

The changes in β -adrenoceptor-mediated cardiac function in experimental hypothyroidism: the possible contribution of cardiac β_3 -adrenoceptors

E. Arioglu · S. Guner · I. Ozakca · V. M. Altan ·
A. T. Ozcelikay

Received: 3 June 2009 / Accepted: 13 August 2009 / Published online: 1 September 2009
© Springer Science+Business Media, LLC. 2009

Abstract Thyroid hormone deficiency has been reported to decrease expression and function of both β_1 - and β_2 -adrenoceptor in different tissues including heart. The purpose of this study was to examine the possible contribution of β_3 -adrenoceptors to cardiac dysfunction in hypothyroidism. In addition, effect of this pathology on β_1 - and β_2 -adrenoceptor was investigated. Hypothyroidism was induced by adding methimazole (300 mg/l) to drinking water of rats for 8 weeks. Cardiac hemodynamic parameters were measured in anesthetised rats *in vivo*. Responses to β -adrenoceptor agonists were examined in rat papillary muscle *in vitro*. We also studied the effect of hypothyroidism on mRNA expression of β -adrenoceptors, $G_{i\alpha}$, GRK, and eNOS in rat heart. All of the hemodynamic parameters (systolic, diastolic and mean arterial pressure, left ventricular pressure, heart rate, $+dp/dt$, and $-dp/dt$) were significantly reduced by the methimazole treatment. The negative inotropic effect elicited by BRL 37344 (a β_3 -adrenoceptor preferential agonist) and positive inotropic effects produced by isoprenaline and noradrenaline, respectively, were significantly decreased in papillary muscle of hypothyroid rats as compared to those of controls. On the other hand, hypothyroidism resulted in increased cardiac β_2 - and β_3 -adrenoceptor, $G_{i\alpha_2}$, $G_{i\alpha_3}$, GRK3, and eNOS mRNA expressions. However, β_1 -adrenoceptor and GRK2 mRNA expressions were not changed significantly in this pathology. These results show that mRNA expression of β_3 -adrenoceptors as well as the signalling pathway components mediated through

β_3 -adrenoceptors are significantly increased in hypothyroid rat heart. Since we could not correlate these alternates with the decreased negative inotropic response mediated by this receptor subtype, it is not clear whether these changes are important for hypothyroid induced reduction in cardiac function.

Keywords β_3 -adrenoceptors · Hypothyroidism · Cardiac contractility · Gi protein · Papillary muscle

Introduction

Cardiac function is mainly regulated by β -adrenoceptors, a member of seven membrane spanning G-protein-coupled receptor superfamily. Stimulation of β_1 - and β_2 -adrenoceptors activates signaling pathway involving stimulatory G-protein (Gs protein), cyclic adenosine monophosphate, (adenylyl cyclase, cAMP), and protein kinase A (PKA) thereby mediates positive inotropic and chronotropic effects in the heart. β_2 -adrenoceptors, however, are also coupled to inhibitory G-protein, (Gi protein) leading to negative inotropic and antiapoptotic effects [1–4]. The third subtype, β_3 -adrenoceptor which is expressed predominantly in adipose tissue, has also been shown in ventricles of nonfailing and failing human hearts [5, 6]. The stimulation of cardiac β_3 -adrenoceptors results in negative inotropic effect. Since this effect was inhibited with pertussis toxine or NOS inhibitors, its mechanism is considered to be involved in the Gi protein–nitric oxide synthase (NOS) pathway [5, 7]. The negative inotropic effect produced by β_3 -adrenoceptor stimulation has been thought as a preventive mechanism against overstimulation of heart muscle by catecholamines [8, 9]. However, in chronic state, this mechanism becomes maladaptive in failing hearts because β_1 - and

E. Arioglu · S. Guner · I. Ozakca · V. M. Altan ·
A. T. Ozcelikay (✉)
Department of Pharmacology, Faculty of Pharmacy,
Ankara University, 06100 Tandogan, Ankara, Turkey
e-mail: ozcelika@pharmacy.ankara.edu.tr

β_2 -adrenoceptors are downregulated or desensitized while β_3 -adrenoceptors are not. Thus, this imbalance may worsen cardiac dysfunction in this pathology [8–11]. Similarly, the expression of cardiac β_3 -adrenoceptors has been found to be increased in diabetes [12] and hypertension [13], which might account for cardiac dysfunction related to these pathologies.

It is well known that number and function of β -adrenoceptors are regulated by thyroid hormones. Therefore, thyroid hormone deficiency causes a reduction in sensitivity and responsiveness to catecholamines as a result of decreased β_1 - and β_2 -adrenoceptor density in heart [19–21]. Consequently, it results in cardiac dysfunction such as impaired cardiac contractility and reduced chronotropy [14–18]. However, the role of cardiac β_3 -adrenoceptors in hypothyroidism has not been studied yet. Thus, the aim of this study was to investigate the molecular and functional changes of β_3 -adrenoceptors in addition to β_1 - and β_2 -adrenoceptors in the heart from hypothyroid rats.

