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Effects of α-, β-Adrenergic, and Calcium Channel Blockers on Renin- Angiotensin System in Perfused Rat Heart

Chang-Gyo Park

Department of Pharmacology, Keimyung University School of Medicine, Taegu 700—712, Korea

α-, β-Adrenergics, and calcium channels were known to be related to inducing cardiac hypertrophy. Recently, it was reported that the cardiac renin-angiotensin system (RAS) was an important factor in ventricular hypertrophy. The present study was aimed to investigate the effects of α-, β-adrenergic, and calcium channel blockers that might be involved in the regulation of cardiac RAS. The reverse transcription-polymerase chain reaction (RT-PCR) was used to detect the expression of renin gene in the perfused rat heart. Changes in angiotensin converting enzyme (ACE) activity and cyclic AMP (cAMP) content which were thought to play a role in inducing cardiac hypertrophy were measured in the perfused rat heart. The expression of renin gene was not only increased by isoproterenol with metoprolol-pretreatment but also increased by vasopressin treatment in the presence of calcium channel blocker, nifedipine or verapamil. Either prazosin alone or norepinephrine with prazosin-pretreatment significantly increased the ACE activity. However, isoproterenol with metoprolol-pretreatment significantly decreased the ACE activity. On the other hand, the ACE activity was not changed by vasopressin, nifedipine, or verapamil treatments. The content of cAMP was significantly increased by either isoproterenol or vasopressin treatment. According to these results, renin gene expression was associated with β_2- adrenoceptor and calcium channel. ACE activity was associated with α- and β_2- adrenoceptor. In conclusion, β_2- adrenoceptor was important in cardiac renin gene expression and ACE activity and α-, β-adrenergic, and calcium channel blockers might be involved in the regulation of cardiac RAS in a complicated way.

Key Words: Ventricular hypertrophy, Renin-angiotensin system

INTRODUCTION

Cardiac hypertrophy is a structural adaptation in response to increased hemodynamic and metabolic demands. This is mediated by mechanical, neurohumoral, autocrine, and paracrine factors. It is well known that a mechanical stretch causes structural and functional changes in cardiac muscles (Komuro et al, 1990; Sadoshima et al, 1993). Numerous studies have suggested that hypertrophic responses may include molecular events such as expression of fetal contractile protein (Bishopric et al, 1987), protooncogene (Starksen et al, 1986; Izumo et al, 1988), cardiac myofibrillar gene (Lee et al, 1988), and atrial natriuretic peptide gene (Lanson et al, 1992). α_1-adrenergic and β-adrenergic agonist also have known to induce a hypertrophy in ventricular myocytes (Simpson, 1983; Buxton & Brunton, 1986; Edwards et al, 1989; Fuller et al, 1990; Clark et al, 1991). The hypertrophic effect of α-adrenergic agonist is thought to be mediated by phosphatidylinositol cascade (Harsdorf et al, 1989). The hypertrophic effects of β-adrenergic agonist are mediated by the stimulation of protein synthesis via cAMP (Xenophontos et al, 1989). Additionally, the β-adrenergic agonist can induce a hypertrophic response through the stimulatory G protein (G_s) that increases the calcium influx by phosphorylation of plasma membrane calcium channels (Katz, 1992). Recently, the renin-angiotensin system (RAS) in the heart (Dzau & Re, 1987;

Corresponding to: Chang-Gyo Park, Department of Pharmacology, Keimyung University School of Medicine, 194 Dongsan-Dong, Chungku, Taegu 700-712, Korea

