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A study of beta-adrenergic myocardial signaling in spontaneously hypertensive rat of transgenic strain SHR-Tg19

Studium beta-adrenergní signalizace v myokardu spontánně hypertenzního potkana transgenního kmene SHR-Tg19

Master of Science Thesis

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Declaration of own work

I declare that this thesis is my own work and all the sources have been quoted and acknowledged by means of complete references.

Prague 27.8.2012 ……

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Abstract

β-Adrenergic signaling plays an important role in regulation of heart function by modulating cardiac frequency and contractility. It may also have a certain role in the development of hypertension and heart hypertrophy. The spontaneously hypertensive rat (SHR) strain is a common model for human essential hypertension, although the origin of blood pressure abnormalities in SHR remains unknown. Dysfunction in the regulation of fatty acid translocase Cd36 was suggested as a link to development of hypertension in SHR. Transgenic strain SHR-Tg19 (also known as SHR-Cd36) was obtained by insertion of a functional wild-type FAT/Cd36 into SHR.

This thesis aimed to investigate key elements of β-adrenergic signaling in the heart of SHR-Tg19 and in the corresponding SHR controls. Expression and distribution of β_1- and β_2-adrenergic receptors (ARs) were measured using radioligand binding and Western blot analysis. In parallel, expression of selected G proteins was accessed and activity of adenylyl cyclase (AC) determined. There were no significant changes in the $G_s\alpha$ and $G_i\alpha$ subunits expression in both the left and right ventricles. Radioligand binding revealed an increase in the quantity of β-ARs, particularly in the β_2 subtype. Adenylyl cyclase activity stimulated with different ligands was significantly higher in SHR-Tg19 compared to SHR controls, and the AC5/6 expression also showed an increase in the left ventricles of transgenic rats. However, no changes in enzyme expression and activity were observed in the right ventricles.

Key words: SHR rats; Cd36; heart; β-adrenergic receptors; adenylyl cyclase

Abstract

β-Adrenergní signalizace hraje důležitou úlohu v regulaci funkce srdci, protože moduluje srdeční frekvenci a kontraktilitu. Může mít také určitou roli v rozvoji hypertenze a srdeční hypertrofie. Kmen spontánně hypertenzního potkana (SHR) je často používaným modelem pro studium lidské esenciální hypertenze, i když její vznik u SHR zůstává zatím neznámý. Dysfunkce v oblasti regulace translokázy mastných kyselin Cd36 byla předpokládaná jako jedna z příčin rozvoje hypertenze u SHR. Transgenní kmen SHR-Tg19 (nazývaný také SHR-Cd36), který má divoký typ genu FAT/Cd36, byl připraven pro studium hypertenze u SHR.

Tato práce je zaměřena na zkoumání β-adrenergní signalizace v srdci spontánně hypertenzního potkana transgenniho kmene SHR-Tg19 v porovnání s SHR. Exprese a distribuce β_1- a β_2-adrenergních receptorů (AR) byla měřena s použitím radioaktivně značených ligandů a Western blotu, pomocí kterého také byla také zjišťována exprese G proteinů a adenylylcyklázy (AC). Také byly pozorovány změny aktivity adenylyl cyklázy pod vlivem různých stimulačních ligandů. Výsledky ukázaly vzestup v expresi β-AR, který byl dán zejména zvýšeným množstvím β_2-AR. Nebyly nalezeny žádné změny v expresi podjednotek Gsα a Giα.. Aktivita AC stimulována různými ligandy byla vyšší v levých komorách SHR-Tg19 než SHR, ale žádné rozdíly nebyly zjištěny v pravých komorách. Současně byla u SHR-Tg19 v levých komorách zvýšena exprese enzymu AC5/6, zatímco v pravých komorách k takové změně nedošlo.

Klíčová slova: SHR potkan; Cd36; srdce; β-adrenergní receptory; adenylylcykláza

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List of Abbreviations

AC	adenylyl cyclase
AR	adrenergic receptors
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
DAG	diacylglycerol
EC	intracellular
eEF-1	eukaryotic elongation factor
FAT	fatty amino acid
GDP	guanosine diphosphate
GEF	guanosine nucleotide exchange factor
GIRK	G protein-coupled inwardly-rectifying potassium channel
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinases
GSKβ	glycogen synthase kinase β
GTP	guanosine triphosphate
IC	extracellular

IP3	inositol-1,4,5-trisphosphate
LV	left ventricle
PI3K	phosphatidylinositol 3-kinases
PIP2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA	phospholipase A
PLC	phospholipase C
PLN	phospholamban
RNA	ribonucleic acid
RV	right ventricle
SHR	spontaneously hypertensive rat
SR	sarcoplasmic reticulum
TM	transmemebrane

1. Introduction

Beta adrenergic receptors (β-ARs) are among the most widely studied cell surface signaling proteins. They mediate the effects of catecholamines in many target tissues. Three subtypes of β-ARs have been found and all of them are expressed in heart, where they increase cardiac frequency and contractility via adenylyl cyclase (AC) stimulation. The β-adrenergic signaling plays a significant role in treatment of heart failure with beta blockers such as propranolol and metoprolol that help to reduce risk of death and lighten the condition. The one of the common causes of the heart failure is arterial hypertension or high blood pressure.

The spontaneously hypertensive rat is the most widely used model of human primary hypertension. One of the causes that is linked to development of hypertension is a deletion in Cd36 gene that encodes a fatty acid transporter. Pravenec et al. created transgenic strain SHR-Tg19, which harbors a functional wild-type Cd36. The insertions of the Cd36 into SHR reduced the hypertension. On the other hand Neckar et al. (2012) found that the SHR-Tg19 transgenic strain had higher arrhythmogenesis when compared to the SHR.

The goal of this work is to compare the important parameters of β-adrenergic signaling pathway in the SHR and SHR-Tg19 strains, such as expression of β_1- and β_2-ARs and adenylyl cyclase expression and activity. This study aims to provide new insights on the transgenic impact on the SHR myocardial processes, specifically β-adrenergic signaling pathway.

2. Literature review

2.1 G protein-coupled receptors

One of the largest groups of cell-surface receptors is the guanine nucleotide-binding protein-coupled receptors (GPCRs) that activate trimeric G proteins. GPCRs are found in all eukaryotic cells and represent the richest source of targets for pharmaceutical industry (Rockman et al., 2002). Detailed analyses have shown that human genome contains over 800 unique GPCRs, 460 of which are olfactory receptors. Based on sequence similarity within the seven transmemebrane (TM) segments, these receptors can be classified into 5 families: the rhodopsin family, the adhesion family, the frizzled/taste family, the glutamate family, and the secretin family (Fredriksson et al., 2003). There is also a large fraction of GPCRs, of which their physiologic function is unknown. These receptors are referred to as orphan GPCRs. However their actual number declines as they are studied (Howard et al., 2001). The rhodopsin-like GPCR family contains the largest number of members (at least 19 subfamilies), including neurotransmitters, hormones and light receptors. All named types of receptors transduce extracellular signals through interaction with G proteins. The adrenergic receptor (AR) subfamily, which mediates the functional effects of epinephrine and norepinephrine, also belongs to rhodopsin family (Warne et al., 2008). The other significant family of GPCRs, the secretin-like receptors contains 34 subfamilies, including receptors for peptide hormones, such as parathyroid hormone, parathyroid hormone-related protein, and calcitonin. This family also contains the vast majority of the orphan GPCRs. The glutamate receptor-like family is comprised of 8 subfamilies that include the metabotropic glutamate receptors, extracellular Ca^{2+}-sensing, gustatory, odorant, and pheromone receptors (Fredriksson et al., 2003). The main focus of this study is the members of adrenergic receptor subfamily expressed in the heart.

The common structural signature of the GPCRs are the seven transmembranic hydrophobic α-helixes (7 TMs), which are connected by three extracellular loops (EL1–3) and three intracellular loops (IL1–3, see fig. 1). The extracellular (EC) amino terminus, responsible for the ligand binding, differs from relatively short and often unstructured sequences as in the rhodopsin-like and bitter taste receptors to large globular EC domains as in metabotropic glutamate GPCRs (Rosenbaum et al., 2009). The EC loops are also rich with disulfide bonds designed to stabilize the receptor structure. The intracellular (IC) domains interact with the G proteins, arrestins and other downstream effectors. The IC domains also include helix VIII and a C-terminus sequence that often carries palmitoylation and other signal sites. For example, a specific cholesterol binding site has been observed in β_2-AR, where cholesterol modulates receptor thermostability and affinity for inverse agonists (Hanson et al., 2008). The opposite placing of termini is given by odd numbers of transmembranic segments and allows glycosylation and ligand binding at the amino terminal segment, and phosphorylation and palmitoylation at the carboxyl terminal segment for desensitization and internalization. The number of TMs is optimal to provide necessary contact sites for G protein and other signal molecules and for a stable yet flexible TM core (Tae et al., 1998). The members of the AR subfamily share 51% sequence homology within themselves, excluding the amino and carboxyl termini and most of cytoplasmic loop. The greatest diversity is observed in the amino terminus, while the greatest homology - within the transmembranic segments (Kobilka et al., 2007; Warne et al., 2008).

All trimeric G-proteins, regardless of whether they are coupled to receptors or not, are composed of three subunits: α, β and γ. Gα and Gβγ subunits regulate the activity of the G-protein, affecting the binding of GTP (Svoboda et al., 2004).

All GPCRs share three main functional features: they distinguish and bind the appropriate ligands; they activate specific G protein-effector systems; they dynamically regulate their functional response resulting in the attenuation of receptor-mediated effects (desensitization) (Rosenbaum et al., 2009).

Agonists ("first messengers"), such as hormones and neurotransmitters, bind to GPCRs from the extracellular side of the cell membrane, that leads to a short-lived increase (or decrease) in the concentration of certain low-molecular-weight intracellular signaling molecules ("second messengers") through interaction with the heterotrimeric G protein complex. These molecules include 3',5'-cyclic AMP (cAMP), 3',5'-cyclic GMP (cGMP), 1,2-diacylglycerol (DAG), and inositol 1,4,5-trisphosphate (IP3). Other important second messengers are Ca^{2+} and various phosphoinositides embedded in cellular membranes.

In spite of the mentioned above structural and functional similarity of GPCRs, their natural ligands show great structural diversity, ranging from a photon, to organic molecules such as peptides and proteins. The location of the ligand binding domains for many GPCRs has been determined (Tae et al., 1998). Generally, it may not depend on the size of a ligand, thus not every small organic agonist bind within TM segments (for example, glutamate, glycoprotein hormones, and Ca^{2+} all bind to relatively large amino terminal domains in their respective receptors), as well as proteins and peptide hormones that often bind to the amino terminus and extracellular sequences joining the TM domains (Kobilka, 2007).