Materials and methods

Animals and experimental model

Male Sprague-Dawley rats (200–250 g) were used in the present study. The rats were housed under the controlled environmental conditions ($22 \pm 1^\circ\text{C}$ ambient temperature, 60% relative humidity, and 12:12 h light cycle) and given free access to food and water. Control rats received normal drinking water. Hypothyroidism was induced by adding methimazole (300 mg/l) to drinking water for 8 weeks. The protocol of this study was approved by the Ethics Committee of Faculty of Veterinary Medicine, University of Ankara.

Experimental procedures

Throughout 8 weeks, the rats were weighed once a week, and average food and fluid intake were recorded twice per week. At the end of 8-week period, blood samples were taken from tail of the rats into the heparinized tubes, centrifuged at 5,000 for 10 min and stored in -20°C until analyzed for cholesterol and triglyceride levels using enzymatic and colorimetric methods (Roche Diagnostic).

For examining the effects of hypothyroidism on the rat heart, experiments were carried out in two series. First, cardiac hemodynamic parameters were measured in anesthetized rats *in vivo*. In the second series, responses to β -adrenoceptor agonists were examined in papillary muscle *in vitro*.

Hemodynamic assessment

The rats were anesthetized with an intraperitoneal injection of xylazine (10 mg/kg) and ketamine (90 mg/kg) combination. The body temperature of rats were stabilized at 37°C with a rectal probe. For hemodynamic assessments, a millar catheter (SPR 249) was inserted into the right carotid artery through proximal arteriotomy. Systolic pressure (SP), diastolic pressure (DP), and mean arterial pressure (MAP) were measured in aorta for 5 min. After arterial pressure measurements, the catheter was inserted to the left ventricle. Left ventricular pressure (LVP), heart rate (HR), and rates of pressure development ($+dp/dt$) and pressure decay ($-dp/dt$) were recorded. All hemodynamic data were obtained online through an analog–digital interface (model MP100; Biopac Systems Inc.) and using a software (Acq-Knowledge 3.5.0 for Windows, Biopac Systems Inc., Santa Barbara, CA). At the end of each experiment, heart and thyroid weights were measured.

Experiments on isolated papillary muscle

The hearts of rats were isolated under ether anesthesia. Papillary muscles were dissected from left ventricle, placed in an experimental chamber and superfused at a flow rate 5 ml/min with Tyrode's solution oxygenated (95% O_2 ; 5% CO_2) and warmed (30°C). The composition of Tyrode's solution was 116 mM NaCl; 5 mM KCl; 2.7 mM CaCl_2 ; 1.1 mM MgCl_2 ; 0.33 mM NaHPO_4 ; 24 mM NaHCO_3 and 5 mM glucose. The tissues were stabilized for 60 min and stimulated at a pacing cycle length of 1,700 ms, stimulus pulse width was 1–2 ms and amplitude was twice the diastolic threshold. Tension was recorded by using a mechanoelectric force transducer. Ventricular tissues were stretched stepwise (10 μm increments) to a length at which contraction force was maximal. The experiments then were performed at 90% of maximal tension. After equilibration, cumulative concentration–response curves of BRL37344 (0.01 nM–1 μM), a preferential β_3 -adrenoceptor agonist, isoprenaline (0.1 nM–30 μM), and a nonselective β -adrenoceptor agonist, were obtained. The concentration–response curve of noradrenaline (0.1 nM–30 μM) a selective β_1 -adrenoceptor agonist was obtained in the presence of prazosin (1 μM) and desipramine (10 μM) and was carried out in each tissue.

At the end of the each experiment, pieces of left ventricle tissue were frozen in liquid nitrogen and stored at -80°C for subsequent PCR experiments.

Total RNA isolation, RT PCR, and PCR experiments

Hearts were powdered with liquid nitrogen and homogenized with an ultrasonic homogenizer (Bandelin) prior to

Table 1 The sequences and product sizes of primers

mRNA	Forward (3'–5')	Reverse (3'–5')	Product size
β_1 -adrenoceptor	GCCGATCTGGTCATGGGA	GTTGTAGCAGCGGCGCG	327
β_2 -adrenoceptor	TAGGTTTTCGAAGAAGACCG	ACCTCCTCCTTGCTATCCA	560
β_3 -adrenoceptor	CGCTTAGCTACGACGAAC	AGTGGGACTCCTCGTAATG	444
eNOS	ATATCTTCAGCCCCAAACGCA	ACCACTTCCATTCTTCGTAGCG	553
Gi α_2	CATCTTCTGTGTCGCCTTGA	CTCAGAAGAGGCCACAGTCC	404
Gi α_3	ACTGATTTGCCTGCTCGTCT	ACACGTGACAAGGTGGAACA	249
GRK2	ATGAGATTGACCGCATGACA	GTTGCGGTACAGTTCCTGGT	326
GRK3	AGCCATCTCTCCAAGAAGCA	TCCTCTCGTTCAAAGCCAGT	322
Beta Aktin	AGCCATGCCAAATGTGTCAT	CGTAAAGACCTCTATGCCAA	387

RNA extraction. Total RNA was extracted with the TRIzol reagent (Sigma) according to the manufacturer's protocol. The optical density values and amounts of RNA were determined spectrophotometrically using Nanodrop (NanoDrop technologies) at wavelength 260 nm (λ_{260}) and 280 nm (λ_{280}) and the OD $\lambda_{260}/\lambda_{280}$ ratio was used as a precursory estimation of RNA quality. In addition, formamide/formaldehyde agarose gels were later used to evaluate RNA integrity.

