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Down-regulation of bradykinin B_2-receptor mRNA in the heart in pressure-overload cardiac hypertrophy in the rat

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Abstract

To determine the potential role of the cardiac kallikrein-kinin system in the development of cardiac hypertrophy, we studied the expression patterns of kallikrein, kininogen, and bradykinin receptor mRNA in the heart by polymerase chain reaction during the development of pressure-overload-induced left ventricular hypertrophy (LVH) in rats. The abdominal aortic constriction produced LVH after 7, 14, and 28 days. Neither mRNA levels for high-molecular-weight (H-) or low-molecular-weight (L-) kininogens and T-kininogen, nor those for tissue kallikreins, changed during LVH. B_2-receptor mRNA levels in the left ventricles decreased 4 and 7 days after aortic constriction, subsequently returning to the levels in sham-operated animals. B_2-receptor densities in cardiac membrane preparations obtained 4 days after aortic constriction significantly decreased compared to preparations from sham-operated rats, whereas the receptor affinity was unchanged. Down-regulation of B_2-receptor mRNA levels was abolished by oral administration of an angiotensin II type 1 (AT1) receptor antagonist, candesartan, for 4 days after aortic constriction. Both cardiomyocytes and nonmyocytes obtained from neonatal rat hearts expressed B_2-receptor mRNA *in vitro*, and the levels were not changed in either cell type by culture with 1 μM angiotensin II (Ang II). However, when a mixture of cardiomyocytes and nonmyocytes was cultured with 1 μM Ang II, B_2-receptor mRNA levels decreased within 12 hr; this *in vitro* effect of Ang II was inhibited by the AT1-receptor antagonist losartan. These results indicate that the mechanical load in the myocardium caused by pressure-overload rapidly produces a down-regulation of B_2-receptor expression during the initial stage of LVH, probably mediated by activating the AT1-receptor.

Keywords: Bradykinin receptor; Kallikrein; Kininogen; Angiotensin; Aortic constriction; Left ventricular hypertrophy

1. Introduction

Clinical and experimental studies have established the therapeutic benefit of ACE inhibitors, not only in treating hypertension and congestive heart failure, but also in reducing reinfarction, limiting infarct size, and preventing reperfusion arrhythmias [1]. These cardioprotective effects of ACE inhibitors are thought to depend upon the ability to attenuate the degradation of endogenous BK and to inhibit the synthesis of Ang II from Ang I [2]. Kinins, such as BK and kallidin, are produced by the limited proteolytic activity of kallikreins on the endogenous substrate kininogens, and exert their actions through the activation of B_1 or B_2 receptors. B_1 receptors are expressed mainly in pathological conditions such as tissue injury and are thought to mediate the inflammatory and pain-producing effects of kinins [3]. B_2 receptors mediate most of the vascular effects of kinins by stimulating nitric oxide and prostacyclin synthesis. The presence of components for the generation and action of kinins, such as kininogen, tissue kallikrein, and B_2 receptors, has been demonstrated in the rat heart [4], suggesting a functional role for the cardiac kallikrein-kinin cascade.

It is well known that Ang II regulates many processes implicated in cardiovascular pathophysiology, including

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Abbreviations: ACE, angiotensin-converting enzyme; Ang, angiotensin; AT1 and AT2, angiotensin II type 1 and type 2, respectively; BK, bradykinin; BNP, brain natriuretic peptide; DME, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LVH, left ventricular hypertrophy; RT–PCR, reverse transcription–polymerase chain reaction; and TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

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growth of vascular cells and cardiac fibroblasts, migration of vascular smooth muscle cells, vascular remodeling, and cardiac hypertrophy [5–8]. On the contrary, several reports have suggested a cardioprotective action of kinins [9–11]. A recent finding by Emanueli *et al*. [12] demonstrated that mice lacking a functional B_2-receptor gene develop hypertension, left ventricular hypertrophy, and functional cardiac impairment, suggesting that kinins play an important role in the functional and structural preservation of the heart. Furthermore, an AT1-receptor antagonist prevented ventricular remodeling and myocardial damage in mice lacking the B_2-receptor gene [13], suggesting that BK inhibits Ang II-induced cardiac remodeling. Thus, the cardiac kallikrein-kinin system has been implicated not only in the action of ACE inhibitors but also in the progression of cardiac hypertrophy. However, little is known about the expression profile of the system in the development of cardiac hypertrophy.

We hypothesized that the cardiac kallikrein-kinin system may be altered during the development of heart failure, and may contribute to the pathophysiology of this disease state. To test this hypothesis, we investigated the mRNA levels of components of the kallikrein-kinin cascade in the left ventricles of rats after pressure-overload caused by aortic constriction. Our results indicate that B_2-receptor expression in the left ventricle transiently decreases within 1 week after pressure-overload and that this decrease is probably mediated by AT1-receptor activation.

2. Materials and methods

2.1. Materials

The following materials were purchased from commercial sources: collagenase II (Worthington); Percoll (Amersham Phamacia Biotech); [^{125}I-Tyr^8]BK (Peninsula); monoclonal antibody against β-myosin heavy chain, captopril, and bacitracin (Sigma Aldrich); Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co.); pancreatin and reverse transcriptase (Invitrogen); CGP42112 [*N*-α-nicotinoyl-Tyr-Lys-(benzyloxycarbonyl-Arg)-His-Pro-Ile], Hoe 140 (D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]-bradykinin), and des-Arg^9-BK (Peptide Institute); osmotic minipump (Alzet); and *Taq* DNA polymerase (Ampli *Taq* Gold; Applied Biosystems). Alacepril, losartan, and candesartan were supplied by the Dainippon Pharmaceutical Co., Ltd., the DuPont Merck Pharmaceutical Co., and the Takeda Pharmaceutical Co., Ltd., respectively.