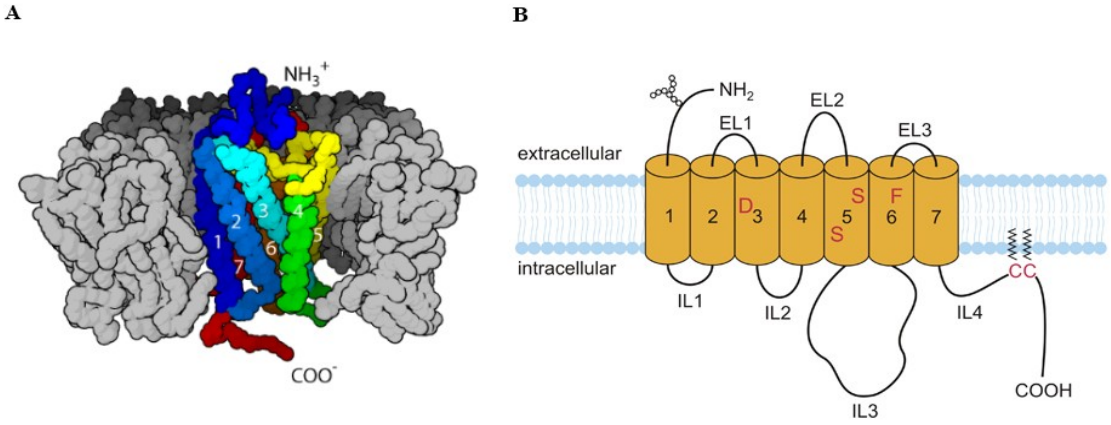

Figure 1: Three dimensional (A) and topographical (B) structure of GPCR. Cylinders numbered 1-7 represent transmembranic α-helixes, EL1-EL3 – extracellular, IL1-IL3 – intracellular loops. Ligand-binding responsible residues are indicated in red: an aspartic acid residue (D) in TM3, serine residues (S) in TM5, and a phenylalanine residue (F) in TM6. A fourth intracellular loop (IL4) is formed in case of posttranslational palmitoylation at cysteine residues (C) in the cytoplasmic tail (after Heller et al, 1993; Bockaert, 1999)

The ternary complex model is the most widely used to describe agonist activation of GPCRs. This model proposes that receptor exists in an equilibrium of active and inactive states. Furthermore, it suggests that principal interactions between the agonist, receptor and stimulatory G protein (G_s) protein forms a ternary complex, accounting possible effects of different classes of drugs (full agonists, partial agonists, neutral antagonists, and inverse agonists) on receptors signal transduction and proposes an equilibrium of active and inactive states of the receptor (De Lean et al., 1980; Gether and Kobilka, 1998). The conversion to the active state is a result of protonation of a highly conserved DRY motif at an intercellular side of TM3, causing the conformational change that ultimately leads to cytoplasmic exposure of buried sequences in the second and third intracellular loops and the diverse movements of TM3 and TM6. Other segments participate in the activation by releasing those sequences from "polar pocket", formed by residues in TMs 1, 2 and 7. Disruption the interaction between those α-helixes renders the receptor constitutively activated (Ballesteros et al., 2001).

Activation of the receptor leads to the changes in heterotrimeric G proteins, bound to the intracellular side of GPCRs. All GPCRs act as guanine nucleotide exchange factors (GEFs) as during the activation cycle they catalyze exchange of GDP bound to the α-subunit of the heterotrimeric G-proteins for GTP. In the inactive state the G_α subunit is bound to GDP and associates with the Gβγ dimer to form an inactive heterotrimer. Receptor activation leads to a decreased affinity of G_α for GDP, and GDP consequently dissociates and becomes replaced by GTP. After GTP is bound, Gα subunit assumes activated state and dissociates from both the receptor and the Gβγ dimer. This state persists until the GTP is hydrolyzed to GDP by the endogenous high-affinity GTPase located in the Gα subunit; this enzyme hydrolyzes the terminal γ-phosphate of Gα-GTP, forming the inactive Gα-GDP, that exhibit the high-affinity binding to the free Gβγ subunits. The Gα and Gβγ subunits reassociate, thus returning itself into initial state (Hamm and Gilchrist, 1996). The activation/deactivation cycle of trimeric G proteins is very rapid process ranging from milliseconds (transducin) to seconds among different G proteins families (Svoboda et al., 2004).

Desensitization (or adaptation) is a process of lowering the receptor-mediated signal transduction, which regulates cellular physiological responses. This might involve the receptor itself, the G protein associated with the receptor, and any of downstream effectors. The most common case of desensitization is impairment of the receptor's ability to activate its G protein, especially within minutes of agonist stimulation. This allows cells to largely diminish the receptor-mediated responses within seconds to minutes, involving phosphorylation of one or more intracellular domains of GPCRs. On a time scale of several hours after ligand binding the described desensitization is complemented by receptor down-regulation which involves the loss of membrane-associated receptor through a combination of protein degradation, transcriptional, and posttranscriptional mechanisms, such as destabilization of mRNA and decrease in receptor production. Receptors can be physically removed from cell membrane and transferred into the cell interior, resulting in their internalization (Svoboda et al., 2004). In this process the receptor separates from its effector molecule and transfers from the functional pool located at the cell surface to an inactive pool located inside the cell. Desensitization feedback mechanism involves phosphorylation of GPCRs by second-messenger-dependent kinases, such as cAMP activated protein kinase A PKA, and DAG activated PKC. Another cellular pathway of desensitizing GPCRs consists of a two-step process where the agonist-occupied receptors are phosphorylated by a G protein coupled receptor kinase (GRK) and then bind an arrestin protein, which prohibits signaling to the G protein. The GRK family includes six members, and the most thoroughly investigated are rhodopsin kinase (GRK1) and βARK1 (GRK2). This process is closely linked to internalization of GPCR, followed by its recycling (Lefkowitz, 1998).

Although the majority of GPCRs mediate signal transduction via G proteins, some of these receptors are also capable of sending signals via alternative signal molecules, e.g. Jak2 kinase, phospholipase Cγ, or PKC, which indicates presence of overall diversity in GPCR superfamily.

2.2 Myocardial adrenergic signaling pathways

Of all GPCRs, the ARs are particularly important for the heart because they function in the regulation of the cardiovascular system. Their stimulation with catecholamines mediates the "fight-or-flight" response to stress that includes many physiological functions involved in circulatory, metabolic, respiratory, and central nervous system homeostasis. ARs are divided into two subfamilies (three α_1-, three α_2- and three β-adrenergic) containing a total of 9 receptors (see Table 1). All adrenergic receptors have been cloned (Machida et al., 1990; Granneman et al, 1991; Liang et al., 1997). The most predominant subtype in the heart is β_1-AR, composing 75–80% of total β-ARs; the amount of α-ARs is significantly smaller, with a ratio of β- to α-ARs of about 70:30 in the atria and 80:20 in the ventricles (Brodde et al., 2006).

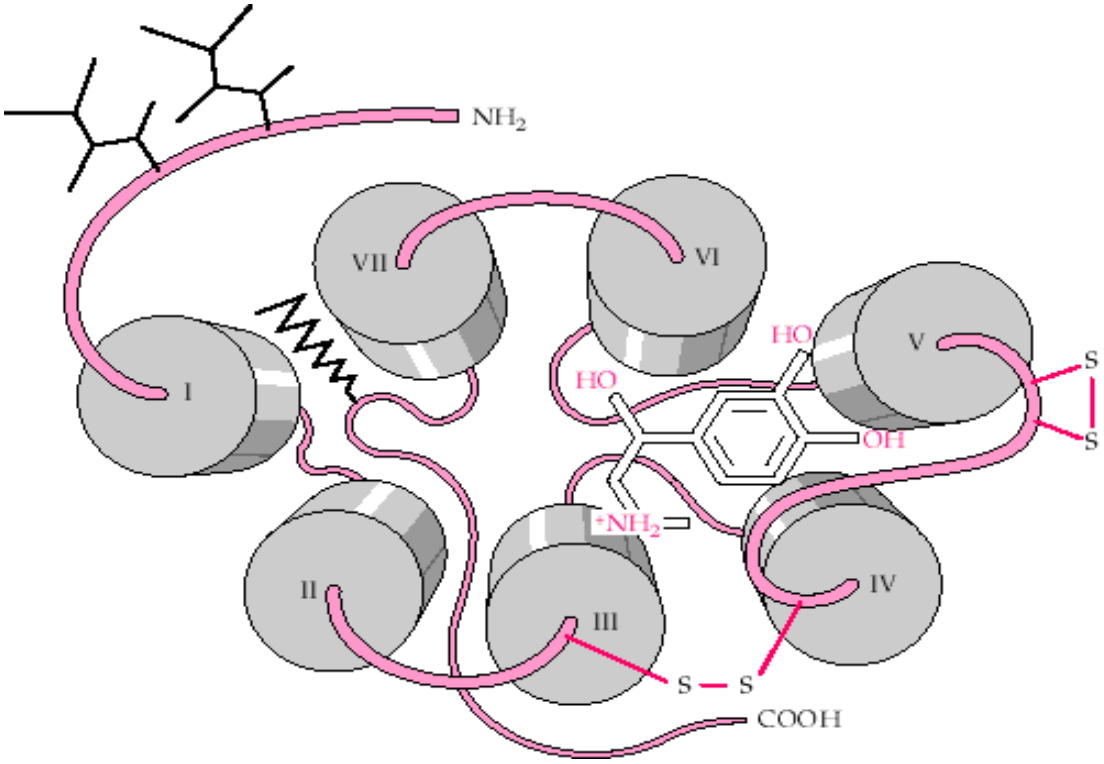

Figure 2: Proposed binding site for epinephrine in the β_2-adrenergic receptor. The binding site is thought to reside within the membrane, in a cleft formed by TM3-6. An aspartic acid residue in TM3 is binding the positively charged amine group of the transmitter, while serine residues in TM5 are forming hydrogen bonds with the hydroxyl groups on the phenyl ring, as was implicated by the site directed mutagenesis experiments (after Ostrowski et al., 1992).

