Role of bradykinin-NO pathway in prevention of cardiac hypertrophy by ACE inhibitor in rat cardiomyocytes

YUTAKA ISHIGAI, TOMOHIRO MORI, TOSHIKO IKEDA,
AKIKO FUKUZAWA, AND TOSHIRO SHIBANO
New Product Research Laboratories II, Tokyo Research and Development Center, Daiichi Pharmaceutical Company, Edogawa-ku, Tokyo 134, Japan

Ishigai, Yutaka, Tomohiro Mori, Toshiko Ikeda, Akiko Fukuzawa, and Toshiro Shibano. Role of bradykinin-NO pathway in prevention of cardiac hypertrophy by ACE inhibitor in rat cardiomyocytes. *Am. J. Physiol.* 273 (*Heart Circ. Physiol.* 42): H2659–H2663, 1997.—To examine whether the bradykinin-nitric oxide (NO) pathway directly participates in the antihypertrophic property of angiotensin-converting enzyme (ACE) inhibitors in congestive heart failure, the effects of bradykinin were studied in rat cultured heart cells. Bradykinin (0.1, 1 nM) prevented the phenylephrine-induced increase in protein/DNA content, an index of hypertrophy of heart cells, and amplified the nitrite/nitrate content in the medium. Perindoprilat (1 μM), an ACE inhibitor, also restrained the progression of cardiac hypertrophy and augmented NO release. These effects of perindoprilat were abolished by HOE-140 (kinin B_2 antagonist), N^{ω}-nitro-L-arginine (NO synthase inhibitor), and methylene blue (guanylate cyclase inhibitor). Furthermore, there was a significant correlation between protein/DNA content and nitrite/nitrate content. These results indicate that bradykinin inhibits the progression of cardiac hypertrophy due to the increase in NO release and that perindoprilat produces beneficial effects on cardiac hypertrophy by stimulating the bradykinin-NO pathway.

rat neonatal cardiomyocytes; nitric oxide; perindopril

CARDIAC HYPERTROPHY is an adaptive response to increasing cardiac overload (14). Several studies have demonstrated that angiotensin-converting enzyme (ACE) inhibitors attenuate the progression of hypertrophy in experimental models of heart failure (5, 18, 25), suggesting a crucial role of the renin-angiotensin system in the induction of cardiac hypertrophy. In addition, bradykinin may participate in the beneficial effects of ACE inhibitors, because HOE-140 (kinin B_2-receptor antagonist) abolished the reduction of cardiac hypertrophy by ramipril in the rat aortic-banding model (8). ACE inhibitors prevent the degradation of bradykinin into nonactive metabolites by inhibition of kininase II (ACE) (9). The activation of kinin B_2 receptors by bradykinin stimulates the release of nitric oxide (NO) in the endothelial cells (11, 21), which can explain the hypotensive actions of this peptide. Furthermore, NO modifies the structure of vascular tissues by inhibiting the growth of vascular smooth muscle cells (16). Recent reports have shown the expression of NO synthases (NOS) in the myocardium (22) and that NO is likely to regulate cardiac contractility (1, 3). However, there is little direct evidence that NO release by bradykinin affects the progression of hypertrophy in cardiomyocytes. Therefore, the present study was designed to examine whether bradykinin induces the production of NO and inhibits the progression of hypertrophy in the cultured heart cells. In addition, we tested the hypothesis that the activation of the bradykinin-NO pathway is involved in the inhibition of cardiac hypertrophy by the ACE inhibitor perindoprilat.

MATERIALS AND METHODS

Cell culture of cardiomyocytes. Animals were treated according to the guidelines for animal experimentation prepared by the Japanese Association for Laboratory Animal Science. Primary culture of rat neonatal cardiomyocytes was prepared by the method of Simpson (24) with modifications. Briefly, the ventricles were separated from 1-day-old neonatal Sprague-Dawley rats (Nihon SLC, Shizuoka, Japan). After the ventricles were digested with 0.1% collagenase and 0.1% trypsin, the dispersed cells were preplated twice for 1 h each to remove nonheart cells. The cells that did not attach to the plate were cultured at a density of 10^6 cells per 35-mm fibronectin-coating dish (Falcon) in Dulbecco's modified Eagle's medium (GIBCO) containing 1 μg/ml transferrin, 1 μg/ml insulin, 500 U/ml penicillin, 500 μg/ml streptomycin, 0.1 mM bromo deoxyuridine, and 5% fetal bovine serum (FBS). After 48 h of incubation, the culture medium was exchanged with a medium without bromo deoxyuridine and FBS, and the cardiomyocytes were incubated a further 24 h.