2.2. Ethical approval for animal experimentation

All animal experiments were performed according to the guidelines of the Kobe Gakuin University Experimental Animal Care and Use Committee.

2.3. Abdominal aorta constriction

Male Wistar rats, weighing 100–120 g, were obtained from SLC Japan. The animals were anesthetized with sodium pentobarbital (40 mg/kg, i.p.), and the aorta was exposed through a midline abdominal incision. For the constriction model, a blunt 22-gauge needle was placed adjacent to the abdominal aorta between the renal arteries just below the renal bifurcations, and a ligature was tightened around the aorta and adjacent needle. The sham procedure for control rats included injection of the same amount of anesthetic, an incision of approximately the same size, and the placement of a loosely tied ligature at exactly the same position on the abdominal aorta. The effectiveness of the aortic constriction was assessed by measuring carotid arterial pressure under pentobarbital anesthesia: systolic blood pressure increased significantly in rats 4 days after aortic constriction compared with that of sham-operated controls (128.3 ± 5.9 mmHg vs 101.7 ± 1.6 mmHg; $P < 0.01$, $N = 5$), and the administration of an AT1-receptor antagonist, candesartan (1 mg/kg/day, p.o.), for 4 days did not affect the elevation of systolic blood pressure following aortic constriction (126.0 ± 5.8 mmHg; $P > 0.1$ compared with aorta-constricted animals not receiving candesartan, $N = 5$).

2.4. Culture of neonatal rat cardiomyocytes and nonmyocytes

Sprague–Dawley rats (2- to 4-day-old) were used for the isolation of cardiomyocytes and nonmyocytes. The ventricular cardiac cells were dispersed in Hanks' balanced salt solution containing 0.04% collagenase II and 0.06% pancreatin as previously reported [14]. Cardiomyocytes and nonmyocytes were collected separately by the discontinuous Percoll gradient method [14]. Purified cells were plated at a density of 3×10^4/cm^2 in DMEM containing 10% FBS. After subculture for 24 hr, the medium was replaced with fresh DMEM containing 10% FBS with or without various reagents. After culture, cells were harvested to extract total RNA. Using this method, we routinely obtained contractile cardiomyocyte-rich cultures with 90–95% myocytes as assessed using immunocytochemistry with a monoclonal antibody against the β-myosin heavy chain.

2.5. Separation of cardiomyocytes and nonmyocytes from rats with aortic constriction

Cardiomyocyte- and nonmyocyte-rich preparations were obtained from rats 4 days after aortic constriction as follows. The excised left ventricle was digested in a salt solution containing 137 mM NaCl, 5.4 mM KCl, 5.6 mM glucose, 4.2 mM $NaHCO_3$, 0.05% collagenase II, and 0.25% trypsin (pH 7.4). The reaction was terminated by adding 1/10 vol. of FBS, and dispersed cells were collected

by centrifugation at 600 *g* for 5 min at 4°. Cardiac cells were suspended in DMEM containing 10% FBS, and then incubated in a culture dish for 1 hr at 37°. The supernatant was transferred to another dish, and then incubated for an additional 1 hr. Cells that adhered to the dish were collected as cardiac fibroblasts, the rest as cardiomyocytes.

2.6. Detection of mRNAs for kininogens, tissue kallikreins, BK receptors, and BNP

Animals were killed under ether anesthesia 4, 7, 14, and 28 days after aortic constriction or sham treatment. Age-matched untreated rats were killed as a control. The excised myocardium was quickly frozen in liquid nitrogen and stored at −80° until RNA extraction. Total RNA of isolated myocardium or cultured cardiomyocytes was extracted with acid guanidinium–phenol–chloroform. To detect mRNAs for kininogens, such as high-molecular-weight (H-), low-molecular-weight (L-), and T-kininogens, and tissue kallikreins, such as *rKLK1* and *rKLK10*, we used RT–PCR followed by Southern blotting with respective specific probes as described previously [15]. B_1- and B_2-receptor mRNAs in myocardium or cardiomyocytes were detected by RT–PCR and then by Southern blotting as follows. Total RNA (1 μg) was reverse-transcribed in a 20-μL reaction mixture containing 20 pmol of the reverse primer, 2 μL of 10 mM dNTP, 2 μL of 10× PCR buffer (0.1 M Tris–HCl, pH 8.3, 500 mM KCl), 4 μL of 25 mM $MgCl_2$, 1 unit of RNase inhibitor, and 20 units of cloned Moloney murine leukemia virus reverse transcriptase. The RT reaction mixture was incubated at 42° for 15 min, at 95° for 5 min, and then at 4° for 5 min to allow synthesis of the first strand of cDNA. The cDNA was amplified in a 100-μL reaction mixture containing 20 pmol of the forward primer, 8 μL of 10× PCR buffer, 4 μL of 25 mM $MgCl_2$, and 2.5 units of *Taq* DNA polymerase. Thirty-five (B_2-receptor) or forty (B_1-receptor) cycles proceeded as follows: denaturation at 95° for 1 min, annealing at 60° for 2 min, and extension at 72° for 3 min. Then 8-μL aliquots were Southern blotted and autoradiographed using a Fujix Bio Imaging Analyzer BAS2000 (Fuji Film). The forward and reverse primers for the B_1-receptor were: 5′-AGAAACCTCCCAAGACAGCA-3′ (nucleotides 26–45) and 5′-AGGAATGTGGGGATGCTCAA-3′ (nucleotides 679–698), respectively. The forward and reverse primers for the B_2-receptor were: 5′- GGACCATGAAGGACTACAGG-3′ (nucleotides 891–910) and 5′-TAGGCCACGTAGGAACTGAT-3′ (nucleotides 1250–1269), respectively. A specific oligonucleotide for the B_1-receptor (5′-AGTACAGGAGGTCAAAT-3′) was used as an internal probe for hybridization. The probe for Southern hybridization of the B_2-receptor was a 400-bp fragment of B_2-receptor cDNA. The *in vivo* experiments were performed two times with 4–6 animals per group, and the *in vitro* experiments four times for each treatment. Representative results of the data are shown on blots in each figure.