RT-PCR experiments were performed using ImProm II Reverse Transcriptase System (Promega). 1 μ l oligo dT (Promega) was added to equivalent amounts of total RNA from control and hypothyroid rats. The mixtures were placed into thermalcycler (Hybaid, UK) and held at 70°C for 5 min. The samples were then transferred into ice for 5 min. Thereafter, 1 μ l 10 mM deoxynucleotide triphosphate (dNTP), 6.1 μ l nuclease free water, 4 μ l 5X MgCl free buffer, 2.4 μ l 25 mM MgCl₂, 1 μ l Reverse transcriptase, and 0.5 μ l RNasin were added, final volume was 20 μ l. The tubes were again placed into the thermocycler and heated for 60 min at 42°C for RT, followed by 15 min at 70°C for denaturation. First strand cDNA samples were then cooled to 4°C and were stored at –80°C until use.

The single strand cDNAs were consequently amplified by PCR using gene specific primers (Table 1) and taq polymerase. For this purpose, 5X MgCl₂ free reaction buffer; MgCl₂ (2.8–3.8 mM), dNTP (0.5 mM); Taq DNA polymerase (5u/ul) (Promega, Madison, WI); cDNA (1 μ l); and 2 μ l (from 10 pmol/ml) of respective sense and antisense primers were added to PCR tubes. Nuclease free water was then added to each tube for a final volume of 50 μ l. The samples were then mixed, placed in the thermocycler, and denatured for 3 min at 94°C. Thereafter, segments were amplified using the sequence 1.5 min denaturation (94°C) followed by 1 min annealing (58–60°C) and 2 min extension (72°C), this sequence was repeated for a total of 35 cycles. Beta-actin was amplified in each set of PCR reactions and served as internal references during quantitation to correct for operator and/or experimental variations. At the

end of the reactions, 30 μ l of each PCR product was then loaded onto a 1% agarose gel containing ethidium bromide and electrophoresed for 1 h at 100 V (Sci-Plas, U.K.). The resulting gels were then visualized using an UV transilluminator (Viber Loumat TFX 20 M UV) and photographed using UV gel camera (Kodak EDAS 290). Band intensities were calculated by using Kodak 1D 3.6 program.

Drugs

All drugs used were purchased from Sigma chemical (St. Louis, MO).

Statistical analysis

The results are expressed as mean $\pm$ SEM. Statistical significance was tested by unpaired Student's *t* test. The probability level of *P* < 0.05 was considered the threshold for statistical significance.

Results

Effects of methimazole treatment on morphological and hemodynamic parameters

Table 2 summarizes some changes in general characteristics of rats. The methimazole treatment markedly decreased food and fluid intakes and body and heart weights of the rats compared with their age-matched controls. The ratio of heart weight to body weight was not different between the groups. As expected, methimazole caused a significant increase in thyroid weight of the rats, thereby enhancing the ratio of thyroid weight to body weight. The cholesterol levels but not the triglyceride levels were significantly increased in methimazole-treated rats in relation to control rats. As seen in Table 3, all of the hemodynamic parameters measured in vivo were significantly reduced by the methimazole treatment.

Table 2 General characteristics of control ($n = 7$) and hypothyroid ($n = 13$) rats at the end of the 8-week methimazole treatment

	Food Intake (g/day)	Fluid Intake (ml/day)	BW (g)	HW (g)	HW/BW (mg/100 g)	TW/BW (mg/100 g)	Cholesterol (mg/dl)	Triglyceride (mg/dl)
Control	25 ± 1	30 ± 1	432 ± 11	1.6 ± 0.1	0.37 ± 0.03	5.5 ± 0.3	96 ± 3	90 ± 3
Hypothyroid	11 ± 1*	23 ± 2*	226 ± 3*	0.8 ± 0.1*	0.36 ± 0.03	31.6 ± 2.3*	129 ± 3*	83 ± 3

BW body weight, TW thyroid weight, HW heart weight. * $P < 0.05$ when compared to control values

Table 3 In vivo hemodynamic parameters of control ($n = 7$) and hypothyroid ($n = 13$) rats at the end of the 8-week methimazole treatment

	MAP (mmHg)	HeartRate (beat/min)	LVP _{max} (mmHg)	+dp/dt _{max} (mmHg/s)	−dp/dt _{max} (mmHg/s)
Control	65 ± 5	242 ± 14	104 ± 3	3515 ± 113	3294 ± 170
Hypothyroid	42 ± 2*	141 ± 5*	80 ± 5*	1939 ± 211*	1789 ± 174*

LVP_{max} maximal left ventricle developed pressure, +dp/dt_{max} maximally developed contraction velocity, −dp/dt_{max} maximally developed relaxation velocity, MAP Mean arterial pressure, * $P < 0.05$, compared to control values

Contractile responsiveness to beta-adrenergic agonists

In isolated ventricular papillary muscles of both groups, BRL 37344 showed biphasic inotropic effect. It caused negative inotropic effect at concentrations ranging from 0.01 nM to 0.01 μM in control group (Fig. 1). The positive inotropic effect was obtained at higher concentrations (0.03 and 0.1 μM). In hypothyroid group, also the negative inotropic effect was observed at the concentrations ranging from 0.01 nM to 1 nM. However, it was found to be markedly attenuated in hypothyroidism ($5.4 \pm 1.4\%$, $P < 0.05$) as compared to control ($16.5 \pm 3.1\%$) without

modifying the pD₂ value (control: 10.1 ± 0.2 , $n = 5$; hypothyroid: 10.0 ± 0.3 , $n = 8$, $P > 0.05$).