Table 1: Characteristics of subtypes of adrenergic receptors (Epi – epinephrine, Iso – isoprenaline, NE - noradrenaline)

Receptor	Agonists	Antagonists	Tissue	Responses
α_1	Epi ≥ NE >> Iso Phenylephrine	Prazosin	Vascular smooth muscle	Contraction
			GU smooth muscle	Contraction
			Liver	Glycogenolysis; Gluconeogenesis
			Intestinal smooth muscle	Hyperpolarization and relaxation
			Heart	Increased contractile force; arrhythmias
α_2	Epi ≥ NE >> Iso Clonidine	Yohimbine	Pancreatic islets (β cells)	Decreased insulin secretion
			Platelets	Aggregation
			Nerve terminals	Decreased release of NE
			Vascular smooth muscle	Contraction
β_1	Iso > Epi = NE Dobutamine	Metoprolol CGP 20712A	Juxtaglomerular cells	Increased renin secretion
			Heart	Increased force and rate of contraction and AV nodal conduction velocity
β_2	Iso > Epi >> NE Terbutaline	ICI 118551	Smooth muscle (vascular, bronchial, GI, and GU)	Relaxation
			Skeletal muscle	Glycogenolysis; uptake of K+
			Liver	Glycogenolysis; gluconeogenesis
β_3	Iso = NE > Epi BRL 37344	ICI 118551 CGP 20712A	Adipose tissue	Lipolysis

Despite the β_1-AR prevalence in the heart, human β_2-adrenoceptor is one of the most studied GPCRs in rhodopsin family. It was first to be structurally determined by crystallography, cloned and characterized by radioligand binding. Those discoveries led to understanding of GPCR activation and signaling.

The ARs, particularly those of a particular type (e.g., α_1, α_2, β) share a high degree of amino acid homology, especially within the TM region forming the ligand binding pocket (68–77 % identity for α_1-, 79–82 % for α_2-, and 63–73 % for β-ARs). All ARs subtypes, except the $\alpha_{2B,}$ display up to two sites for N-glycosylation in the amino tterminal region. N-linked carbohydrates may account for as much as a quarter of the apparent weight of the adrenergic receptor proteins. Partial or complete disruption of glycosylation does not seem to alter ligand binding or signal transmission in any of examined GPCRs. Carbohydrates may play a role in receptor trafficking. Palmitoylation of Cys residues in carboxyl terminus and their insertion into the cytoplasmic membrane forms a fourth intracytoplasmic loop resulting in active conformation for G protein coupling. Absence of this fourth loop was associated with constitutively increased phosphorylation, rendering receptors accessible to regulatory mechanisms (Strosberg, 1993).

The binding site is formed by the seven receptor's TMs and is located approximately 11 Å below the extracellular surface. It contains at least four critical receptor\ligand moiety contacts (Tota and Strader, 1990).

Each receptor shows a preference for a particular G protein class. In particular, all three α_1-adrenoceptor subtypes couple to the $G_{q/11}$ signaling pathway, resulting in the generation of the second messengers IP_3 and DAG, the mobilization of intracellular Ca^{2+} and the activation of PKC. All three β-AR subtypes are G_s-coupled receptors. Stimulation of these receptors activates the G_s protein, which is responsible for AC activation and cAMP formation (Xiao et al., 1999). In the human heart cAMP is preferentially activated by β_1-adrenoceptor stimulation (Bristow et al., 1989). Subsequently, activation of cAMP dependent PKA leads to phosphorylation of regulatory proteins involved in cardiac EC coupling and energy metabolism, including L-type Ca^{2+} channels, sarcoplasmic reticulum membrane protein phospholamban

(PLB), myofilament proteins, and glycogen phosphorylase kinase, resulting in positive contractile response (see Fig. 3). PKA also activates an endogenous protein phosphatase inhibitor I by its phosphorylation, which inhibits protein phosphatases, ensuring the PKA-mediated protein phosphorylation. Stimulation of β-ARs modulates virtually all important components of the cardiac excitation-contraction (EC) coupling cascade and therefore plays a prominent role in the regulation of cardiac performance. Cardiac EC coupling is initiated by a Ca^{2+} influx through voltage-dependent sarcolemmal L-type Ca^{2+} channels during an action potential. This Ca^{2+} influx *per se* is insufficient to produce a contraction, but it triggers a large Ca^{2+} release from the sarcoplasmic reticulum via ryanodine receptors through a Ca^{2+}-induced Ca^{2+} release mechanism. The resultant intracellular Ca^{2+} transient activates proteins responsible for contraction. It is subsequently removed from the cytoplasm by the sarcoplasmic reticulum Ca^{2+}-ATPase and the sarcolemmal Na^{+}-Ca^{2+} exchanger (Xiao et al., 1999).

Although it has been shown in *in vitro* experiments that both β-ARs can mediate positive inotropic effects in atrial and ventricular preparations, in ventricles only β_1-adrenoceptor stimulation can evoke maximum positive inotropic effects, and β_2-adrenoceptors only submaximal positive inotropic effects (Bristow et al., 1989). Despite the dominant role of the Gs/AC/cAMP pathway in β-AR signaling, different subtypes of β-ARs are capable of coupling to other G proteins, thereby activating more than one intracellular signaling pathway. For example, persistent stimulation of β-AR subtypes may trigger a time-dependent switch of signaling pathways and elicit different functional roles in regulating cardiac structure and function. Specifically, prolonged β_2-AR activation switches the receptor G-protein coupling from G_s to G_i (pertussis-toxin sensitive pathway), resulting in activation of MAP kinase in a Src- and Ras-dependent pathway (Kilts et al., 2000). The time dependence of β_2-AR–G_i coupling could be attributed to the agonist-induced translocation of β_2-adrenoceptors out of caveolae.

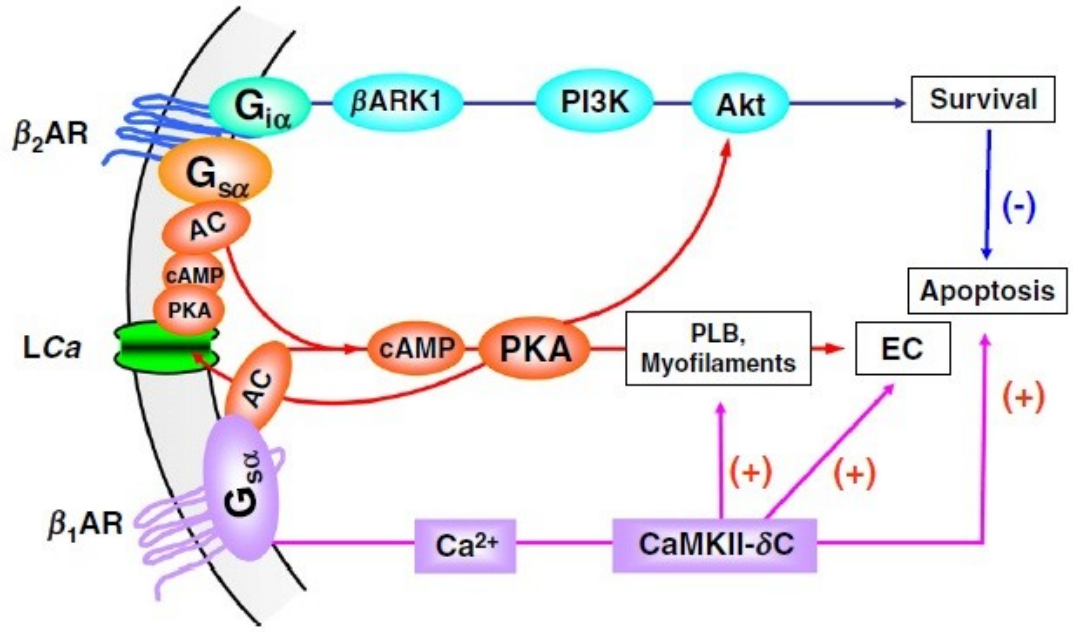

Figure 3: Subtype-specific G protein coupling and function of β_1- and β_2-AR in cardiac myocytes. β_2-AR has dual coupling to G_s and G_i, which activates cell survival signals by the G_s-cAMP-PKA-Akt pathway and the G_i-Gβγ-PI3K-Akt pathway respectively. In contrast, β_1-AR couples exclusively to G_s, which activates PKA-independent, CaMKII-mediated apoptotic signaling (after Talan et al., 2010) .

Persistent β_1-AR stimulation changes the receptor signaling pathway from PKA to Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) predominance, leading to myocyte apoptosis and maladaptive remodeling (Xiao et al., 1993).

The last type of β-adrenergic receptors, the β_3-AR is expressed primarily in adipose tissues and may be important in regulation of body weight. In human atrial myocytes β_3-AR is coupled to stimulatory G proteins, and stimulates L-type calcium current. However, some controversial data show that in human ventricle β_3-ARs reduce the cardiac contractility through coupling to G_i and inhibition of AC (Gauthier et al., 1996). β_3-ARs also stimulate the production of nitric oxide (NO) through the activation of endothelial constitutive NO synthase (NOS) in human ventricular myocytes. In heart, NO is able to reduce contractile force, stimulated via the cAMP pathway through inhibition of PDE3 and/or activation of PDE2 (Skeberdis, 2004).

It was shown by Yi-Tang Tseng et al. (2003) that the tonic activation of β-AR signaling plays a critical role in cardiomyocyte proliferation during early postnatal development.

All three subtypes of α_1-ARs (α_{1A}, α_{1B} and α_{1D}) are expressed in the heart. Activation of these receptors results in positive inotropic effect.The binding studies in the rat heart have revealed an α_{1A}:α_{1B}-AR ratio of 20:80 (Pönicke et al., 2001). α_{1B}-AR is the α-AR, mainly involved in the regulation of contractile function and cardiac growth.. The mediated effect of this receptor is largely depending on the developmental stage of the organism. In neonatal cardiomyocytes stimulation of α_{1B}-AR leads to positive chronotropy, while in adult it has a negative effect on both chronotropy and inotropy. Extended stimulation of cardiac α_1-ARs can also cause the development of a hypertrophic phenotype (Barki-Harrington et al., 2004). α_{1B}-AR is coupled with G_q protein, which stimulates the formation of IP_3 from PIP_2 through PLC activation. Increased IP_3 stimulates Ca^{2+} release by the sarcoplasmic reticulum, thereby increasing inotropy as one of its actions. It was however shown that in adult cardiomyocytes α_{1B}-AR is bound with G_i thus creating a negative inotropic effect (Han et al., 1989). A transgenic strain TG α43 was obtained by Akhter et al (1997) harboring an overexpression of wild type α_{1B}-AR. Their data shows increased PLC activity together with damping of basal and agonist-stimulated AC activity, which can be reversed by pertussis toxin. Additionally, α-ARs couple to multiple intracellular calcium mobilization pathways via voltage-dependent and independent calcium channels (Minneman, 1988). Although the α_2-AR type is not present in heart, it plays a key role in regulating neurotransmitter release in the central and peripheral sympathetic nervous systems, affecting the blood pressure. Its deletion increases sympathetic activity, leading to a hypertensive, hyperadrenergic state (Lerman et al., 2005).