For quantitative analysis of BNP mRNA, RNA samples were subjected to Northern blotting using BNP cDNA as a probe, and the levels were quantified with a Fujix Bio Imaging Analyzer BAS2000. Values represent the ratio of BNP mRNA to 18S rRNA in each sample normalized to the mean value of the ratio in untreated rats on each day.

2.7. Myocardial membrane preparation from rat left ventricles

Myocardial membranes were prepared from left ventricles of sham-operated or aorta-constricted rats 4 days after the operation. The tissue was trimmed free of atria, great vessels, and connective tissue, and then was homogenized three times for 15 sec on ice with a Polytron homogenizer (Kinematica AG) at half-maximum speed in 20 vol. of homogenization buffer (25 mM TES buffer, pH 6.8, containing 300 mM sucrose, 1 mM 1,10-phenanthroline, and 140 μg/mL bacitracin). The homogenate was sedimented twice at 500 *g* for 10 min at 4° to remove unbroken cells, nuclei, and cell debris. The supernatant was centrifuged at 40,000 *g* for 20 min at 4°, and the membrane fraction in the precipitate was washed in 10 vol. of homogenization buffer and resedimented. The final pellet was resuspended in homogenization buffer supplemented with 10 μM captopril and 0.2% BSA (incubation buffer) to obtain approximately 1–3 mg of membrane protein/mL.

2.8. BK binding assay

[^{125}I-Tyr^8]BK was used to assay the myocardial membrane preparations for specific BK binding sites. Approximately 0.2 mg of myocardial membrane protein was incubated in polyethylene tubes at 4° with increasing concentrations of [^{125}I-Tyr^8]BK between 50 and 4000 pM in a total volume of 0.5 mL of incubation buffer for 2 hr. Nonspecific binding, described as the amount of [^{125}I-Tyr^8]BK bound in the presence of 1 μM unlabeled BK, was subtracted from all counts to yield the specific binding. After equilibrium was reached, the binding reactions were terminated by rapid filtration over Whatman GF/B glass fiber filters (presoaked for >2 hr in 0.2% BSA). The test tubes and filters were washed three times with 2–3 mL of ice-cold 25 mM TES buffer, pH 6.8, and the filters were then placed in a gamma counter. The Scatchard equation bound/free $= (B_{max}/K_d) - (\text{bound}/K_d)$, where B_{max} and K_d are maximal binding site density and affinity, respectively, was used to calculate B_{max} and K_d. Competitive binding experiments were performed as follows: myocardial membrane was incubated with 200 pM [^{125}I-Tyr^8]BK in the presence or absence of increasing concentrations of unlabeled BK, the B_2-receptor antagonist Hoe 140, or the B_1-receptor agonist des-Arg^9-BK for 2 hr at 4° in 0.5 mL of incubation buffer.

2.9. Ang II infusion

An osmotic minipump filled with Ang II or vehicle only (0.01 M acetic acid) was implanted subcutaneously through a l-cm midline incision of the dorsal skin of male Wistar rats, weighing 100–120 g, under ether anesthesia; the calculated infusion rate of Ang II was 100 ng/100 g body weight/min. Two and four days after implantation, the animals were killed, and total RNA was extracted from left ventricles.

2.10. Statistical analysis

Group data are expressed as means ± SEM. Statistical analysis was performed by ANOVA. Differences were considered significant at a value of $P < 0.05$.

3. Results

3.1. Induction of LVH after aortic constriction

The effects of aortic constriction on left ventricular weight and BNP mRNA expression in left ventricles are shown in Fig. 1. An apparent hypertrophy shown by the ratio of left ventricular weight to heart weight developed at 7, 14, and 28 days, which was supported by increased amounts of BNP mRNA, generally accepted as a marker for ventricular hypertrophy. Treatment with the ACE inhibitor alacepril (10 mg/kg/day, p.o.) or the AT1-receptor antagonist candesartan (1 mg/kg/day, p.o.) for 7 and 28 days significantly inhibited the development of LVH (data not shown).

3.2. Changes in the mRNA levels of kininogens, kallikreins, and BK-receptors after aortic constriction

Detection of mRNAs of the kallikrein-kinin components in left ventricles was carried out using RT–PCR of RNA samples extracted from left ventricles 4, 7, 14, and 28 days after aortic constriction or sham-operation followed by Southern blotting. Untreated rats were used as the control. As shown in Fig. 2, no signal for H-kininogen or B_1-receptor mRNA was observed in the left ventricle of untreated, sham-operated, or aorta-constricted animals. For detection of T- and L-kininogen mRNAs, we used a set of primers common to both kininogen mRNAs, and thus the RT–PCR products were a mixture of cDNAs amplified from T- and L-kininogen mRNAs as described previously [15,16]. Faint signals corresponding to a mixture of T- and L-kininogen mRNAs were observed in left ventricles, but there was no difference in their levels between the three groups of animals. We also could not find any differences in two tissue kallikrein mRNA signals, *rKLK1* and *rKLK10*, in left ventricles between these groups of animals. However, the signal intensity for B_2-receptor mRNA apparently decreased at 4 and 7 days after aortic constriction, whereas no differences were observed in these levels between sham-operated and aorta-constricted animals after 14 and 28 days (Fig. 2). In contrast to B_2-receptor mRNA levels in left ventricles, B_2-receptor mRNA levels in right ventricles were not changed by aortic constriction (Fig. 3).