In the control group, isoprenaline (0.1 nM–10 μM), induced a concentration-dependent positive inotropic effect which was significantly reduced by hypothyroidism. The maximum negative inotropic effect of isoprenaline was markedly lower in hypothyroid group ($15.4 \pm 2.8\%$, $P < 0.05$) than in control group ($93.8 \pm 3.9\%$). In the presence of prazosin (1 μM) and desimipramine (10 μM), noradrenaline (0.1 nM–30 μM) induced a concentration-dependent increase in contractility. The positive inotropic effect of noradrenaline was decreased dramatically in hypothyroid rats (control, $148.2 \pm 17.4\%$; hypothyroid, 21.5 ± 5.1 , $P < 0.05$). However, pD₂ values for isoprenaline and noradrenaline were found to be similar in control and hypothyroid group (isoprenaline; control: 7.1 ± 0.1 , $n = 5$; hypothyroid: 7.0 ± 0.3 , $n = 8$, $P > 0.05$; noradrenaline; control: 5.9 ± 0.2 , $n = 5$; hypothyroid: 6.6 ± 0.6 , $n = 8$, $P > 0.05$).

Effect of hypothyroidism on of β_1 -, β_2 -, and β_3 -adrenoceptor, Giα₂, Giα₃, eNOS, GRK2, and GRK3 mRNA expression in rat heart

RT-PCR technique was used to detect mRNA expression of β_1 -, β_2 -, and β_3 -adrenoceptors, Giα₂, Giα₃, eNOS (endothelial nitric oxide synthase), GRK2 (G-protein receptor kinase 2), and GRK3 in the rat heart. There was no change in mRNA expression of β_1 -adrenoceptors, whereas significant increase was observed in the mRNA expressions of β_2 -adrenoceptors and β_3 -adrenoceptors in the hypothyroid group compared to control group (Fig. 2). Hypothyroidism also significantly resulted in a rise in the mRNA expressions of Giα₂, Giα₃, eNOS, GRK2, and GRK3 in comparison with control (Fig. 2).

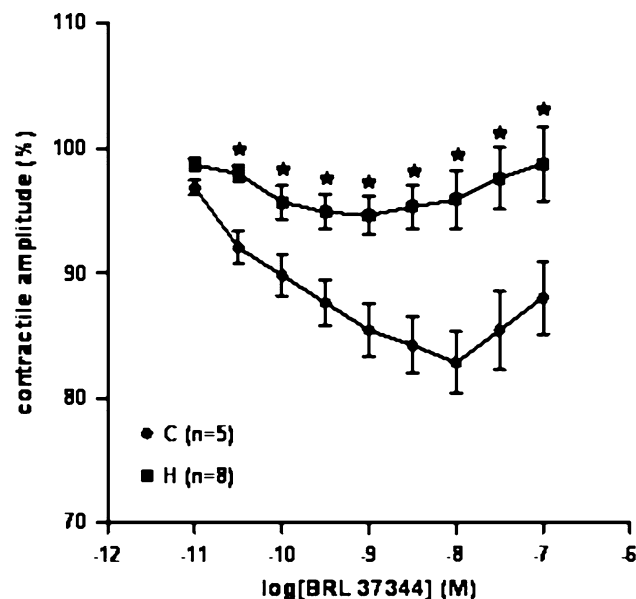


Fig. 1 Concentration dependent effects of BRL 37344 on papillary muscles of control (C) and hypothyroid (H) rats. * $P < 0.05$