Dostal et al, 1992a & 1992b) provides particular interest on cardiac hypertrophic mechanism. Angiotensin converting enzyme (ACE) inhibitors are effective in heart failure with cardiac hypertrophy (Friedrich et al, 1994). The RAS exerts its modulatory influences on circulatory homeostasis not only through circulating components but also through local components in various tissues such as kidney, lung, heart, and vascular smooth muscle cells (Campbell & Habener, 1986; Dzau, 1988; Lindpaintner et al, 1990; Schunkert et al, 1990; Danser et al, 1994; Pieruzzi et al, 1995). It has been reported that there is a significant increase of angiotensinogen mRNA after initiation of left ventricular hypertrophy in the rat (Baker et al, 1990). Schunkert et al (1990) have reported that pressure overloading in the heart resulted in increases in cardiac ACE gene expression and intramyocardial conversion from angiotensin I to angiotensin II (Ang II). Ang II can stimulate protein synthesis and cell growth in cardiac tissue by increasing the intracellular calcium and inositol triphosphate ($InsP_3$) through the activation of voltage sensitive calcium channel and phospholipase C (Baker et al, 1989; Aceto & Baker, 1990; Baker & Aceto, 1990). Sadoshima et al (1993) have reported the release of Ang II in cultured cardiomyocyte. Additionally, Ang II may influence cardiac function by increasing adrenergic input to the heart because Ang II receptor binding sites are localized to the myocardium and cardiac adrenergic nerves (Urata et al, 1989). Both Ang II cardiac receptors and β-adrenergic receptors are coupled to G proteins (Allen et al, 1988). However, the effects of Ang II on the heart is likely to be mediated by different pathway because Ang II retains its positive inotrophic activity in the presence of β-adrenoceptor blockade (Kobayashi et al, 1978).

α-adrenergic agonists (Knowlton et al, 1993), β-adrenergic agonists (Clark et al, 1991), and Ang II (Sadoshima & Izumo, 1993) which were known to induce cardiac hypertrophy have been described extensively for their roles in the cardiac hypertrophy, but none of these have provided clear explanations. Therefore, This study was aimed to elucidate the relation of α-, β-adrenergic, and calcium channel blockers in the regulation of the cardiac RAS which was known to an important factor in ventricular hypertrohpy.

METHODS

Experimental animals and isolation of heart

Five male Sprague-Dawley rats weighing 250 to 300 g were used for each group. They were kept at room temperature under a 12 hour light regimen of 6 a.m. to 6 p.m. and fed regular pelleted rat chow and tap water ad libitum. The rats were sacrificed by cervical dislocation after the i.p. injection of 300 unit/kg heparin sodium. The heart was rapidly isolated after opening the thoracic cavity. The ascending aorta was cannulated with a perfusion cannula and mounted in a Langendorff apparatus. Perfusion was begun immediately using Krebs-Henseleit solution (120 mM NaCl, 20 mM $NaHCO_3$, 4.63 mM KCl, 1.17 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 8 mM glucose) at a constant flow of 10 ml/min. The buffer was equilibrated with 95% O_2-5% CO_2, held at a constant temperature of 37°C, and titrated to pH of 7.4. Experimental animals were divided into four groups. The first group was control treated with saline of equal volume. The second group was treated with norepinephrine (1 μM), prazosin (1 μM), and norepinephrine with prazosin-pretreatment. The third group was treated with isoproterenol (1 μM), metoprolol (1 μM), and isoproterenol with metoprolol-pretreatment. The fourth group was treated with vasopressin (5 unit), nifedipine (1 μM), verapamil (1 μM), and vasopressin with pretreatment of nifedipine or verapamil.

Experimental protocol

Infusion of drug was started after equilibrating the system with Krebs-Henseleit solution for 30 minutes. The duration of drug pretreatment or treatment was 10 minutes. The heart was finally dissected for analysis of renin mRNA expression, ACE activity, and cAMP assay on three hours after drug infusion.

RNA isolation and analysis

Total cellular RNA was extracted with acid guanidinium thiocyanate-phenol-chloroform from dismembrated tissue according to a modified single-step method of RNA isolation (Chomczynski & Sacchi, 1987). Left ventricular tissues were homogenized individually by a tissue homogenizer (Ultra-Turrax T25, Janke & Kunkel Co) with the RNAzol B

(Biotecx Lab Inc). The 1/10 volume of chloroform was added to the homogenate. After a vigorous vortexing, they were centrifuged at 12,000×g, 4°C for 15 minutes and the upper aqueous phase was carefully recovered. The RNA was precipitated with an equal volume of isopropanol and stored for 2 hours at −20°C. Subsequently, the precipitates were centrifuged for 15 min at 12,000×g, 4°C. The pellet was washed with cold 75% ethanol and dried under vacuum. The RNA pellet was dissolved in dimethylpyrocarbonate-treated water and measured absorbance at 260 nm by UV spectrophotometer (DU 650, Beckman).