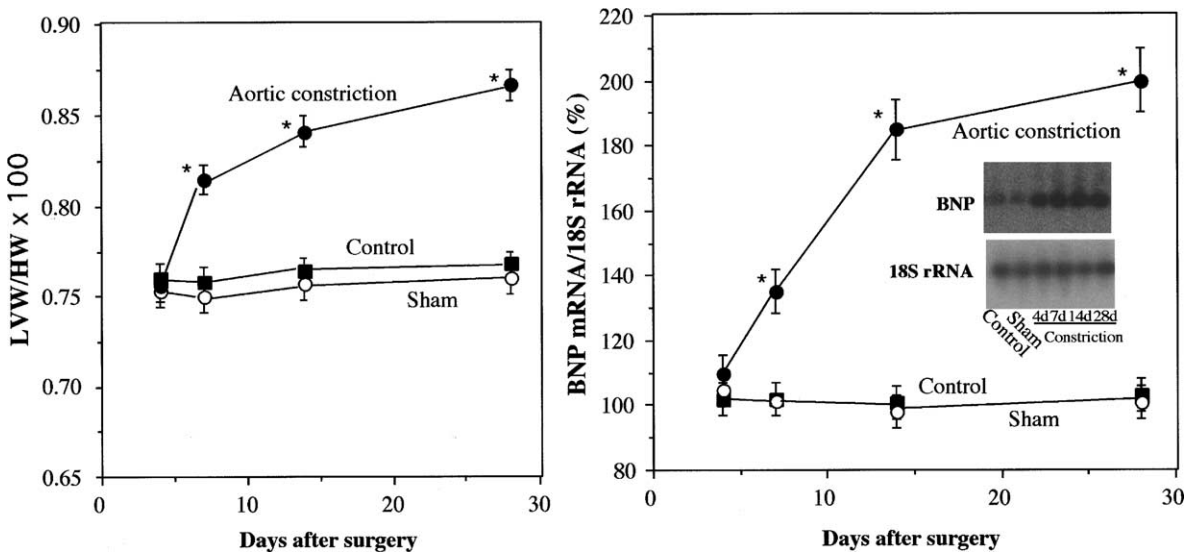

Fig. 1. Left ventricular weight to heart weight ratios and brain natriuretic peptide (BNP) mRNA levels in left ventricles in rats after aortic constriction. Left ventricular weight to heart weight ratios (left panel) and left ventricular BNP mRNA to 18S rRNA ratios (right panel) were measured in rats with aortic constriction (closed circles), sham-operated age-matched animals (open circles) and untreated age-matched control (closed squares). Data are means ± SEM (N = 6). The inset in the right panel shows a representative Northern blot of BNP mRNA and 18S rRNA in left ventricles of rats after aortic constriction or 14 days after sham-operation. Key: (*) $P < 0.05$ when compared with the control.

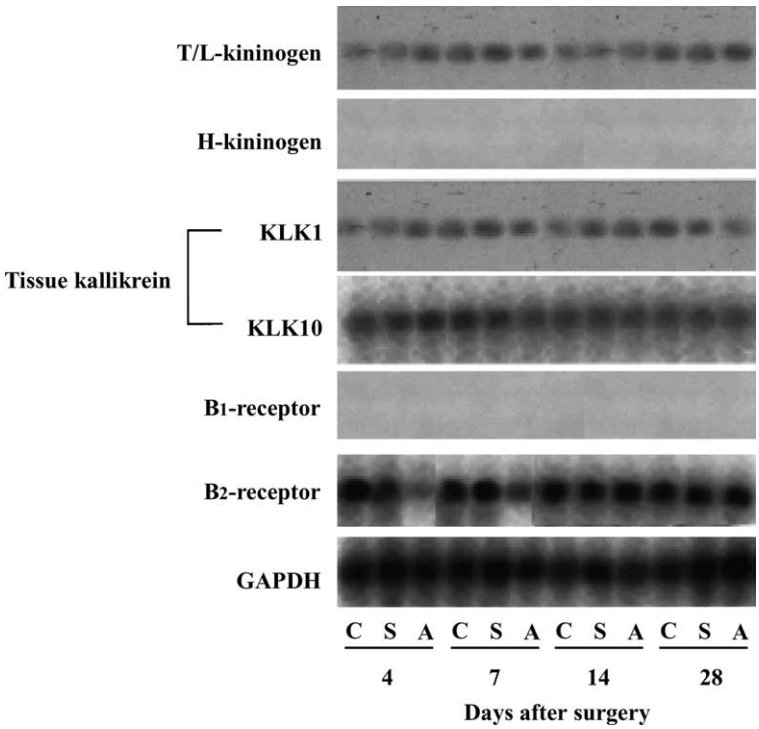

Fig. 2. Representative Southern blots of RT–PCR products amplified from mRNAs of kininogens, kallikreins, and BK receptors in rat left ventricles. The mRNAs for kininogens (T- and/or L- and H-kininogens), kallikreins (*rKLK1* and *rKLK10*), BK receptors (B_1- and B_2-receptors), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in left ventricles from untreated control (C), sham-operated (S), and aorta-constricted (A) rats were reverse-transcribed and amplified with specific primers; then amplified products were detected by Southern blotting using cDNAs. Data show representative blots of 5 control, 5 sham-operated, and 5 aorta-constricted animals.