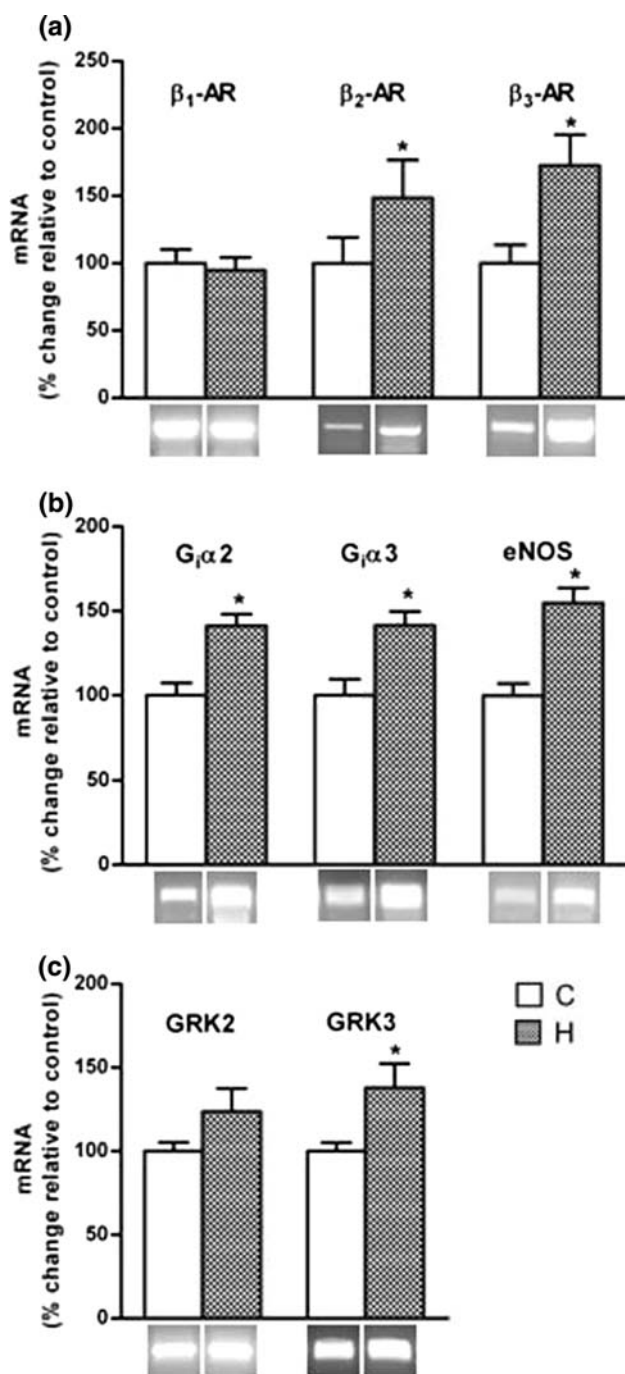


Fig. 2 mRNA expressions of **a** β_1 , β_2 , β_3 -adrenoceptors; **b** eNOS, $G_{i\alpha 2}$, $G_{i\alpha 3}$; **c** GRK2 and GRK3 in control ($n = 5$) and hypothyroid ($n = 6$) rats. * $P < 0.05$

Discussion

Consistent with the previous findings [22–25], many symptoms of hypothyroidism induced by methimazole treatment were also observed in our study. It is well known that adrenergic receptors are regulated by thyroid hormones. However, this regulation seems to depend on tissue

type, receptor subtype, and duration of hypo- or hyperthyroidism. In general, it has been shown that thyroid hormone deficiency leads to a decrease in both β_1 - and β_2 -adrenoceptor numbers and expressions in different tissues including heart [19–21, 26–28]. On the other hand, there are a limited number of studies that thyroid hormones affect β_3 -adrenoceptors. Hypothyroidism decreases and increases β_3 -adrenoceptor expressions in white and in brown adipose tissue, respectively [29]. In rat ileum, it has been found that although hypothyroidism has no effect on β_3 -adrenoceptor mRNA expression, it caused decreased relaxation responses to isoprenaline and CL316234 (selective β_3 -adrenoceptor agonist) [30]. In the present study, we examined for the first time the effect of hypothyroidism on β_3 -adrenoceptor function and mRNA expression in rat heart and found that mRNA expression of β_3 -adrenoceptors was increased in hypothyroidism, which was not correlated with the decreased functional response of this receptor subtype.

Our findings showing decreased positive inotropic responses to both isoprenaline and noradrenaline in isolated papillary muscle despite unchanged β_1 -adrenoceptor mRNA expressions in hypothyroid state are in disagreement with the literature. In most of the studies hypothyroidism caused a decrease in both number and function of β -adrenoceptors [19–21, 26–28]. On the other hand, Brown et al. [31] found that β_1 - and β_2 -adrenoceptor densities were enhanced whereas positive inotropic response to noradrenaline was decreased in hypothyroid state.

A few possible factors can account for this discrepancy between functional and molecular results in hypothyroid state in our study. First, thyroid hormones exert important effects on the heart through modulating the expressions of genes coding key proteins related to contraction and relaxation such as myosin heavy chain- α , L-type calcium channel, ryanodine channel, SERCa2 (sarcoplasmic reticulum Ca^{++} -ATPase), and phospholamban [14, 23, 32–34]. Therefore, changes in proteins regulating Ca^{++} homeostasis as a result of thyroid hormone deficiency could contribute to decreased contractile responses to isoprenaline and noradrenaline in papillary muscle despite of unchanged β_1 -adrenoceptor mRNA expressions.

Heterolog/homolog desensitization mechanism is another important factor to be considered. It is well known that activities or expressions of cytosolic G-protein-coupled receptor kinases (GRK2, GRK3, and GRK5) are increased in many pathophysiological situations such as heart failure and hypothyroidism [35, 36]. This situation causes desensitization to beta-adrenergic stimulation, thereby leading to a decrease in contractile response to catecholamines [26]. Penela et al. [37] have suggested that a marked decrease in the β -adrenergic receptor/GRK2 ratio predicts alterations in adrenergic receptor desensitization.

From this point of view, in the present study despite of being statistically not significant, a decrease in β_1 -adrenoceptor and an increase in GRK2 mRNA expressions and a significant increase in GRK3 mRNA expression in hypothyroid rats as compared to controls may have caused the inhibited response to isoprenaline and noradrenaline.