The expression of the renin gene was determined by the reverse transcription-polymerase chain reaction (RT-PCR; RNA PCR core kit, Perkin Elmer Co). Sense and antisense primer oligonucleotides were designed from the rat renin gene (GenBank accession number: J02941 M16984, sense primer: nucleotides 433 through 450, 5'-CGGTGGTCCTCACCAAG-3', antisense primer: nucleotides 783 through 800, 5'-GCCCATGCCCAGACCCCC-3') and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (GenBank accession number: X02231 X00972, sense primer: nucleotides 318 through 342, 5'-ATCAAATGGGGTGATGCTGGTGCTG-3', antisense primer: nucleotides 798 through 822, 5'-CTGACATGCCGCCTGGAGAAACCTG -3'). After the addition of 2 μg of total RNA, 50 unit reverse transcriptase, 0.5 μM oligo d (T), 1.0 mM dNTPs, and 20 unit ribonuclease inhibitor, the reaction mixtures were placed in a thermocycler (GeneAmp system 2400, Perkin Elmer Co) at 37°C for 60 minutes, followed by incubation at 95°C for 5 minutes. The cDNA of interest was amplified with 2.0 unit of *Taq* DNA polymerase and oligonucleotide primers in a total reaction volume of 50 μl. The reaction mixtures were overlayed with 50 μl of light mineral oil to prevent evaporation. The amplification profile for renin involved denaturation at 94°C for 1 minute, primer annealing at 45°C for 1 minute, and primer extension at 72°C for 1.5 minute for 35 cycles. The amplification profile for GAPDH gene involved denaturation at 94°C for 1 minute, primer annealing at 56°C for 1 minute, and primer extension at 72°C for 1.5 minute for 30 cycles. The 10 μl of each PCR product was separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, visualized by UV transilluminator and photographed. The expression level of renin mRNA was measured using gel-document system (Biorad Co).

Measurement of ACE activity

ACE activity was measured by the method of Pinto et al (1993). Ventricular tissue was homogenized in 50 mM K_2HPO_4 homogenation buffer (pH 7.5) using tissue homogenizer (Ultra-Turrax T25, Janke & Kunkel Co). As a substrate, 100 μl of 12.5 mM hippuryl-His-Leu, which is converted by ACE into His-Leu, was added and incubated at 37°C for exactly 10 minutes. The conversion of the substrate was stopped by adding 1.45 ml of 280 mM NaOH and 100 μl of 1% phtaldialdehyde which adheres to the His-Leu product. The amount of tagged His-Leu was fluorimetrically determined at an excitation wavelength of 364 nm and an emission wavelength 486 nm using spectrofluorophotometer (RF-5301PC, Shimadzu Co).

Measurement of cAMP content

Ventricular tissures were homogenized in cold 6% trichloroacetic acid at 4°C to give 10% (w/v) homogenate, and extracts were centrifuged for 15 minutes at 2,000×g in a refrigerated centrifuge. Trichloroacetic acid in supernatants were washed three times with five volume of water-saturated ether, and the remaining ether was evaporated under a stream of nitrogen at 60°C. Dried extracts were resuspended in 500 μl of 0.05 M acetate buffer prior to analysis. cAMP content was determined by Biotrak cAMP[^{125}I] assay system (Amersham Life Science) and expressed as fmol cAMP per mg protein.

Measurement of protein content

All samples were assayed for protein content by the method of Lowry et al (1951). Bovine serum albumin was used as a standard.

Statistical analysis

Results were expressed as mean ± SEM. Statistical analysis was performed with one-way ANOVA. Scheffe's multiple comparison procedure was used as post hoc comparison. The p value less than 0.05 was considered statistically significant.