2.3 Spontaneous hypertensive rats

Hypertension is a polygenic disease that involves complex interactions between genetic and environmental factors. The latter include obesity and increased dietary sodium intake. Although it is thoroughly studied, the event sequence leading from genetic predisposition to hypertension is still not defined, as there are no positionally cloned genes identified to date in either rodent models or the human hypertension phenotype (Mein et al., 2004). Animal models used to study various factors associated with development of hypertension and according cellular responses. Models are divided into two groups, based on the approach in inducing the changes of blood pressure. The phenotype-driven approach is taking advantage of selective breeding of animals (primarily rats) that exhibit a desired phenotype. Genotype-driven models include transgenic techniques, in which mice are the most successful for selective deletion or overexpression of target genes (Lerman et al., 2005).

Among other animal models, the spontaneously hypertensive rat (SHR/NIH strain) is one of most widely used for study of essential hypertension. The SHR strain was obtained by Okamoto and Aoki (1963) by continuous breeding Wistar-Kyoto (WKY) rats with high blood pressure and signs of spontaneous hypertension, thus it belongs to a phenotype-driven genetic models. The WKY strain is considered a control for the SHR. The SHR is not a strictly inbred strain as it mimics a subtype of human primary hypertension that is inherited in a Mendelian fashion. The SHR blood pressure rises around 5-6 weeks of age, reaching and exceeding systolic pressure of 200 mmHg (Okamoto and Aoki, 1963). The SHR develop strict features of hypertensive end-organ damage such as cardiac hypertrophy, cardiac failure and renal dysfunction. They are also known to have left ventricle hypertrophy and impaired endothelium dependent relaxations (Pinto et al., 1998). Concomitant to that, SHR also spontaneously develop marked islet hyperplasia and hyperglycemia. The presence of abnormal hypothalamic pituitary-adrenal activity in SHR mediates those pathogenic effects (Wexler et al., 1976).

Erythrocytes of SHR possess increased Na^+ unidirectional flux. De Mendonca et al. (1980) suggested that these abnormalities of Na^+ flux are genetically associated

with hypertension. Human essential hypertension has been associated with decreased serum ionized calcium and increased serum parathormone levels. SHR has been reported to have several abnormalities in calcium metabolism, including reduced serum ionized calcium levels, increased serum parathormone levels along with hypercalciuria, and abnormalities in intestinal calcium transport and vitamin D metabolism. Elevated intracellular calcium may lead to functional changes in vascular smooth muscle cell resulting in increased peripheral resistance with the development of hypertension (Shibata and Ghishan, 1990).

SHR exhibit renal vascular wall thickening that could be a primary alteration to the hypertension development, as was suggested by morphometric studies of renal vasculature of SHR. These studies have indicated that the increase of cross-sectional area of the renal blood vessels in SHR compared to WKY before the hypertension development (Nordborg et al., 1983). However, the blood pressure in SHR is elevated at birth without a prehypertensive phase. Treating SHR with hydralazine in utero and onward from birth did lower the blood pressure, but wasn't able to prevent vascular wall changes, suggesting that vascular wall thickening in the renal vasculature of SHR over WKY is not dependent on the elevation of blood pressure (Smeda et. al., 1988). It was show that treatment with hydralazine had no effect on ventricular weight of SHR, unlike a-methyldopa. Methyldopa and hydralazine were equally successful in controlling the hypertension in the older rats or in preventing its development in the younger spontaneously hypertensive rats. Same study has shown significant increase in ventricular weight in very young SHR, and the degree of hypertrophy did not increase as the difference in blood pressure between normal rats and SHR became more pronounced (Sen et. al., 1974).

Both neural and vascular alterations contribute to the genetic mechanisms of hypertension in SHR. Variations in the genetic background of the SHR may have an effect on evolution of hypertension and end-organ damage in this model, but at least three major genes are known to be involved in early development of hypertension, whereas an additional gene identified on chromosome 10 contributes to development and maintenance of hypertension during aging in SHR (Lerman et al., 2005). The

hypertension and age-related renal injury in SHR can be modulated by androgens, as they alter pressure natriuresis and tubulo-glomerular feedback in a manner that potentially resembles human hypertension (Reckelhoff et al., 1999).

The SHR strain became a useful model for exploring the relationship between metabolic and hemodynamic dysregulation, since it has increased fasting levels of insulin consistent with metabolic insulin resistance. It ultimately leads to hyperinsulinemia, which increases sympathetic activity and serves the cause of hypertension. Along with decreased high-density lipoprotein level it results in heightening of the cardiovascular risk. This suggests that insulin resistance and compensatory hyperinsulinemia are primary events, and enhanced sympathetic activity and diminished adrenal medullary activity are important links between the defect in insulin action and the development of hypertension and the associated metabolic abnormalities (Epstein, 1996). SHR harbors both low insulin-stimulated glucose transport and decreased adipocyte glucose uptake (Reaven et. al., 1989).

After the establishment of hypertension SHR shows the reduced chronotropic response, which is developed in the course of a high blood pressure state, but is not the inherent cause of hypertension (Masuda and Matsuoka, 1997). Inotropic response to β-adrenergic stimulation of the myocardium is decreased in hypertension. A biochemical basis for this decrease was provided by the observation that the number of β-adrenergicreceptors — as reflected in specific [3H]dihydroalprenolol binding — was diminished in the myocardium of spontaneously hypertensive rats without a change in the affinity of dihydroalprenolol for the binding sites or in the capacity of isoproterenol to displace dihydroalprenolol.

β-Adrenergic stimulation of the myocardium in SHR appears to be decreased. It was observed using specific [^{3}H]dihydroalprenolol binding by Limas et al. (1978). They also state that the decline in the amount of β-AR is not secondary to hypertension and may be related to increased sympathetic drive in SHR. It was shown that SHR have lower isoprenaline-stimulated adenylyl cyclase activity and contractile responses to β-agonists. A functional impairment in β-adrenergic-mediated responses is apparent in cultured single cell vascular systems in SHR. It was suggested that in a

hypertensive state, the defect in β-AR coupling to AC activation indicates a generalized impairment in signaling via both short-term vasoregulatory pathways as well as longer term pathways mediating the effects on vascular smooth muscle cell growth and development (Gros et al., 2005). Administration of various β-blockers resulted in the increase of the number of β-AR binding sites in the ventricular myocardium in SHR. However, no increase was found in the steady state mRNA level of β-ARs suggesting that the level of β-AR protein was regulated post-transcriptionally (Masahiko et al., 1998).

Myocardial hypertension is often accompanied by a state of over-stimulation of the sympathetic nervous system and it has been stated that there is dysfunction of a central adrenergic signaling mechanism in SHR that leads to an increase in noradrenaline release and subsequent blood pressure increase. The development of cardiac hypertrophy is influenced by a variety of interacting genetic, haemodynamic, neurohumoral, trophic and dietary factors. Studies indicate that the inotropic responsiveness to catecholamines is diminished in the hypertrophied myocardium (Zucker et al., 1980; Hein et al., 1979).

Experiments with the use of cDNA microarrays with characterization of a congenic strain and RH mapping led to the identification of a defective Cd36 gene in the SHR. It was also shown that overexpression of Cd36 in transgenic mice reduces blood triglycerides and FA, suggesting that Cd36 deficiency underlies insulin resistance, defective fatty acid metabolism and hypertriglyceridemia in SHR (Aitman et al., 1999). Cd36, sometimes referred as FAT/CD36, is a membrane scavenger receptor that facilitates long chain fatty acid (FA) uptake by different tissue e.g. muscle. A response to insulin and contraction was shown to be an increase in expression of membrane Cd36 and FA uptake in heart. FA is one of the main energy sources used by oxidative muscle and heart and Cd36 deficiency, leading to its reduced transport, has shown to predispose the SHR with hypertrophy (Hajiri et al. 2002). Transgenic mice that overexpress Cd36 in heart and muscle exhibit increased FA utilization and glucose reserving. Deficiency in Cd36 is associated with a defect in

FA uptake that is most pronounced in the heart and that is compensated for by increased glucose utilization (Klevstig et al., 2011).

Transgenic rescue of Cd36 in SHR ameliorates insulin resistance and improves dyslipidemia (Pravenec et al., 2001). However, transgenic expression of wild type Cd36 on the SHR background showed little or no effect on blood pressure regulation. Only one transgenic line to exhibit a modest decrease in blood pressure was SHR-Tg19 due to the marked overexpression of the Cd36 transgene observed in the kidney. This strain was obtained by rescuing the SHR that harbored a deletion variant of Cd36 transporter by transgenic expression of wild-type Cd36 under the control of the EF-1α promoter. The resulting founder line SHR/Ola-TgN(EF1aCd36)19Ipcv (abbreviated as SHR-Tg19, also known as SHR-Cd36) harbored a single copy of the transgene (Pravenec et al., 1999). Although this transgenic expression of wild type Cd36 improved insulin resistance and defective fatty acid metabolism, the blood pressure remained unaffected, therefore leading to the conclusion that Cd36 deficiency itself is no major determinant of hypertension in the SHR (Pravenec et al., 2003).

3. Aims of the thesis

1. To investigate the expression of major components of the β-adrenergic signaling cascade (β_1- and β_2-ARs, trimeric G proteins, AC5/6) in the heart of SHR and SHR-Tg19 rat strains.

2. To determine the activity of adenylyl cyclase in the heart of SHR and SHR-Tg19 strains.

4. Materials and methods

4.1 Animals

All procedures were performed in conformity with the Animal Protection Law of the Czech Republic (311/1997) and were approved by the Ethics Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic. Two groups of animals were used to conduct the experiments: SHR/Ola and transgenic SHR/Ola-TgN(EF1aCd36)19Ipcv (also referred as SHR-Tg19) line harboring the wild type of Cd36 transgene. Both groups contained five 24 week old males in average weight of 350 g. Rats were kept on stock chow in a constant 12 hours light/dark cycle.

After animals were sacrificed by cervical dislocation the hearts were quickly disserted into the left ventricle, right ventricle, and septum. Each part was weighted and momentary frozen in liquid nitrogen and stored at -80°C until use.