3.3. B_2-receptor density and affinity of myocardial membranes from rats with or without aortic constriction

Figure 4 shows the saturation binding study of [^{125}I-Tyr^8]BK and ventricular membranes obtained from rats with or without aortic constriction. Myocardial membranes of rats with aortic constriction were prepared from left ventricles 4 days after surgery. Saturation was attained at approximately 2000 pM [^{125}I-Tyr^8]BK in membrane preparations from both sham-operated and treated rats.

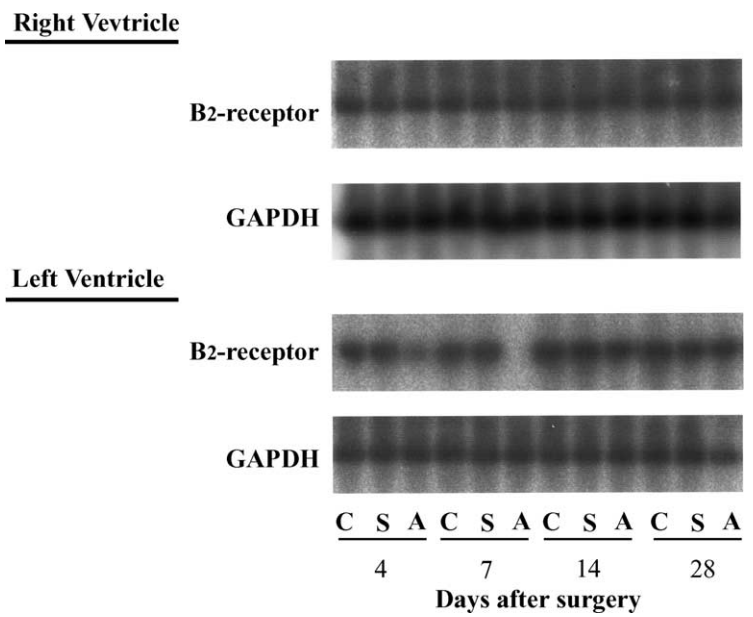

Fig. 3. Decreased levels of B_2-receptor mRNA in left ventricles but not in right ventricles after aortic constriction. Total RNA from left and right ventricles from sham-operated or aorta-constricted rats was amplified by RT–PCR using specific primers for B_2-receptors. Data show representative Southern blots of 4 sham-operated and 4 aorta-constricted rats. C, untreated control animals; S, sham-operated animals; A, aorta-constricted animals.

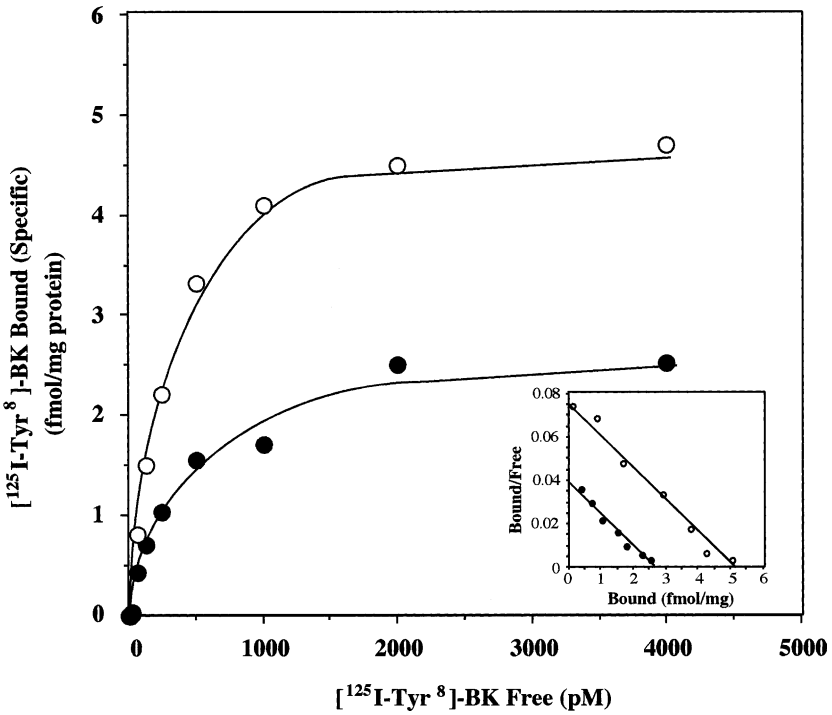

Fig. 4. Saturation of [^{125}I-Tyr^{8}]BK binding to myocardial membranes isolated from left ventricles of rats with or without aortic constriction. Myocardial membranes were prepared from left ventricles of rats 4 days after sham-operation (○) or aortic constriction (●). The inset shows Scatchard analysis of the same data. These results are from a single experiment performed in duplicate for each membrane preparation from sham-operated or aorta-constricted rats, which are representative of four independent assays for either 4 sham-operated or 4 aorta-constricted rats. The K_d was calculated as 0.25 ± 0.04 nM in the 4 sham-operated vs 0.24 ± 0.04 nM in the 4 aorta-constricted rats. The $B_{max}$was 5.1 ± 0.5 fmol/mg protein in the 4 sham-operated vs 2.7 ± 0.3 fmol/mg protein in the 4 aorta-constricted rats.