RESULTS

Expression of renin gene

In control experiments, no PCR products were generated from RNA samples in which the reverse transcriptase was omitted, indicating that the RNA samples were not contaminated by genomic DNA. The cardiac renin gene was not obtained in samples that were treated with norepinephrine, isoproterenol, or vasopressin. On the other hand, renin gene expression was increased by isoproterenol with metoprolol-pretreatment. Renin gene expression was also increased by vasopressin treatment in the presence of calcium channel blocker, nifedipine or verapamil. The patterns of cardiac renin gene expression were shown in Fig. 1 and Fig. 2.

Changes in ACE activity

To investigate the significance of α-adrenergic stimulation in cardiomyocyte ACE activity, the effects of norepinephrine, prazosin, and norepinephrine with prazosin-pretreatment were assessed. Although norepinephrine alone did not increase the ACE activity, norepinephrine with prazosin-pretreatment significantly ($p < 0.05$) increased the ACE activity compared to control (Table 1). Prazosin alone also in-

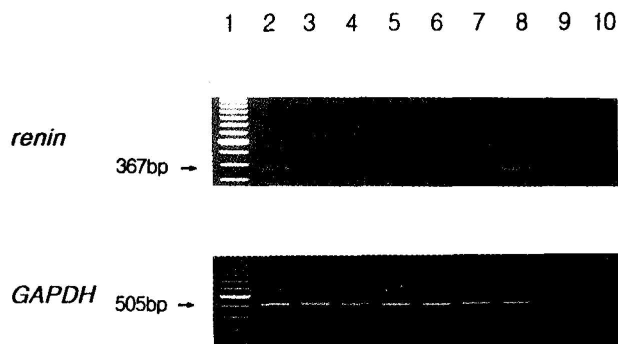

Fig. 1. Ethidium bromide-stained gel of PT-PCR products for renin mRNAs by norepinephrine, prazosin, isoproterenol, and metoprolol treatment in rat heart. Rat GAPDH mRNA was amplified for internal control. Lane 1: size marker, Lane 2: control, Lane 3: norepinephrine, Lane 4: prazosin, Lane 5: prazosin+norepinephrine, Lane 6: isoproterenol, Lane 7: metoprolol, Lane 8: metoprolol+isoproterenol, Lane 9: RT-PCR control (no mRNA), Lane 10: RT-PCR control (no reverse transcriptase).

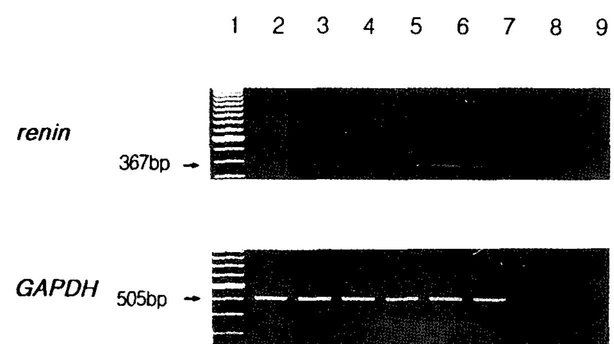

Fig. 2. Ethidium bromide-stained gel of PT-PCR products for renin mRNAs by vasopressin, nifedipine, and verapamil treatment in rat heart. Rat GAPDH mRNA was amplified for internal control. Lane 1: size marker, Lane 2: control, Lane 3: vasopressin, Lane 4: nifedipine, Lane 5: verapamil, Lane 6: nifedipine+vasopressin, Lane 7: verapamil+vasopressin, Lane 8: RT-PCR control (no m RNA), Lane 9: RT-PCR control (no reverse transcriptase).

Table 1. Changes in angiotensin converting enzyme (ACE) activity and cAMP content by norepinephrine and prazosin treatment in rat heart

	Control	Norepinephrine (1 μM)	Prazosin (1 μM)	Prazosin (1 μM) + Norepinephrine (1μM)
ACE(unit/mg protein)	13.4±0.6	16.2±1.1	18.9±0.9*	19.5±1.3*
cAMP(fmol/mg protein)	124.0±9.2	132.2±3.2	137.0±3.1	145.9±20.8