β_2 -adrenoceptors are coupled both to G_s and G_i proteins differently from β_1 -adrenoceptors. Xiao et al. [38] reported that the receptor-G-protein coupling was switched from G_s to G_i due to the persistent agonist stimulation in HEK293 cells and rat cardiomyocytes. On the other hand, PKA-mediated β_2 -adrenoceptor phosphorylation was a prerequisite for β_2 -adrenoceptor- G_i coupling [39, 40]. It has been shown that antiapoptotic effect of β_2 -adrenoceptors in some conditions such as heart failure and hypertrophy arises from β_2 -adrenoceptor- G_i protein coupling [38, 41, 42]. Our results also showed that β_2 -adrenoceptor expression was enhanced in hypothyroidism. In addition, we found that $G_{i\alpha}$ mRNA expression was increased in hypothyroid rats, which was consistent with previous results found in this pathology [43, 44]. Therefore, the increases in expressions of β_2 -adrenoceptor and G_i might be result of a homeostatic mechanism against hypothyroidism-induced apoptosis as shown in brain [45]. On the other hand, the negative inotropic effect observed at high doses of isoprenaline (3 μ M and higher) but not noradrenaline (β_1 -adrenoceptor agonist in the presence of prazosin and desimipramine) on papillary muscles of hypothyroid rats could be associated with β_2 -adrenoceptor- G_i protein coupling since ICI 118551, selective β_2 -adrenoceptor antagonist, was able to inhibit this response (unpublished observation).

In the mammalian heart, stimulation of β_3 -adrenoceptors with a selective agonist such as BRL 37344 has been found to lead to a negative inotropism through a signal transduction pathway coupling with G_i /eNOS/NO/cGMP/PKG [8].

Cardiac β_3 -adrenoceptors were found to be upregulated in some pathological conditions such as heart failure [10]. Similar to heart failure, also in diabetes, β_3 -adrenoceptors have been demonstrated to have been upregulated [12]. This change has been considered a preventive mechanism against increased sympathetic activation at the early stages of heart failure. Moniotte et al. [10] have found that β_3 -adrenoceptor-mediated cardiac responses were abolished although protein levels of β_3 -adrenoceptors were increased in heart failure. They suggested that these results were related to postreceptor changes in this pathology since decreased protein level of eNOS was found in that study [10]. Using selective β_3 -adrenoceptor agonist BRL 37344, we investigated the change of β_3 -adrenoceptor-mediated cardiac responses in hypothyroid rats. The negative inotropic effect of BRL 37344 was decreased in hypothyroid rats compared to control rats. Inconsistent with the functional response, β_3 -adrenoceptor, G_i and eNOS mRNA

expressions were increased markedly in hypothyroid state. Therefore, in the present study, increased signal transduction pathway related to β_3 -adrenoceptors seems not to be associated with decreased negative inotropic response to BRL 37344 in hypothyroid state.

It has been demonstrated that intracellular calcium level is a critical determinant of eNOS activity [46]. As mentioned above thyroid hormones regulate cardiac gene expressions of proteins affecting intracellular calcium cycling. Therefore, alteration of calcium cycling by hypothyroidism may cause decreased eNOS activity, leading to reduced β_3 -adrenoceptor response. Besides Ca^{2+} -dependent eNOS activation in cardiac tissue, especially in left ventricular myocardium, it has been established that PI-3 kinase/Akt/PKB-dependent phosphorylation pathways can mainly regulate eNOS activity. Brixius et al. [47] indicated for the first time that an activation of the PI-3 kinase/Akt kinase dependent signal transduction pathway may contribute to the BRL 37344 induced eNOS activation. On the other hand, thyroid hormones activate Akt signaling in myocardium [48]. Taken together, it could be suggested that PI-3 kinase/Akt/PKB-dependent phosphorylation pathway of eNOS impaired due to the thyroid hormone deficiency results in the reduced BRL 37344-induced negative inotropic responses in the present study.

In conclusion, in addition to β_1 - and β_2 -adrenoceptor, we showed for the first time that mRNA expression and function of β_3 -adrenoceptor were also changed by hypothyroidism. However, it is not clear whether these changes are important for reduced cardiac function.

Limitations

In this study western blot studies could not be performed because of the technical difficulties.

References

1. Xiao RP, Ji X, Lakatta EG (1995) Functional coupling of the beta 2 adrenoceptor to a pertussis toxine sensitive G protein in cardiac myocytes. *Mol Pharmacol* 47:322–329
2. Xiao RP, Avdonin P, Zhou YY, Cheng H, Akhter SA, Eschenhagen T, Lefkowitz RJ, Koch WJ, Lakatta EG (1999) Coupling of beta2-adrenoceptor to G_i proteins and its physiological relevance in murine cardiac myocytes. *Circ Res* 84:43–52
3. Steinberg SF (1999) The molecular basis for distinct β -adrenergic receptor subtype actions in cardiomyocytes. *Circ Res* 85:1101–1111
4. Kuschel M, Zhou YY, Cheng H, Zhang SJ, Chen Y, Lakatta EG, Xiao RP (1999) G_i protein mediated functional compartmentalization of cardiac beta 2 adrenergic signaling. *J Biol Chem* 274:22048–22052
5. Gauthier C, Tavernier G, Charpentier F, Langin D, Le marec H (1996) Functional beta 3 adrenoceptor in the human heart. *J Clin Invest* 98:556–562