4.2 Homogenization and fractionation of myocardium

Solutions used:

Homogenization buffer TMES (pH 7.4): 20 mM Tris, 3 mM $MgCl_2$, 1 mM EDTA, 250 mM saccharose

Buffer TME (pH 7.4): 20 mM Tris, 3 mM $MgCl_2$, 1 mM EDTA

Tissue samples of left or right ventricles were immersed in 12 ml TMES buffer with 300 µl of Complete Protease Inhibitor Cocktail (Roche) and roughly sliced. The samples were homogenized on an Ultra-Turrax blending device at 24000 rpm for 15 seconds and passed 10 times through a glass-Teflon homogenizer at 1200 rpm. Afterwards the suspension was centrifuged on Hettich Universal R30 centrifuge at 600 g (2100 rpm) for 10 minutes, 4°C. The pellet was ressuspended in 12 ml TMES

buffer and 300 µl Complete Protease Inhibitor Cocktail and then processed 10 times through a glass-Teflon homogenizer (1200 rpm) and once again centrifuged at 600 g (2 100 rpm) for 10 minutes. The two supernatants were mixed together and centrifuged at 50 000 g (2 7000 rpm) for 30 minutes, 4°C, at maximum acceleration and slowdown, using Ti 50.2 Beckman rotor. Pellet fraction (crude membranes) was resuspended in 7 ml TME buffer. Supernatant fraction was centrifuged at 300 000 g for an hour, 4°C using MLA-80 rotor (ultracentrifuge Beckman Optima). Pellet from second centrifugation was resuspended in 20 ml TME and sonicated two times for 10 seconds. All obtained fractions were snap-frozen in liquid nitrogen and stored at –80°C.

4.3 Protein assay

Protein concentration of individual samples was measured with Bicinchoninic acid protein assay (BCA) method.

Solutions used:

Standard BSA (beef serum albumin) c = 1 µg/µl and c = 0.1 µg/µl

Reagent A (pH 11.25): 8 mg of sodium carbonate monohydrate, 1.6 mg sodium tartrate add. 100 ml

Reagent B: 4 mg BCA (Bicinchoninic acid) diluted in 100 ml H2O

Reagent C: 0.4 mg copper sulphate pentahydrate diluted in 10 ml H2O

Working solution: 1 part of reagent C mixed with 25 parts of reagent B and with 26 parts of reagent A

Array of BSA standards was prepared on microtiter plate, containing 0.2 – 50 µg of protein in 100 µl.

Samples were diluted in water in proportion of 1:1000 and were pipetted to the plate in triplicates. 100 µl of working solution was added to all wells, standard and

sample, and the microtiter plate was incubated for 30 minutes at 60 °C. Then the plate was read at 562 nm on Synergy plate multireader. The results were processed by Gen 5 program.

4.4 Adenylyl cyclase enzymatic activity assay

Enzymatic activity of adenylyl cyclase was observed on cardiomyocital crude membranes from SHR-Tg19 strain and SHR controls. Ligands used to examine stimulated activity are listed below.

Solutions used:

Stimulator ligands:

$1 \cdot 10^{-5}$ M Forskolin

$1 \cdot 10^{-5}$ M $MnCl_2$

$1 \cdot 10^{-5}$ M GTPγS

$1 \cdot 10^{-5}$ M NaF

$1 \cdot 10^{-5}$ M Isoprenaline

Reaction mixture:

240 mM Tris-HCl (pH 8,0)

2 mM $MgCl_2$

20 μM GTP

0.8 mg/ml BSA

40 μM isobutylmethylxantin

5 mM fosfoenolpyruvate kalium salt

3.2 units of pyruvate kinase

100 mM NaCl.

All crude membranes samples were diluted to 0.5 µg/µl. Samples for basal activity detection contained 40 µl reaction mixture, 20 µl tissue and 30 µl H_2O. Samples for stimulated activity detection were mixed in same way, except 10 µl H_2O was replaced by 10 µl of stimulating agent. Samples were incubated in a water bath at 30 °C for 1 min and the reaction was initiated by adding 10 µl 0.4 mM ATP. Samples were then incubated for 20 minutes in water bath at 30°C and the reaction was stopped by adding 200 µl 0.15 M HCl, after that samples were mixed and stored on ice. The level of produced cAMP was detected with Monoclonal Anti-cAMP Antibody Based Direct cAMP ELISA kit (NewEast Biosciences), spectrometric measurements were done on Synergy Microplate Reader and the adenylyl activity was calculated by using Gen 5 program.

4.5 Electrophoresis and Immunoblotting

The expressions of selected GPCRs, adenylyl cyclase and β-ARs were examined with the use of electrophoresis combined with Western immunoblotting. β-Actin was used as a loading control for all named proteins.

Solutions used

Laemmli sample buffer: 2.4 ml 1 M Tris-HCl 1 ml glycerol, 0.8 g sodium dodecyl sulfate, 0.8 g dithiothreitol and 1 mg bromphenol blue add H2O to 10 ml

TBS buffer (pH 8.0) 10x concentrated: 45 ml 4 M NaCl, 12 ml 1 M Tris-HCl (pH 8.0), 63 ml H_2O

Blocking buffer: TBS, 5% milk, 0.1% Tween-20

Diluting buffer: TBS, 1% milk, 0.1% Tween-20

Washing buffer: TBS, 0.3% Tween-20

Crude membrane samples, concentration 2 µg/µl, were diluted in Laemmli sample buffer in proportion of 3:1 and boiled at 100°C for 2 minutes. The proteins were electrophoretically separated by 10% polyacrylamide gel at 200 V for 1 hour,

and blotted to a nitrocellulose membrane at 100 V for 1 hour. The membrane was blocked in blocking buffer for 1 hour and then incubated in primary antibodies, diluted in proportion of 1:1 000-1:10 000 (depended on detected protein) in diluting buffer for more than 2 hours at 4 °C. Afterwards membrane was rinsed 3x10 minutes in washing buffer and incubated in secondary antibodies marked with horseradish peroxidase, diluted in diluting buffer in proportion of 1:40 000 for anti-rabbit or 1:80 000 for anti-goat. Then the membrane was again washed 3x10 minutes in washing buffer, and prepared for evocation by applying substrate for horseradish peroxidase (Pierce Super Signal) for 1 minute. Intensity of chemoluminescence was detected by exposing a medical film (Agfa Healthcare NV Medical X-Ray film) to a membrane and developing it on Optimax (Fomei) device. Results were evaluated in ImageQuant program.

4.6 Saturation and competition binding experiments

Quantity and affinity of β_1- and β_2-AR was measured with saturation experiments and β_1- to β_2-AR ratio with competition binding experiments.

Solutions used

Incubation medium (pH 7.4): 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ascorbic acid

Washing buffer (pH 7.4): 50 mM Tris-HCl, 10 mM MgCl2

[^{3}H]-CGP12177: radioactive labeled agonist of β_1- and β_2-AR, 50 Ci/mmol

ICI 118.551: 10^{-3} nM, diluted in water

0.3% Polyethyleneimine (PEI): PEI diluted to 0.3% in water

Saturation measurement was carried out in triplicates with six concentrations of the radioligand [^{3}H]-CGP12177 (3 nM, 1.5 nM, 0.75 nM, 0.375 nM, 0.1875 nM, 0.09375 nM). The reaction mix contained 100 µl of crude membrane samples (concentration 1 µg/µl), 100 µl of [^{3}H]-CGP12177 radioligand and 300 µl of

incubation medium. Nonspecific binding was measured by adding 10 nM L-propranolol to the reaction mix and was measured in duplets. Test-tubes with the reaction mix were incubated in water bath at 37 °C until equilibrium was reached (appr. 1 hour). The reaction was stopped by adding 3 ml of cooled washing buffer and the end product was filtrated through glass-fiber filters (Watman) which have been pretreated with cooled polyethylenimine (PEI). After that, filters were washed two times with 3 ml of washing buffer.

Competitive binding was carried out with [^{3}H]-CGP12177 radioligand (1.5 nM). The specific β_2-AR antagonist ICI 118.551 was used as competitor. Measurement was done in duplicates and consisted of sample set with increasing competitor concentrations, 10^{-10}…10^{-4} nM. Nonspecific binding was evaluated with use of 10 nM L-propranolol. Reaction mix was composed, incubated and filtrated same way as described above.

Captured radioactivity was measured with liquid scintillation spectrometry on Ray-Test device in 4 ml of scintillated cocktail (CytoScint) for 5 minutes each sample.

Results were evaluated using Excel and GraphPad Prizm version 5.00 programs.

4.7 Data analysis

All described experiments were conducted at least three times. Obtained results were statistically analyzed using Student's two-tailed unpaired t-test ($p<0.05$) to determine the presence of significant differences between groups. Data was expressed as mean ± standard deviation.

5. Results

5.1 Body and heart weights

Body and heart weight of SHR-Tg19 and SHR strains are summarized in Table 2. Both groups showed no statistically significant difference in their body (BW) and heart weights (HW), although average values of the left ventricle weight (LV) and HW in SHR-Tg19 was higher. Relative heart weight (HW/BW) was significantly higher in transgenic rats. The right ventricles (RV) weights remain unchanged in SHR-Tg19 as well as the relative RV (RV/BW) and LV weights (LV/BW).

Table 2: Body and heart weight parameters of SHR and SHR-Tg19 (* $p < 0.05$).

	Body weight, g	Heart weight, mg	LV, mg	RV, mg	HW/BW, mg/g	LV/BW, mg/g	RV/BW. mg/g
SHR	350 ± 6	1.14 ± 0.03	0.672 ± 0.024	0.208 ± 0.008	3.27 ± 0.046	1.92 ± 0.046	0.59 ± 0.017
SHR-Tg19	341 ± 5	1.19 ± 0.02	0.705 ± 0.022	0.211 ± 0.006	3.48 ± 0.04*	2.07 ± 0.05	0.62 ± 0.02

5.2 Basal and stimulated activity of adenylyl cyclase

Function of myocardial adrenergic signaling system largely depends on adenylyl cyclase (AC). The activity of AC was measured at basal level and under different stimulatory conditions (Forskolin, $MnCl_2$, GTPγS, NaF, and Isoprenaline). Figure 4 shows data obtained for the LV in SHR and SHR-Tg19.

Basal activity has not experienced a significant change in both left and right ventricles of transgenic rats. Direct stimulation with forskolin showed a significant increase of AC activity in the SHR-Tg19 LV, by 50 % in comparison to SHR controls. Activity stimulated with $MnCl_2$ was by 35 % significantly higher in the LV of SHR-Tg19. Under stimulation of GTPγS the activity of AC was increased significantly by

25 %. Significant increase by 20 % was also observed in the isoprenaline stimulated SHR-Tg19 LV. NaF-stimulated activity showed no significant change in SHR-Tg19 left ventricle.

Figure 5 shows the activity of AC in the RV. In SHR-Tg19 it has not changed significantly under the stimulation of forskolin, NaF and GTPγS. Stimulation with MnCl2 gave a 41 % increase in AC activity. Isoprenaline stimulation significantly decreased activity of AC by 30 %.

