthesiology 97*, 15-23.

17. Munford, R. S. and Pugin, J. (2001) Normal responses to injury prevent systemic inflammation and can be immunosuppressive, *Am. J Respir. Crit Care Med. 163*, 316-321.

18. Lin, E., Calvano, S. E., and Lowry, S. F. (2000) Inflammatory cytokines and cell response in surgery, *Surgery 127*, 117-126.

19. Chang, Q. and Tepperman, B. L. (2003) Effect of selective PKC isoform activation and inhibition on TNF-alpha-induced injury and apoptosis in human intestinal epithelial cells, *Br. J. Pharmacol. 140*, 41-52.

20. Bayer, A. L., Heidkamp, M. C., Patel, N., Porter, M., Engman, S., and Samarel, A. M. (2003) Alterations in protein kinase C isoenzyme expression and autophosphorylation during the progression of pressure overload-induced left ventricular hypertrophy, *Mol. Cell. Biochem. 242*, 145-152.

21. Braun, M. U., LaRosee, P., Schon, S., Borst, M. M., and Strasser, R. H. (2002) Differential regulation of cardiac protein kinase C isozyme expression after aortic banding in rat, *Cardiovasc. Res. 56*, 52-63.

22. Sedmera, D., Thompson, R. P., and Kolar, F. (2003) Effect of increased pressure loading on heart growth in neonatal rats, *J. Mol. Cell. Cardiol. 35*, 301-309.

23. Kolar, F., Papousek, F., Pelouch, V., Ostadal, B., and Rakusan, K. (1998) Pressure overload induced in newborn rats: effects on left ventricular growth, morphology, and function, *Pediatr. Res. 43*, 521-526.

24. Tuominen, R. K., Werner, M. H., Ye, H., McMillian, M. K., Hudson, P. M., Hannun, Y. A., and Hong, J. S. (1993) Biphasic generation of diacylglycerol by angiotensin and phorbol ester in bovine adrenal chromaffin cells, *Biochem. Biophys. Res. Commun. 190*, 181-185.

25. Baker, K. M., Chernin, M. I., Wixson, S. K., and Aceto, J. F. (1990) Renin-angiotensin system involvement in pressure-overload cardiac hypertrophy in rats, *Am. J. Physiol. 259*, H324-H332.

26. Brilla, C. G., Janicki, J. S., and Weber, K. T. (1991) Cardioreparative effects of lisinopril in rats with genetic hypertension and left ventricular hypertrophy, *Circulation 83*, 1771-1779.

27. Chen, L., Hahn, H., Wu, G., Chen, C. H., Liron, T., Schechtman, D., Cavallaro, G., Banci, L., Guo, Y., Bolli, R., Dorn, G. W., and Mochly-Rosen, D. (2001) Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC, *Proc. Natl. Acad. Sci. U. S. A 98*, 11114-11119.

28. Hahn, H. S., Yussman, M. G., Toyokawa, T., Marreez, Y., Barrett, T. J., Hilty, K. C., Osinska, H., Robbins, J., and Dorn, G. W. (2002) Ischemic protection and myofibrillar cardiomyopathy: dose-dependent effects of in vivo deltaPKC inhibition, *Circ. Res. 91*, 741-748.

29. Mochly-Rosen, D., Wu, G., Hahn, H., Osinska, H., Liron, T., Lorenz, J. N., Yatani, A., Robbins, J., and Dorn, G. W. (2000) Cardiotrophic effects of protein kinase C epsilon: analysis by in vivo modulation of PKCepsilon translocation, *Circ. Res. 86*, 1173-1179.

30. Syed, F., Odley, A., Hahn, H. S., Brunskill, E. W., Lynch, R. A., Marreez, Y., Sanbe, A., Robbins, J., and Dorn, G. W. (2004) Physiological growth synergizes with pathological genes in experimental cardiomyopathy, *Circ. Res. 95*, 1200-1206.

31. Bowman, J. C., Steinberg, S. F., Jiang, T., Geenen, D. L., Fishman, G. I., and Buttrick, P. M. (1997) Expression of protein kinase C beta in the heart causes hypertrophy in adult mice and sudden death in neonates, *J. Clin. Invest. 100*, 2189-2195.

32. Roman, B. B., Geenen, D. L., Leitges, M., and Buttrick, P. M. (2001) PKC-beta is not necessary for cardiac hypertrophy, *Am. J. Physiol. Heart. Circ. Physiol 280*, H2264-H2270.

33. Bowling, N., Walsh, R. A., Song, G., Estridge, T., Sandusky, G. E., Fouts, R. L., Mintze, K., Pickard, T., Roden, R., Bristow, M. R., Sabbah, H. N., Mizrahi, J. L., Gromo, G., King, G. L., and Vlahos, C. J. (1999) Increased protein kinase C activity and expression of Ca2+-sensitive isoforms in the failing human heart, *Circulation 99*, 384-391.

34. Takeishi, Y., Ping, P., Bolli, R., Kirkpatrick, D. L., Hoit, B. D., and Walsh, R. A. (2000) Transgenic overexpression of constitutively active protein kinase C epsilon causes concentric cardiac hypertrophy, *Circ. Res. 86*, 1218-1223.

35. Wu, G., Toyokawa, T., Hahn, H., and Dorn, G. W. (2000) Epsilon protein kinase C in pathological myocardial hypertrophy. Analysis by combined transgenic

expression of translocation modifiers and Galphaq, *J. Biol. Chem 275*, 29927-29930.

36. Braz, J. C., Gregory, K., Pathak, A., Zhao, W., Sahin, B., Klevitsky, R., Kimball, T. F., Lorenz, J. N., Nairn, A. C., Liggett, S. B., Bodi, I., Wang, S., Schwartz, A., Lakatta, E. G., Paoli-Roach, A. A., Robbins, J., Hewett, T. E., Bibb, J. A., Westfall, M. V., Kranias, E. G., and Molkentin, J. D. (2004) PKC-alpha regulates cardiac contractility and propensity toward heart failure, *Nat. Med. 10*, 248-254.

37. Braz, J. C., Bueno, O. F., De Windt, L. J., and Molkentin, J. D. (2002) PKC alpha regulates the hypertrophic growth of cardiomyocytes through extracellular signal-regulated kinase1/2 (ERK1/2), *J. Cell. Biol. 56*, 905-919.

38. Adams, J. W., Sakata, Y., Davis, M. G., Sah, V. P., Wang, Y., Liggett, S. B., Chien, K. R., Brown, J. H., and Dorn, G. W. (1998) Enhanced Galphaq signaling: a common pathway mediates cardiac hypertrophy and apoptotic heart failure, *Proc. Natl. Acad. Sci. U. S. A 95*, 10140-10145.

39. Heidkamp, M. C., Bayer, A. L., Martin, J. L., and Samarel, A. M. (2001) Differential activation of mitogen-activated protein kinase cascades and apoptosis by protein kinase C epsilon and delta in neonatal rat ventricular myocytes, *Circ. Res. 89*, 882-890.

40. Goni, F. M. and Alonso, A. (1999) Structure and functional properties of diacylglycerols in membranes, *Prog. Lipid Res. 38*, 1-48.

41. Novak, F., Tvrzicka, E., Hamplova, B., Kolar, F., and Novakova, O. (2006) Postnatal development of phospholipids and their fatty acid profile in rat heart, *Mol. Cell. Biochem. 293*, 23-33.