Scatchard transformation of the data (Fig. 4 inset) revealed two parallel lines, indicating that both membrane preparations have a single class of receptors with the same K_d (0.25 ± 0.04 nM in 4 sham-operated vs 0.24 ± 0.04 nM in 4 aorta-constricted rats). The data also demonstrated that the binding sites on membranes from treated rats (2.7 ± 0.3 fmol/mg protein; $N = 4$) numbered about half of those on membranes from sham-operated rats (5.1 ± 0.5 fmol/mg protein; $N = 4$). [^{125}I-Tyr^{8}]BK was displaced from these membrane preparations by BK (10^{-12}–10^{-7} M) and Hoe 140 (10^{-12}–10^{-6} M), but not by des-Arg^{9}-BK (10^{-12}–10^{-7} M), suggesting that the binding is attributable to the B_2-receptor (data not shown).

3.4. B_2-receptor mRNA levels in cardiomyocytes and nonmyocytes prepared from rats with aortic constriction

Myocardium is composed of cardiomyocytes, fibroblasts, vascular smooth muscle cells, and endothelial cells. To determine which type of cell is responsible for the down-regulation of the B_2-receptor, cardiac cells prepared from rats 4 days after aortic constriction were separated into cardiomyocyte- and nonmyocyte-rich preparations; then B_2-receptor mRNA levels were compared with those in respective preparations from sham-operated rats. As shown in Fig. 5, decreased levels of B_2-receptor mRNA were observed in cardiomyocyte-rich but not in nonmyocyte-rich preparations, suggesting that cardiomyocytes are responsible for the down-regulation of cardiac B_2-receptor after aortic constriction.

3.5. Effect of candesartan on the aorta constriction-induced down-regulation of B_2-receptor mRNA expression

Aortic constriction decreases blood pressure distal to the constriction and stimulates the kidneys to secrete renin,

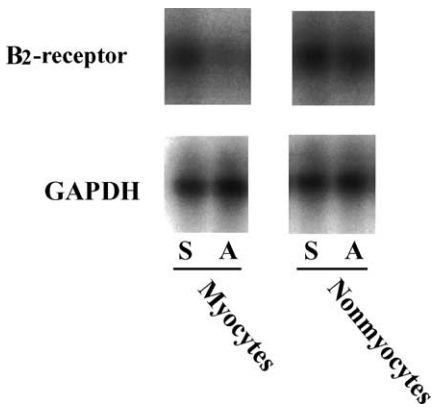

Fig. 5. Decreased levels of B_2-receptor mRNA in cardiomyocyte-rich but not nonmyocyte-rich preparations from rat myocardium after aortic constriction. Left ventricular myocardium obtained from rats 4 days after sham-operation or aortic constriction was separated into cardiomyocyte- and nonmyocyte-rich preparations, and B_2-receptor mRNA was detected by RT–PCR followed by Southern blotting. Data show representative blots of 4 sham-operated (S) and 4 aorta-constricted (A) animals.

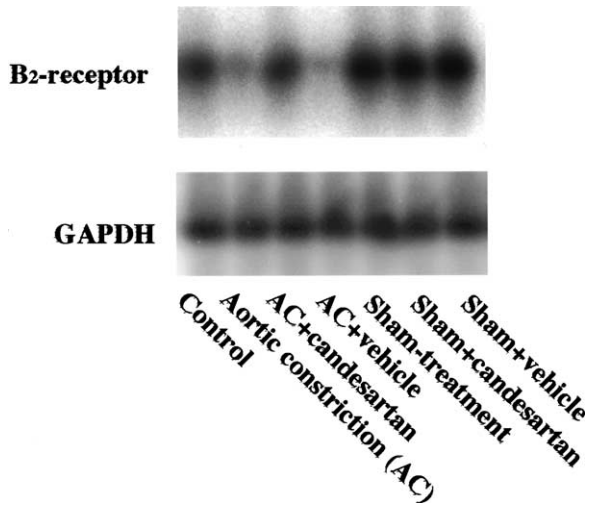

Fig. 6. Effect of candesartan, an AT1-receptor antagonist, on the aorta constriction-induced decrease in left ventricular B_2-receptor mRNA levels. Candesartan (1 mg/kg/day, p.o.) was administered to sham-operated and aorta-constricted (AC) rats for 4 days after the operation; then left ventricular B_2-receptor mRNA was detected by RT–PCR followed by Southern blotting. Experiments were carried out using 6 animals in each group, and data are representative of the results.

resulting in increased circulating levels of Ang II in a period of 3–4 days [17,18], suggesting that circulating Ang II may be responsible for the down-regulation of the cardiac B_2-receptor. To test this possibility, the effects of the AT1-receptor antagonist candesartan on the aorta constriction-induced reduction in B_2-receptor mRNA and of Ang II-infusion on cardiac B_2-receptor mRNA levels were examined. When candesartan (1 mg/kg/day, p.o.) was administered for 4 days after aortic constriction, no reduction in cardiac B_2-receptor mRNA levels was observed (Fig. 6). An infusion of Ang II (100 ng/100 g body weight/min) for 2 or 4 days in untreated rats did not alter B_2-receptor mRNA levels in the left ventricles (data not shown).