The values are mean±SEM of five rat hearts.
*$p < 0.05$ vs. control, # $p < 0.05$ vs. norepinephrine

creased the ACE acitivity significantly ($p < 0.05$) compared to control (Table 1). On the other hand, norepinephrine with prazosin-pretreatment did not significantly increase the ACE activity compared to norepinephrine alone. To investigate the significance of β-adrenergic stimulation in cardiomyocyte ACE activity, the effects of isoproterenol, metoprolol, and isoproterenol with metoprolol-pretreatment were assessed. Treatment with either isoproterenol or metoprolol did not have any effect on ACE activity of cardiomyocytes. However, isoproterenol with metoprolol-pretreatment significantly ($p < 0.05$) decreased the activity of ACE in comparison with that of control (Table 2). The ACE activity was also significantly ($p < 0.05$) decreased by isoproterenol with metoprolol-pretreatment in comparison with that of isoproterenol alone (Table 2). To study the significance of calcium channel blocker in cardiomyocyte ACE actvity, the effects of vasopressin, nifedipine, verapamil, and vasopressin with pretreatment of nifedipine or varapamil were assessed. Vasopressin did not alter the ACE activity comparing with control (Table 3). Nifedipine or verapamil had no effects on basal ACE activity of the cardiomyocytes. In the presence of nifedipine or verapamil, the response to vasopressin in the ACE activity was not altered.

Changes in cAMP content

The effects of norepinephrine, prazosin, and norepinephrine with prazosin-pretreatment were assessed to investigate the significance of α-adrenergic stimulation in cardiomyocyte cAMP content. Neither norepinephrine nor prazosin alone affected the cAMP content of the cardiomyocytes. The cAMP content by norepinephrine treatment was not significantly increased in the presence of prazosin (Table 1). The effects of isoproterenol, metoprolol, and isoproterenol with metoprolol-pretreatment were assessed to investigate the significance of β-adrenergic stimulation in

Table 2. Changes in angiotensin converting enzyme (ACE) activity and cAMP content by isoproterenol and metoprolol treatment in rat heart

	Control	Isoproterenol (1 μM)	Metoprolol (1 μM)	Metoprolol (1 μM) + Isoproterenol (1 μM)
ACE(U/mg protein)	13.4±0.6	14.6±0.1	15.8±0.3	8.8±1.0*#
cAMP(fmol/mg protein)	124.0±9.2	204.7±23.1*	132.0±10.4	138.0±22.9#

The values are mean±SEM of five rat hearts.
*$p < 0.05$ vs. control, #$p < 0.05$ vs. isoproterenol

Table 3. Changes in angiotensin converting enzyme (ACE) activity and cAMP content by vasopressin, nifedipine, and verapamil treatment in rat heart

	Control	Vasopressin (5 unit)	Nifedipine (1 μM)	Verapamil (1 μM)	Nifedipine (1 μM) + Vasopressin (5 unit)	Verapamil (1 μM) + Vasopressin (5 unit)
ACE(unit/mg protein)	13.4±0.6	16.5±1.1	16.1±1.3	16.7±0.8	16.8±1.1	15.7±0.9
cAMP(fmol/mg protein)	124.0±9.2	183.6±23.1*	116.8±6.6	150.3±8.5	191.6±4.9*	180.6±8.4*

The values are mean±SEM of five rat hearts.
*$p < 0.05$ vs. control, #$p < 0.05$ vs. vasopressin

cardiomyocyte cAMP content. Isoproterenol alone significantly ($p < 0.05$) increased the cAMP content in comparison with control (Table 2). Metoprolol alone had no effect on basal cAMP content of the cardiomyocytes. However, isoproterenol with metoprolol-pretreatment significantly ($p < 0.05$) decreased the cAMP content in comparison with that of isoproterenol alone (Table 2). Metoprolol blocked the isoproterenol-induced increase of cAMP content of the cardiomyocytes. To study the significance of calcium channel blocker in cardiomyocyte cAMP content, the effects of vasopressin, nifedipine, verapamil and vasopressin with pretreatment of nifedipine or verapamil were investigated. Vasopressin significantly ($p < 0.05$) increased the cAMP content in comparison with that of control (Table 3). In the presence of calcium channel blocker, nifedipine or verapamil, the response to vasopressin was not altered (Table 3). Nifedipine or verapamil alone had no effects on basal cAMP content of the cardiomyocytes.