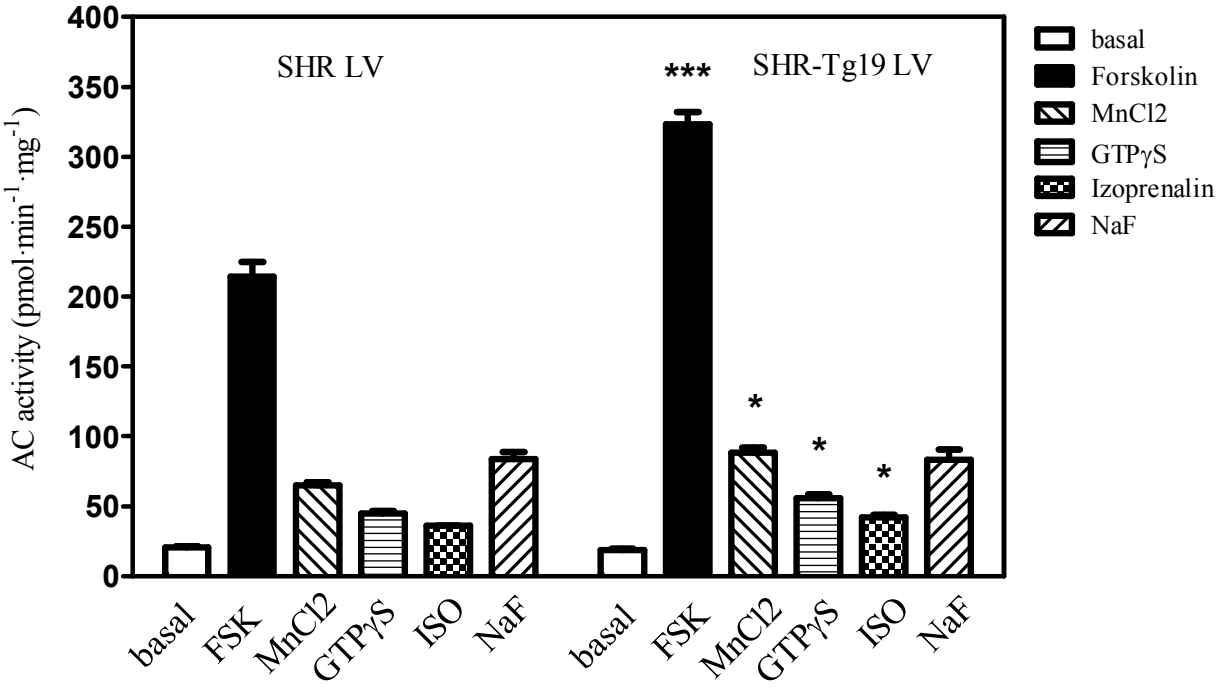

Figure 4: Basal and stimulated AC activity in the LV of the SHR and SHR-Tg19,
* $p < 0.05$ (FSK – forskolin, ISO – isoprenaline).

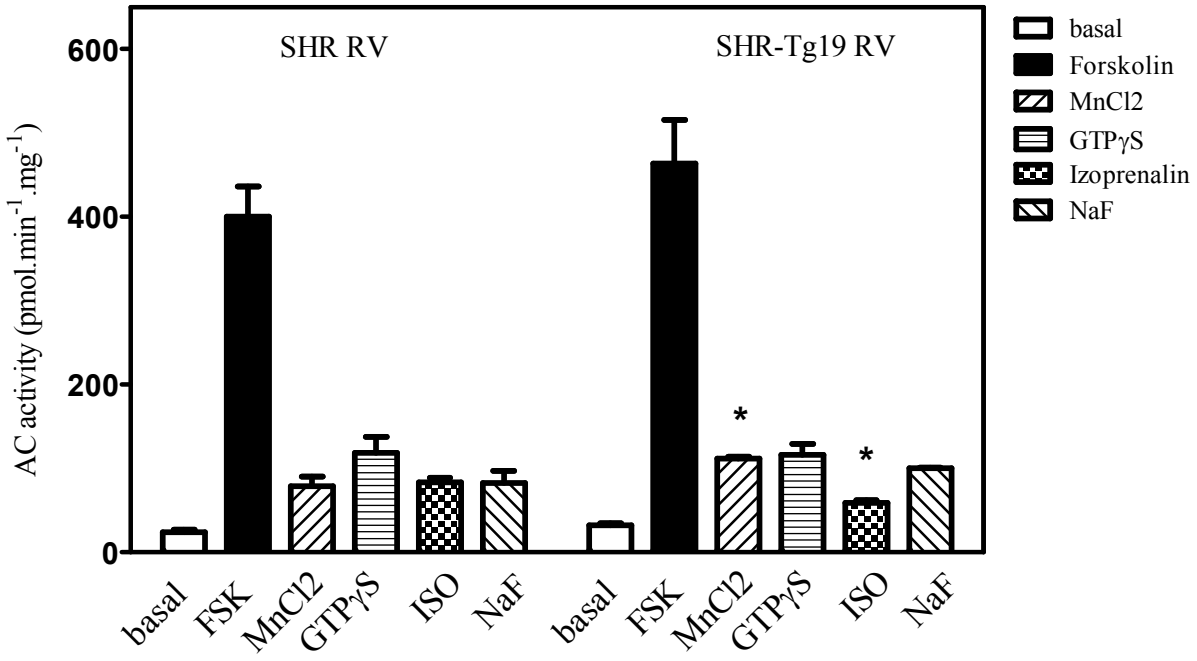

Figure 5: Basal and stimulated AC activity in the RV of the SHR and SHR-Tg19, * $p < 0.05$. (FSK – forskolin, ISO – isoprenaline)

5.3 Adenylyl cyclase expression

Expression of adenylyl cyclase was evaluated using Western blot analysis. Two AC isoforms predominant in heart were studied – AC5 and AC6. In SHR-Tg19 left ventricle was observed an increased amount of AC5/6 protein (Fig. 6A). Although no significant changes were found in the RV (Fig. 6B), the level of AC expression appears to be slightly lower in SHR-Tg19 than in SHR. The detection of AC5/6 was difficult to perform and it may be necessary to conduct additional studies in order to clarify these results.

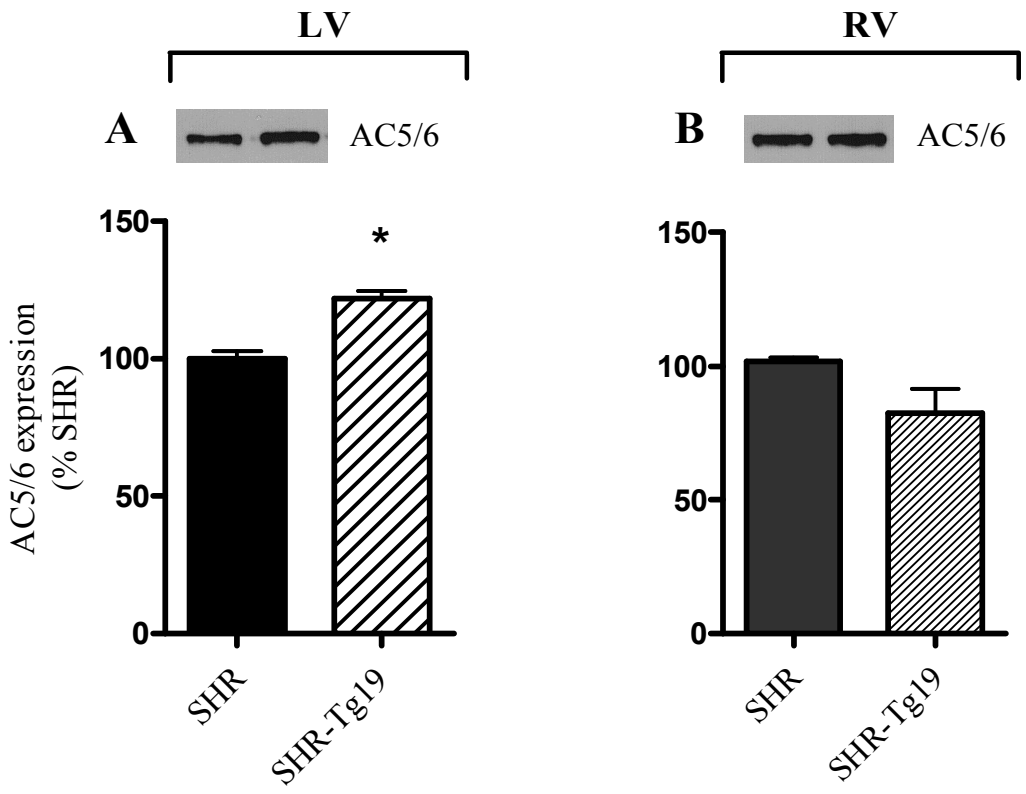

Figure 6: Expression of AC5/6 in the LV (A) and the RV (B) of SHR-Tg19 compared to SHR (100 %). Measured with SDS-PAGE and Western blot analysis using specific antibodies (A cyclase V/VI Antibody (C-17), sc-590). * $p < 0.05$

5.4 G protein expression

The expression of $G_s\alpha$, examined using SDS-PAGE electrophoresis and Western immunoblotting did not experienced any significant changes in both ventricles of SHR-Tg19, as shown in Figure 7.

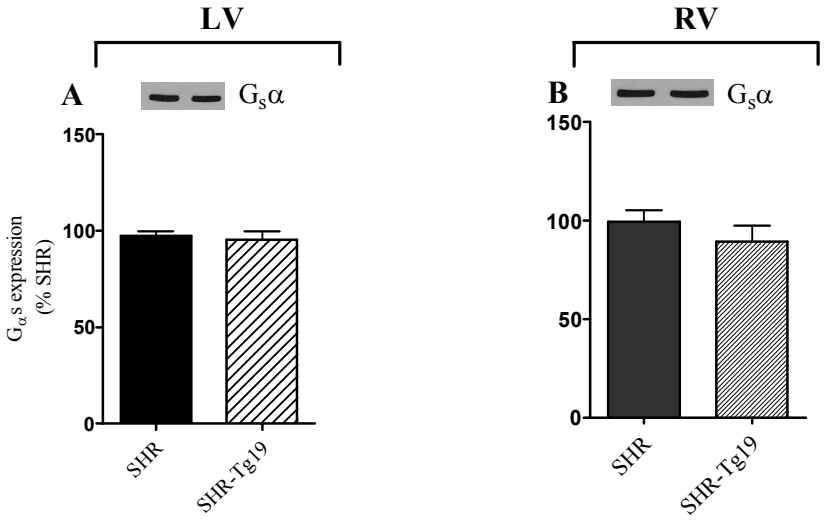

Figure 7: Expression of $G_s\alpha$ protein in the LV (A) and the RV (B) of SHR-Tg19 compared to SHR (100 %). Data obtained using SDS-PAGE and Western blot analysis with specific antibodies for $G_s\alpha$.