3.6. Down-regulation of B_2-receptor mRNA expression in cultured myocytes by Ang II in vitro

The effect of Ang II on B_2-receptor expression was studied *in vitro* using cultures of cardiomyocytes and nonmyocytes from neonatal rats. As shown in representative Southern blots in Fig. 7, both cultures expressed B_2-receptor mRNA. The addition of Ang II at 1 μM did not alter B_2-receptor mRNA levels in cardiomyocytes and nonmyocytes for up to 48 hr in cultures. However, when cardiomyocytes and nonmyocytes were mixed at a ratio of 3:1, respectively, and were cultured with 1 μM Ang II, B_2-receptor mRNA levels in these cells decreased at 6 and 12 hr, and slightly at 24 hr. The Ang II-induced down-regulation of B_2-receptor mRNA in cocultured cells was abolished by the AT1-receptor antagonist losartan (1 μM) but not by the Ang II type 2 (AT2-) receptor antagonist CGP42112 (1 μM). Neither antagonist alone affected B_2-receptor mRNA levels.

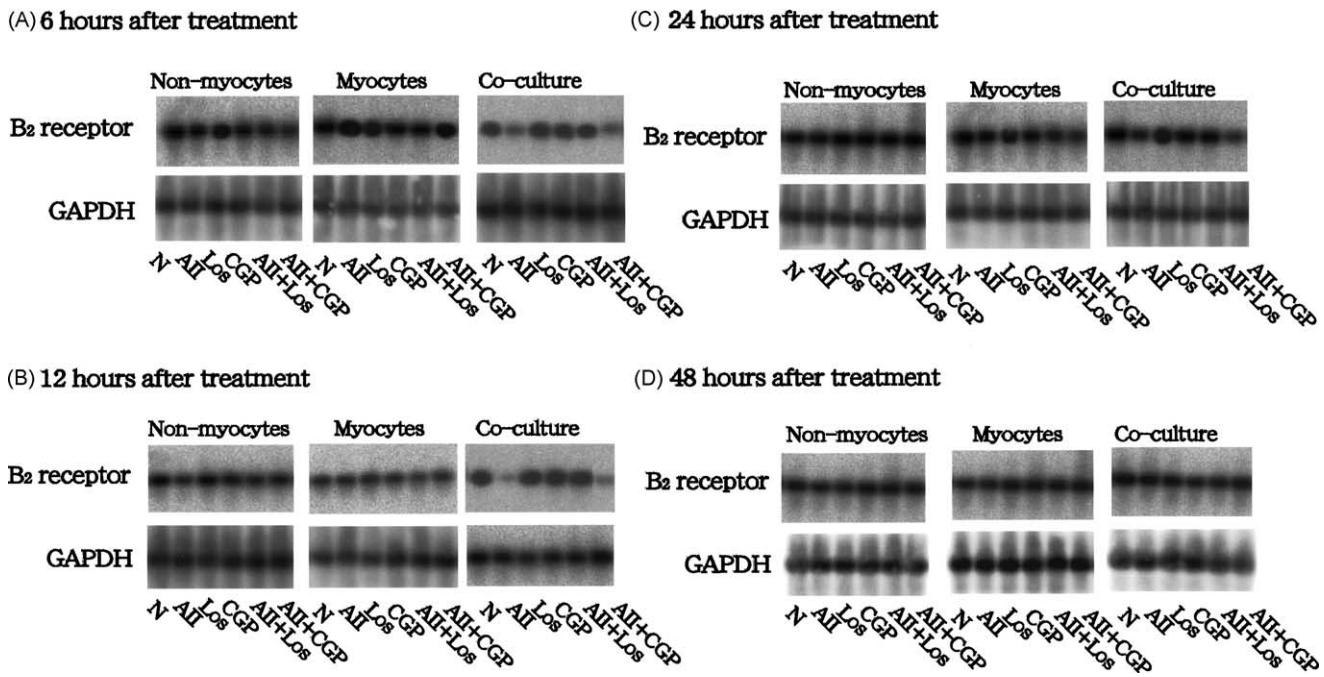

Fig. 7. Effect of Ang II on B_2-receptor mRNA expression in cultured cardiomyocytes, nonmyocytes, and their combination *in vitro*. Cardiomyocytes and nonmyocytes were prepared from neonatal rats as described in the text. These cells or a mixture of them (3:1, cardiomyocytes to nonmyocytes) were cultured with or without 1 μM Ang II for 6, 12, 24, and 48 hr (A–D) in the presence or absence of the AT1-receptor antagonist losartan (1 μM), or the AT2-receptor antagonist CGP42112 (1 μM). B_2-receptor mRNA levels were determined by RT–PCR followed by Southern blotting. Experiments were carried out using 4 cultures for each treatment, and data are representative of the results. N, non-treated; AII, angiotensin II; Los, losartan; and CGP, CGP42112.

4. Discussion

The present study demonstrates that the acute pressure-overload induced by aortic constriction results in a down-regulation of the B_2-receptor in the left ventricle. The findings that an AT1-receptor antagonist inhibited its down-regulation *in vivo* and that exogenous Ang II caused a decrease in B_2-receptor mRNA levels in cultured cardiac cells *in vitro* suggest a potential role for Ang II in the regulation of cardiac B_2-receptor expression after an acute pressure-overload.

As demonstrated by previous reports [17,18] and the present study, rats develop significant left ventricular hypertrophy 7 days after aortic constriction. In contrast, the levels of B_2-receptor mRNA were decreased at 4 and 7 days, subsequently returning to normal. Thus, a reduction in cardiac B_2-receptor mRNA was seen only in the initial stages of pressure-overload-induced hypertrophy, and not in subsequent stages characterized by remodeling processes such as an increase in both contractile and extracellular matrix protein synthesis, depressed cardiac output, a redistribution of regional blood flow, and renal sodium avidity [18,19]. Interestingly, the down-regulation of B_2-receptor mRNA was not found in the right ventricle, suggesting that the mechanical stress on the left ventricular wall directly or through a liberation of local paracrine and autocrine factors influences the gene expression of the B_2-receptor in the myocardium.