DISCUSSION

The present study was aimed to investigate the effects of α-, β-adrenergics, and calcium channel blockers that might be involved in the regulation of intracardiac RAS and their integration into contractile and growth processes. Renin gene expression in the heart could confirm that cardiac renin activities reflect local renin synthesis rather than circulating renin (Soubrier et al, 1988; Schunkert et al, 1990). ACE which plays a role in local generation of Ang II was found largely in cardiovascular tissues composed of fibrillar collagen such as heart valves, the adventitia of great vessel and intramyocardial coronary arteries, and the scar by myocardial infarction (Yamada et al, 1991).

In the present study, although norepinephrine did not increase the ACE activity, but prazosin alone and norepinephrine with prazosin-pretreatment increased the ACE activity compared with control. ACE activity was increased by α-adrenoceptor blocker. The cAMP content was not increased by either prazosin alone or norepinephrine with prazosin-pretreatment. This suggested that the α-adrenoceptor was not linked to adenylate cyclase, instead, might be coupled to the activation of phosphoinositide degradation in changes of ACE activity (Katz, 1992). On the other hand, either β agonist and selective β_1 antagonist did not affect the ACE activity, however, isoproterenol with metoprolol-pretreatment significantly decreased the ACE activity in the isolated perfused rat heart. cAMP content was also decreased by isoproterenol with metoprolol-pretreatment in comparison with that of isoproterenol alone. Though neither β agonist nor β_1 antagonist increased the renin gene expression, expression of cardiac renin gene was increased when β_2-action by isoproterenol in the presence of β_1-adrenoceptor blocker. According to these results, ACE activity was related to β_2 adrenoceptor through cAMP as second messenger and renin gene expression was especially related to β_2-adrenoceptor because neither β agonist nor β_1 antagonist affected the renin gene expression. However, it needed β_1-adrenoceptor blocking at the same time. These results suggested that β_2-adrenoceptor was important in cardiac renin gene expression and ACE activity.

Ang II increases vasopression secretion (Ramsay et al, 1978). Vasopressin which potentiates ACTH secretion may be related to inducing cardiac hypertrophy. Vasopressin was known to act by increasing cytosolic free calcium in the cardiomyocyte (Xu & Gopalakrishnan, 1991). In this experiment, vasopressin did not affect the ACE activity. However, vasopressin increased the cAMP content. Increased intracellular content of cAMP has been thought to play a role in inducing cardiac hypertrophy resulting from a variety of pharmacological and physiological stimuli. Dzau and Re (1987) have detected a significant increase of renin activity in cultured cardiomyocyte by incubation with calcium channel blockers. In this experiment, renin gene expression was increased by calcium channel blockers only when growth stimulus like vasopressin, which was mediated by cAMP, was existed. When the cAMP content was increased, a decrease of calcium content by calcium channel blocker might be related to the existence of a feedback regulation to activate renin gene expression because calcium is intracelluar mediater of the Ang II action.

On the other hand, these results suggested that α-, β-adrenoceptors, and calcium channels could co-regulate the cardiac RAS. This agrees with findings, reported by Ferry and Kaumann (1987), that the density of both β-adrenoceptors and calcium channels were under co-regulation on the RAS. It is well known that calcium channel blocker could exert an α-adrenergic receptor blocking effect (Frishman et

al, 1989; Lubic et al, 1989). On the contrary, α- and β-adrenoceptor blockers could decrease slow inward calcium current (Bruckner & Scholz, 1984).

In summary, renin gene expression was associated with β_2-adrenoceptor agonist when β_1-adrenoceptor blocking existed at the same time and with calcium channel blocker when the elevation of cAMP content coexisted by vasopressin. ACE activity was associated with α-and β_2-adrenoceptor. So, β_2-adrenoceptor was important in cardiac renin gene expression and ACE activity and α-, β-adrenergic, and calcium channel blockers which were known to be related to cardiac hypertrophy coregulate the cardiac RAS in a complicated way.

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