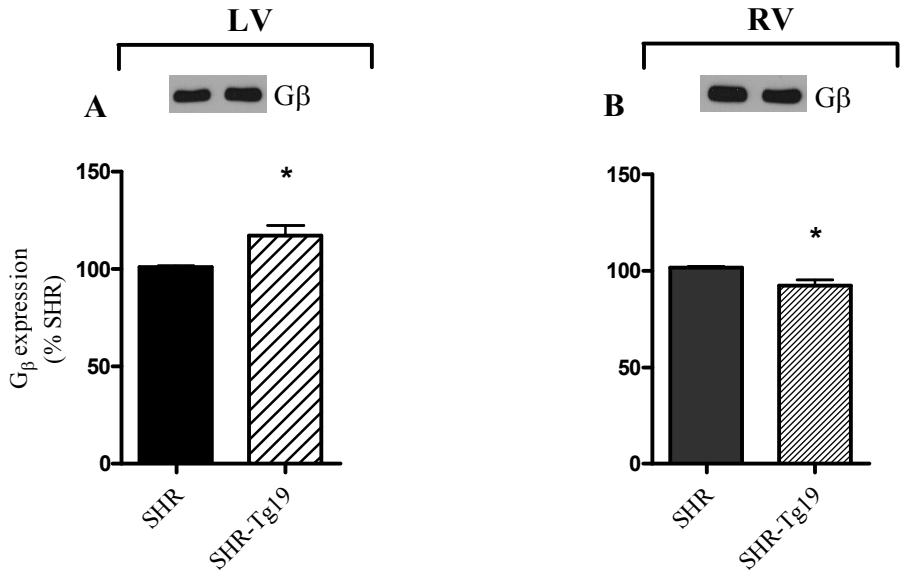

Figure 8: Expression of Gβ protein in the LV (A) and the RV (B) of SHR-Tg19 compared to SHR (100 %). Data obtained using SDS-PAGE and Western blot analysis with specific antibodies (Gβ Antibody (T-20), sc-378). * $p < 0.05$

Figure 8 shows data obtained of Gβ protein expression in myocardium membrane. In both ventricles of SHR-Tg19 it was significantly different from SHR. Expression of Gβ in the SHR-Tg19 LV (Fig. 8A) was significantly higher, while in the RV (Fig. 8B) it showed a significant decrease in comparison to SHR.

The expression of $G_i\alpha(1,2)$ in SHR-Tg19 appeared to be on the same level as in SHR in both LV and the RV (Fig. 9A and 9B respectively).

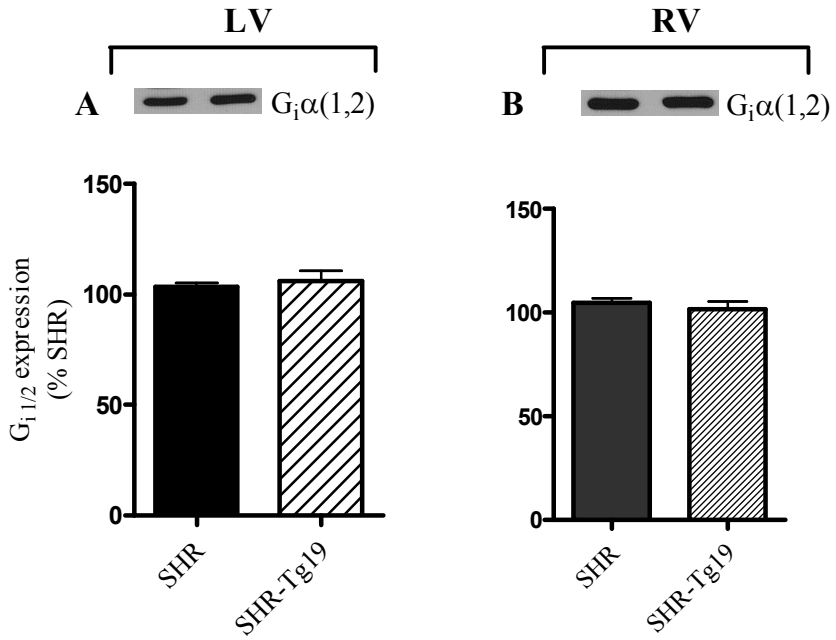

Figure 9: Expression of $G_i\alpha(1,2)$ protein in the LV (A) and the RV (B) of SHR-Tg19 compared to SHR (100 %). Data obtained using SDS-PAGE and Western blot analysis with specific antibodies.

5.5 β-Adrenergic receptors expression

According to the Western blot analysis, the expression of β_1-AR in heart of SHR-Tg19 strain was unaltered compared to SHR controls in both the left and right ventricles (Fig. 10). The expression of β_2-AR in transgenic rats was significantly higher compared to SHR controls in the RV, but showed no alteration in the LV (Fig. 11)

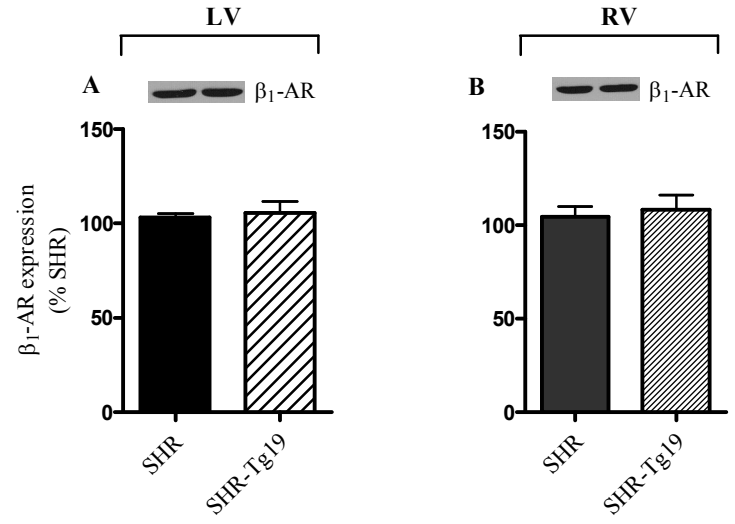

Figure 10: Expression of β_1-AR in the LV (A) and the RV (B) of SHR-Tg19 compared to SHR (100 %). Data obtained using SDS-PAGE and Western blot analysis with specific antibodies (β_1-AR antibody (V-19): sc-568).

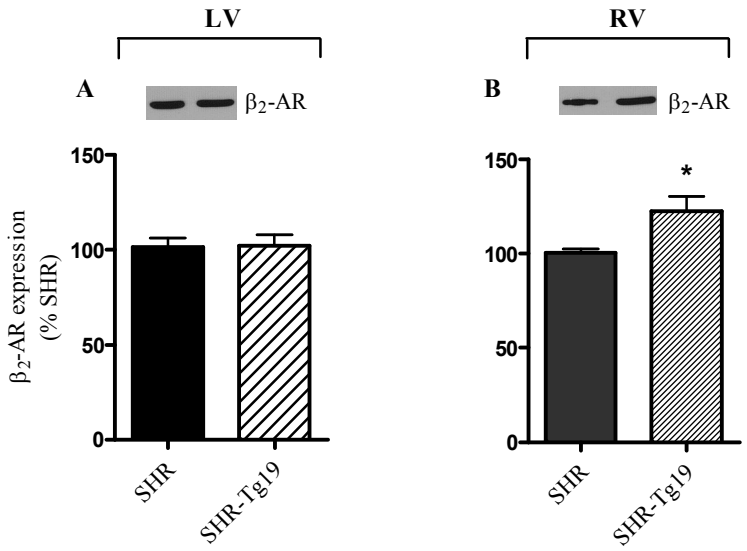

Figure 11: Expression of β_2-AR in the LV (A) and the RV (B) of SHR-Tg19 compared to SHR (100 %). Data obtained using SDS-PAGE and Western blot analysis with specific antibodies (β_2-AR antibody (H-20): sc-569). * $p < 0.05$

5.6.1 Saturation binding experiments

Saturation binding experiments were used to determine the expression and quantity of two types of β-adrenergic receptors. In order to do that the specific binding of [^{3}H]-CGP12177 ligand were compared between SHR and SHR-Tg19 (Fig 12).

Results of the saturation binding curves showed a significantly higher number of β-ARs in SHR-Tg19 cellular membrane preparations when compared to those from SHR controls (28.6 ± 0.8 vs. 21.0 ± 2.0 fmol/mg protein). No significant difference was observed in the binding affinity of β-adrenergic receptors (KD: 0.58 ± 0.11 vs. 0.68 ± 0.12 nM).

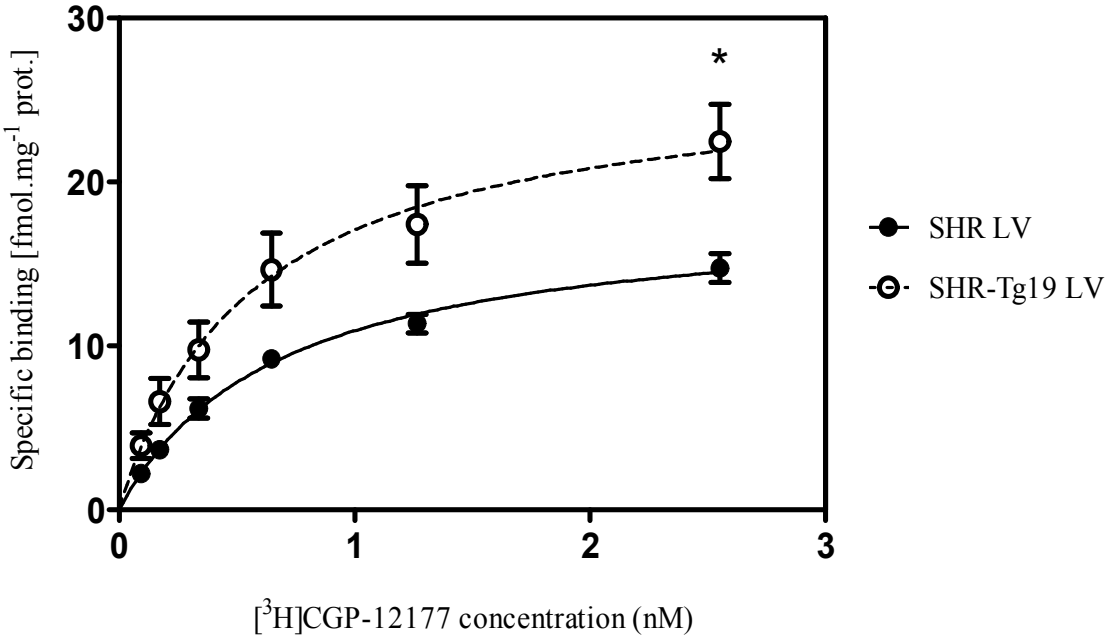

Figure 12: Saturation binding curves of SHR-Tg19 compared to SHR control in the LV, showing specific binding of [^{3}H]CGP-12177 radioligand to β-ARs on myocardial membranes.