In animal models of aortic constriction, the decrease in blood pressure distal to the constriction causes renin secretion from the kidneys in a period of just 3–4 days [17,18]. Therefore, it is possible that the increase in circulating Ang II secondary to aortic constriction stimulates the myocardium to down-regulate B_2-receptor expression. The finding that the administration of the AT1-receptor antagonist candesartan inhibited the down-regulation of the cardiac B_2-receptor after aortic constriction supports this possibility. However, an infusion of Ang II for 2 or 4 days produced no significant reduction in B_2-receptor mRNA levels in the myocardium. In addition, no down-regulation of the B_2-receptor was seen in the right ventricle, which had also been exposed to increased levels of circulating Ang II after aortic constriction. Thus, it seems unlikely that the increase in circulating Ang II secondary to aortic constriction contributes to the down-regulation of the B_2-receptor in the left ventricle. Moreover, there were no differences in carotid arterial pressure in candesartan-treated or untreated aorta-constricted animals, indicating that the ability of the AT1-receptor antagonist to prevent the down-regulation of the B_2-receptor following aortic constriction is not due solely to changes in afterload.

It has been well documented that there is an intracardiac renin-angiotensin system, which may play an important role in the development of cardiac hypertrophy through the AT1-receptor [20]. In rat cardiomyocytes, exposure to mechanical stretch results in an increased expression of cardiac renin-angiotensin components, such as angiotensinogen and AT1-receptor [17,21–23], suggesting a possible involvement of locally generated Ang II in the down-regulation of cardiac B_2-receptors. Although an infusion of Ang II for 2 or 4 days did not influence cardiac levels of B_2-receptor mRNA, we cannot exclude the possibility that the dose of Ang II (100 ng/100 g body weight/min) that we examined was insufficient to elevate the intracardiac Ang II concentration required for B_2-receptor down-regulation. In fact, Ang II induced a reduction of B_2-receptor mRNA levels in mixed cultures of cardiomyocytes and nonmyocytes, and the effect was inhibited by the AT1-receptor antagonist losartan but not by the AT2-receptor antagonist CGP42112. This evidence suggests that the mechanical stress on the ventricular wall results in the activation of the intracardiac renin-angiotensin system, which, in turn, causes the down-regulation of the cardiac B_2-receptor through the AT1-receptor.

Down-regulation of B_2-receptors was confirmed not only from mRNA levels but also from the capacity to bind ligand. The ventricular preparations for these measurements contained, in addition to RNA or membrane preparations from cardiomyocytes, those from nonmyocytes with the majority representing cardiac fibroblasts. Since B_2-receptors are localized in both cardiomyocytes and nonmyocytes [24,25], the B_2-receptor mRNA and the BK binding sites that we identified on the left ventricle could have come from several different cell types. However, as shown in the present study, the decreased levels of B_2-receptor mRNA were observed in cardiomyocyte- but not in fibroblast-enriched preparations obtained from the left ventricles of aorta-constricted rats, suggesting an involvement of cardiomyocytes in the down-regulation of B_2-receptors.

An unexpected finding was that Ang II was ineffective or only partially effective in reducing the levels of B_2-receptor mRNA in either nonmyocyte or cardiomyocyte cultures, respectively. The discrepancy suggests the potential involvement of a paracrine mechanism in Ang II-induced B_2-receptor down-regulation between cardiomyocytes and nonmyocytes. As discussed above, our *in vivo* data indicated an involvement of cardiomyocytes in the down-regulation, suggesting a potential role for nonmyocytes in the Ang II-induced paracrine mechanisms of cardiac B_2-receptor down-regulation. In fact, AT1-receptors are nearly absent in neonatal cardiomyocytes and are localized predominantly on cardiac fibroblasts [20]. Ang II has been shown to stimulate cardiac fibroblasts to release transforming growth factor-β1, endothelin-1 [26,27], and the interleukin-6 family of cytokines, such as interleukin-6, leukemia inhibitory factor (LIF), and cardiotrophin-1 [28]. Of these factors, endothelin-1, LIF, and cardiotrophin-1 have been demonstrated to mediate Ang II-induced cardiomyocyte hypertrophy [27,28]. The role of these factors in the pressure overload- and/or Ang II-induced down-regulation of B_2-receptors needs to be determined.

It is well known that Ang II has positive inotropic and chronotropic effects on the heart [29,30]. In contrast, BK has negative inotropic and chronotropic effects on cultured neonatal rat cardiomyocytes [31]. One could speculate that mechanical stress on the heart results in the local liberation of Ang II, which might depress BK signaling in cardiomyocytes via the down-regulation of the B_2-receptor. Since endogenous BK seems to antagonize Ang II activities, such as the increase in collagen synthesis and oxygen consumption and the promotion of cardiomyocyte hypertrophy, a transient suppression of B_2-receptor levels in the myocardium after acute pressure-overload may be one of the compensatory mechanisms for maintaining cardiac functions. Although it is necessary to perform further experiments in order to clarify the exact mechanism of B_2-receptor down-regulation, this study reveals the interaction and importance of the cardiac renin-angiotensin system and the kallikrein-kinin system in the pathophysiological responses of the heart.

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