6. Lowell BB, Spiegelman BM (2000) Towards a molecular understanding of adaptive thermogenesis. *Nature* 404:652–660
7. Gauthier C, Leblais V, Kobzik L, Trochu JN, Khandoudi N, Bril A (1998) The negative inotropic effects of beta 3 adrenoceptor stimulation is mediated by activation of a nitric oxide synthase pathway in human ventricle. *J Clin Invest* 102:1377–1384
8. Rozec B, Gauthier C (2006) Beta 3 adrenoceptors in the cardiovascular system: putative roles in human pathologies. *Pharmacol Ther* 111:652–673
9. Moniotte S, Balligand JL (2002) Potential use of beta 3 adrenoceptor antagonists in heart failure therapy. *Cardiovasc Drug Rev* 20:19–26
10. Moniotte S, Kobzik L, Feron O, Trochu JN, Gauthier C, Balligand JL (2001) Upregulation of beta (3)-adrenoceptors and altered contractile response to inotropic amines in human failing myocardium. *Circulation* 103:1649–1655
11. Cheng HJ, Zhang ZS, Onishi K, Ukai T, Sane DC, Cheng CP (2001) Upregulation of functional beta 3 adrenergic receptor in the failing canine myocardium. *Circ Res* 89:509–606
12. Dincer UD, Bidasee KD, Guner S, Tay A, Ozelikay AT, Altan VM (2001) The effect of diabetes on expression of beta1, 2-, 3-adrenoceptors in rat hearts. *Diabetes* 50:455–461
13. Mallem MY, Toumaniantz G, Serpillon S, Gauthier F, Gogyn M, Desfontis JC (2004) Impairment of the low affinity state beta 1 adrenoceptor induced relaxation in spontaneously hypertensive rats. *Br J Pharmacol* 143:599–605
14. Bilezikian JP, Loeb JN (1983) The influence of hyperthyroidism and hypothyroidism on alpha and beta adrenergic receptor systems and adrenergic responsiveness. *Endocr Rev* 14:378–387
15. Biondi B, Palmieri EA, Lombardi G, Fazio S (2002) Effects of thyroid hormone on cardiac function: the relative importance of heart rate, loading conditions, and myocardial contractility in the regulation of cardiac performance in human hyperthyroidism. *J Clin Endocrinol Metab* 87:968–974
16. Klein I (2003) Thyroid hormone and cardiac contractility. *Am J Cardiol* 91:1331–1332
17. Franklyn JA, Gammage MD, Ramsden DB, Sheppard MC (1984) Thyroid status in patients after acute myocardial infarction. *Clin Sci* 67:585–590
18. Polikar R, Kennedy B, Ziegler M, O'Connor DT, Smith J, Nicod P (1990) Plasma norepinephrine kinetics, dopamin beta hydroxylase and chromogranin A in hypothyroid patients before and following replacement therapy. *J Clin Endocrinol Metab* 70:277–281
19. Dowell RT, Atkins FL, Love S (1994) Beta adrenergic receptors, adenylyl cyclase activation and myofibril enzyme activity in hypothyroid rats. *Am J Physiol* 266:H2527–H2534
20. Revelli JP, Pescini R, muzzin P, Seydoux J, Fitzgerald MG, Fraser CM, Giacobino JP (1991) Changes in beta 1 and beta 2 adrenergic receptor mRNA levels in brown adipose tissue and heart of hypothyroid rats. *Biochem J* 277:625–629
21. Wibo M, Kilar F, Zheng I, Godfraind T (1995) 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
22. Wieshammer S, Keck FS, Waitzinger J, Henze E, Loos U, Hombach V, Pfeiffer EF (1989) Acute hypothyroidism slows the rate of left ventricular diastolic relaxation. *Can J Physiol Pharmacol* 67:1007–1010
23. Klein I, Ojamaa K (2001) Thyroid hormone and the cardiovascular system. *N Engl J Med* 344:501–509
24. Moolman JA (2002) Thyroid hormone and the heart. *Cardiovasc J S Afr* 13:159–163
25. Iwata T, Honda H, Matsuda H, Kondo M, Taniguchi J, Miwa T, Kumasaka K, Moroe H, Notoya Y (2005) Hypothyroidism changes adrenoceptor and muscarinic receptor mediated blood pressure responses. *Eur J Pharmacol* 507:311–316
26. Ungerer M, Bohm M, Elce JS, Erdman E, Lohse MJ (1993) Altered expression of beta-adrenergic receptor kinase and beta 1-adrenergic receptors in the failing human heart. *Circulation* 87:454–463
27. Whitsett JA, Pollinger J, Matz S (1982) Beta-adrenergic receptors and catecholamine sensitive adenylate cyclase in developing rat ventricular myocardium: effect of thyroid status. *Pediatr Res* 16:463–469
28. Fox AW, Juberg EN, May JM, Johnson LD, Abel PW, Minneman KP (1985) Thyroid status and adrenergic receptor subtypes in the rat: comparison of receptor density and responsiveness. *J Pharmacol Exp Ther* 235:715–723