5.6.2 Competition binding experiments

Competition binding experiments with the β_2-AR-selective antagonist ICI 118.551 were conducted on the LV to determine the percentage of each β-AR subtype. Unfortunately, there was not enough research material to perform these studies on the RV, so additional experiments will be required. Data obtained from these experiments showed a higher percent of β_2-AR subtype in the SHR-Tg19 LV. The ratio of β_1 : β_2 was 59 : 41 in transgenic rats and 72 : 28 in SHR controls (Fig 13).

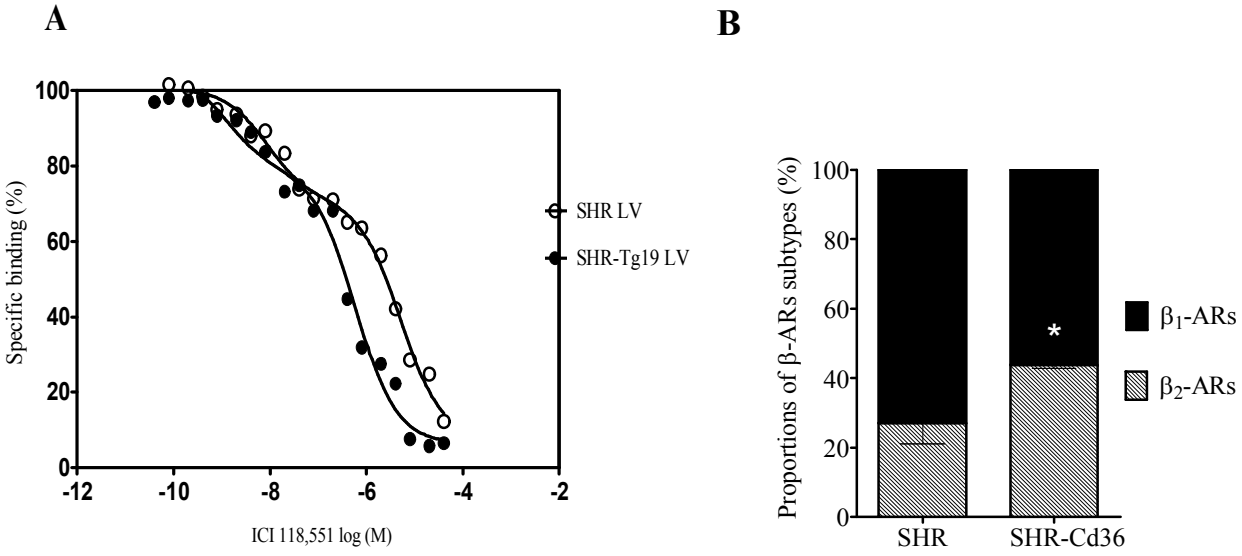

Figure 13: Representative competition binding curves of the LV of SHR-Tg19 and SHR were plotted as a percentage of total binding against the log concentration of the competing ligand (A). This data was expressed as the relative proportion of β_1- and β_2-ARs in myocardial crude membranes of SHR and SHR-Tg19 (B).

5. Discussion

The spontaneously hypertensive rat strain is a widely used model for human essential hypertension. It carries a deletion variant of FAT/Cd36, resulting in hyperinsulinemia and insulin resistance. Providing SHR with short-chain FA that do not require CD36 for transport eliminates those symptoms as well as decreases heart hypertrophy, but does not alter the hypertension (Hajri et al., 2001). The same effects can be seen in the transgenic strain SHR-Tg19 that harbors wild type of Cd36 gene (Pravenec et al., 2003). Along with the mentioned disorders, SHR develops left ventricular hypertrophy from 4 months of age in conjunction to hypertension that ultimately causes oxidative damage (Adams et al., 1989). It was shown that overexpression of β-ARs leads to cardiac hypertrophy (Ligget et al., 2000). Experiments on transgenic mice with heart-specific overexpression of β_1-ARs showed a short-lived improvement of cardiac function, followed by progressive heart failure (Engelhardt et al., 1999). However, it is not clear if hypertrophy serves as a primary cause for the hypertension or develops alongside with it (Alvarez et al., 2008).

Several studies suggest a link between the development of hypertension and cardiac contractility, mediated by the β-AR activity (Taquini et al., 1991; Liu et al., 1993). It is known that contractile response is regulated mainly due to β_1-AR stimulation and following AC activation (Lissandron et al, 2006). Studies of isoprenaline stimulated β-ARs on isolated cardiomyocytes from β_2-AR knockout mice showed an increased cAMP production along with sustained PKA activity, leading to increased contractile response (Devic et al., 2001; Soto et al, 2009). It has been reported that the blockade of β-ARs reduces fatal arrhythmias and myocardial infarct size induced by coronary artery ligation (Zhang et al, 2010). The increase in β_2-AR density in the LV of SHR-Tg19 determined in our study by radioligand binding experiments may be the main factor contributing to heart hypertrophy, along with increased AC activity. For instance, β_2-adrenoceptor stimulation was increased in patients with hypertension (Leenen et al., 1998). Other studies also suggest that

stimulation of β_2-ARs activates antiapoptotic pathways, while β_1-ARs activate proapoptotic signaling pathways (Talan et. al., 2011).

Although no increase in β_1-AR expression in the SHR-Tg19 heart was observed compared to SHR controls, the activity of AC was heightened, especially in the LV of SHR-Tg19. This may be due to the increase in β_2-AR relative percentage in the LV, shown with competitive radioligand binding. However, no significant increase in β_2-AR expression was determined. It is known that β_2-ARs can mediate anti-apoptotic signaling through activation of G_i, PI3K and PKB (Zhu et al, 2001; Bernstein et al, 2011). Thus, the higher quantity of these receptors could play role in lowering the infarct size in the heart of SHR-Cd36 (Neckar et al, 2012). More research will be needed to clarify the mechanisms behind these observations.

Neckar et al. (2012) showed the increased susceptibility of the transgenic SHR to arrhythmias using a coronary artery occlusion in an open-chest model. According to their data, transgenic rescue of Cd36 gene heightens duration and incidence of ischemic arrhythmias when compared to the SHR control. Those changes were observed in isolated perfused hearts as well, and were eliminated by catecholamine depletion using reserpine.

Our study showed an increase in the relative heart weight in SHR-Tg19 strain compared to SHR controls. Our unpublished data also showed significant left ventricular hypertrophy in transgenic rats, which, along with an unaltered weight of the SHR-Tg19 RV allows us to assume that only the LV is affected by hypertrophic changes.

These observations are corresponding with AC activity, which expressed significant increase under the stimulation with different ligands in the LV of SHR-Tg19 compared to SHR controls. Among them was $MnCl_2$ that stimulates enzyme directly, forskolin that affects both G_s and AC and isoprenaline that stimulates β-ARs as well as stimulation of G_s protein with GTPγS. The increment of AC activity may be related to the increased expression of AC5/6 observed in SHR-Tg19 heart. The data obtained from the RV showed the lack of increase in the AC activity in transgenic group under the stimulation of forskolin, GTPγS and NaF, compared to SHR.

However, a significant increase was observed in activity stimulated by $MnCl_2$. Surprisingly, the stimulation with isoprenaline reduced AC activity in SHR-Tg19 despite the overexpression of the β-ARs and AC5/6. No clear link between the function of Cd36 and β-AR signaling in the heart has been reported so far. However, some earlier studies indicated that SHR express lower cardiac membrane-bound β-AR quantity as compared to normotensive WKY rats and that those WKY rats have higher myocardial AC activity (Matsumori et al., 1989).

Trimeric G proteins are an essential link in β-AR signaling. During this study, the expression of stimulatory $G_s\alpha$, inhibitory $G_i\alpha$ and Gβ was investigated. No significant changes in SHR-Tg19 compared to controls were observed in both $G_\alpha s$ and $G_i s$ protein subunits expressions in myocardial crude membranes. However, expression of G_β in transgenic rats was significantly higher in the LV and lower in the RV. This is consistent with the activity of AC, particularly with the decrease observed in enzymatic activity under the stimulation of isoprenaline in the RV.

Data obtained from SHR-Tg19 showed that transgenic rescue of wild-type Cd36 gene increases AC activity cardiac β-AR responsiveness and sensitization of AC in heart of SHR. A parallel increase in expression of PKA was observed as well (our unpublished data), which led to a higher susceptibility to ischemia-induced arrhythmias. However, complete understanding of molecular mechanisms behind these effects will require more research in future.

Conclusion

Transgenic strain SHR-Tg19 was obtained to investigate the effects of FAT/Cd36 transgenic rescue on spontaneously hypertensive rats. It showed a decrease in FA levels and a higher glucose tolerance than SHR alongside with reduced hypertension due to an increased renal Cd36 expression.

Our study was dedicated to investigate the β-adrenergic signaling pathway in transgenic strain SHR-Tg19 of spontaneously hypertensive rats. Data obtained by the experiments conducted during our study showed significant changes in the expression of proteins involved in myocardial β-adrenergic signaling, as well as in stimulated AC activity. In the left ventricle of SHR-Tg19 significant increase was observed in the expressions of Gβ and AC5/6, while in the right ventricle was significantly increased the expression of β_2-AR. The expression of $G_i\alpha(1,2)$ and $G_s\alpha$ showed no changes in transgenic rats. Data obtained with radioligand binding experiments showed higher density of β-ARs along with higher relative β_2-AR amount in LV of SHR-Tg19 compared to SHR. Adenylyl cyclase activity was significantly increased when stimulated with forskolin, GTPγS, $MnCl_2$ and isoprenaline in the LV, while in the RV only stimulation with $MnCl_2$ showed an increase in AC activity, and stimulation with isoprenaline lowered the activity of enzyme compared to SHR. These observations indicate the up-regulatory effect of wild-type Cd36 expression on β-adrenergic signaling in SHR-Tg19, which underlies the increased cardiac adrenergic responsiveness in transgenic rats compared to SHR progenitors. Additional studies of SHR-Tg19 will be required to reveal all molecular processes behind the effects of Cd36 on development of hypertension in spontaneously hypertensive rats and the role of β-adrenergic signaling in it. .

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