29. Germack R, Starzec A, Perret GY (2000) Regulation of beta 1- and beta 3-adrenergic agonist stimulated lipolytic response in hyperthyroid and hypothyroid rat white adipocytes. *Br J Pharmacol* 129:448–456
30. Brown KJ, Summers RJ (2001) Beta 1 and beta 3 adrenoceptor mediated smooth muscle relaxation in hypothyroid rat ileum. *Eur J Pharmacol* 415:257–263
31. Brown I, Nankernis R, Kerr D, Sernia C (1994) Adrenoceptor mediated cardiac and vascular responses in hypothyroid rats. *Biochem Pharmacol* 47:281–288
32. Klein I, Danzi S (2007) Thyroid disease and the heart. *Circulation* 116:1725–1735
33. Hawthorn MH, Genqo P, Wei XY, Ruhedge A, Moran JF, Gallant S, Triggle DJ (1988) Effect of thyroid status on beta-adrenoceptors and calcium channels in rat cardiac and vascular tissue. *Naunyn Schmiedebergs Arch Pharmacol* 337:539–544
34. Brodde OE, Michel MC (1999) Adrenergic and muscarinic receptors in the human heart. *Pharmacol Rev* 51:651–690
35. Aragay AM, Ruiz-Gomez A, Penela P, Sarnago S, Elorza A, Jimenez-Sainz MC, JrF Mayor (1998) G protein coupled receptor kinase 2: mechanisms of regulation and physiological functions. *FEBS Lett* 430:37–40
36. Mayor F Jr, Penela P, Ruiz-Gomez A (1998) Role of G protein coupled receptor kinase 2 and arrestins in beta adrenergic receptor internalisation. *Trends Cardiovasc Med* 8:234–240
37. Penela P, Barradas M, Alvarez-Dolado M, Munoz A, JrF Mayor (2001) Effect of hypothyroidism on G protein coupled receptor kinase 2 expression levels in rat liver, lung, and heart. *Endocrinology* 142:987–991
38. Xiao RP, Zhu W, Zheng M, Chakir K, Bond R, Lakatta EG, Cheng H (2006) Subtype specific beta adrenoceptor signaling pathways in the heart and their potential clinical implications. *Trends Pharmacol Sci* 25:358–365
39. Daaka Y, Luttrell LM, Lefkowitz RJ (1997) Switching of the coupling of the beta 2 adrenergic receptor to different G proteins by protein kinase A. *Nature* 390:88–91
40. Zou Y, Komuro I, Yamazaki T, Kudoh S, Uozumi H, Kadowaki T, Yazaki Y (1999) Both Gs and Gi proteins are critically involved in isoproterenol induced cardiomyocyte hypertrophy. *J Biol Chem* 274:9760–9770
41. Chesley A, Lundberg MS, Asai T, Xiao RP, Ohtani S, Lakatta EG, Crow MT (2000) The beta(2)-adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through G(i)-dependent coupling to phosphatidylinositol 3'-kinase. *Circ Res* 87:1172–1179
42. Foerster K, Groner F, Matthes J, Koch WJ, Birnbaumer I, Herzig S (2003) Cardioprotection specific for the G protein Gi2 in chronic adrenergic signaling through beta 2 adrenoceptors. *Proc Natl Acad Sci* 100:14475–14480
43. Zwaveling J, Batink HD, Taguchi K, De Jong J, Michel MC, Pfaffendorf M, Van Zwieten A (1998) Thyroid status affects the rat cardiac beta adrenoceptor system transiently and time dependently. *J Auton Pharmacol* 18:1–11
44. Novotny J, Bourova I, Malkova O, Svoboda P, Kolar F (1999) G proteins, beta adrenoceptors, and beta adrenergic responsiveness

- in immature and adult rat ventricular myocardium: influence of neonatal hypo- and hyperthyroidism. *J Mol Cell Cardiol* 31:761–772
45. Singh R, Upadhyay G, Kumar S, Kapoor A, Kumar A, Tiwari M, Godbole MM (2003) Hypothyroidism alters the expression of Bcl-2 family genes to induce enhanced apoptosis in the developing cerebellum. *J Endocrinol* 176:39–46
46. Chen PF, Wu KK (2000) Characterization of the roles of the 594–645 region in human endothelial nitric-oxide synthase in regulating calmoduline binding and electron transfer. *J Biol Chem* 275:13155–13163
47. Brixius K, Bloch W, Pott C, Napp A, Krahwinkel A, Ziskoven C, Koriller M, Mehlhorn U, Hescheler J, Fleischmann B, Schwinger RH (2004) Mechanisms of beta 3-adrenoceptor-induced eNOS activation in right atrial and left ventricular human myocardium. *Br J Pharmacol* 143:1014–1022
48. Kuzman JA, Gerdes AM, Kobayashi S, Liang Q (2005) Thyroid hormone activates Akt and prevents serum starvation-induced cell death in neonatal rat cardiomyocytes. *J Mol Cell Cardiol* 39:841–844