Protocol. This study consisted of four experimental sets. *1*) To determine the effects of phenylephrine (agonist of α_1-adrenergic receptors), bradykinin, and perindoprilat, cardiomyocytes were divided into seven groups treated with vehicle, phenylephrine (10 μM), phenylephrine plus bradykinin (1, 10 nM), phenylephrine (10 μM) plus perindoprilat (1 μM), phenylephrine (10 μM) plus perindoprilat (1 μM) plus N^{ω}-nitro-L-arginine (L-NNA; 30 μM), phenylephrine (10 μM) plus perindoprilat (1 μM) plus methylene blue (10 μM), and bradykinin (10 nM), respectively. *2*) To study the effects of perindoprilat, cardiomyocytes were divided into four groups treated with vehicle, phenylephrine (10 μM), phenylephrine (10 μM) plus perindoprilat (0.01, 0.1, 1 μM), and perindoprilat (1 μM), respectively. *3*) To determine the effects of bradykinin B_2-receptor antagonist and inhibitors of NOS, cardiomyocytes were divided into five groups treated with vehicle, phenylephrine (10 μM), phenylephrine (10 μM) plus perindoprilat (1 μM), phenylephrine (10 μM) plus perindoprilat (1 μM) plus HOE-140 (kinin B_2-receptor antagonist; 0.1, 1 μM), and phenylephrine (10 μM) plus perindoprilat (1 μM) plus L-NNA (NOS inhibitor; 30 μM), respectively. *4*) To study the effects of an NO donor, cardiomyocytes were divided into four groups treated with vehicle, phenylephrine (10 μM), phenylephrine (10 μM) plus perindoprilat (1 μM), and phenylephrine (10 μM) plus NOC-18 (3, 30 μM). After 48 h of incubation with these drugs, each dish was rinsed three times with phosphate-buffered saline (PBS). The cell layer was scraped with 1 ml of 15 mM sodium citrate containing 0.25% (wt/vol) sodium dodecyl sulfate and 0.154 M NaCl. These extracts were frozen at −40°C before determination of protein and DNA contents.

Protein/DNA content. Protein content was measured with bicinchoninic acid (micro-BCA protein assay kit, Pierce), and

DNA content was assayed fluorometrically using Hoechst dye 33258 (Sigma Chemical, St. Louis, MO) (19). The total protein content of the dishes was corrected by the DNA content to indicate the hypertrophy of cardiomyocytes.

Nitrite/nitrate production. At the end of the experiments, 100 μl of the culture medium was collected and incubated for 5 min with 40 μM NADPH and 14 mU/ml nitrate reductase from *Aspergillus niger* (Sigma) in 20 mM tris(hydroxymethyl)-aminomethane buffer (pH 7.6). After the reduction of nitrate, 20 μl 2,3-diaminonaphthalene (50 μg/ml 0.62 N HCl) was added to the samples to form fluorescent products (13). Ten minutes later, the reaction was stopped with the addition of 10 μl of 2.8 N NaOH, and 100 μl of the samples were diluted to a total of 2 ml with distilled water. The final products (200 μl) were transferred to a 96-well plate, and the fluorescent products were determined using a fluorometer (Fluoroskan II; Dainippon Pharmaceutical, Osaka, Japan) with excitation at 355 nm and emission at 460 nm. Sodium nitrite (Sigma) was used as a standard.

Reverse transcription-polymerase chain reaction for bradykinin B_2 receptors. Total RNA was isolated from the cardiomyocytes treated with vehicle or phenylephrine using the methods of Chomczynski and Sacchi (6). Reverse transcription (RT) of the RNA was accomplished using a standard protocol. Briefly, after 100 ng of the total RNA was denatured at 90 °C for 5 min, the RNA was moved to an RT mixture containing 5 mM $MgCl_2$, 1 mM dNTP, 1 U/μl ribonuclease inhibitor, 2.5 U/μl reverse transcriptase, and 2.5 μM random hexamers. The mixture was incubated at 42°C for 30 min, 99°C for 5 min, and 5°C for 5 min. Amplification of the RT products was accomplished by 35 cycles of polymerase chain reaction (PCR) (95°C for 1 min, 46°C for 1 min, and 72°C for 1.5 min for each cycle) in the presence of 0.025 U/μl of Ampli *Taq* polymerase in a standard buffer containing 2 mM $MgCl_2$. The sense oligonucleotide 5′-TACCGTCTAGACTCCCTACAACACAGA-ACC-3′ corresponds to base pairs (bp) 74–94 of the open reading frame of bradykinin B_2-receptor cDNA, and the antisense oligonucleotide 5′-CTGAGAATTCACGACAGCCT-GTGTGCTTCGG-3′ is complementary to bp 368–388 (17). The PCR products were electrophoresed in 2% agarose gel to confirm molecular size.

Histochemistry. At the end of the experiments, the dishes were rinsed three times with PBS and fixed in methanol for 10 min. The cultured cells were incubated with a blocking solution [diluted normal horse serum, Vectorstain, avidin-biotin complex-peroxidase (ABC-PO) kit] for 30 min at room temperature. The cells were then incubated with primary antibody at 4°C overnight. Monoclonal antibody against titin (T-9030, Sigma; diluted 100×) was used for cardiomyocytes, monoclonal anti-α-smooth muscle actin (1A4, Sigma; diluted 200×) was used for smooth muscle cells, monoclonal anti-cellular fibronectin (DH1, Biohit, diluted 200×) was used for fibroblasts, and monoclonal anti-human factor VIII-related antigen goat immunoglobulin G (IgG; Instar; diluted 100×) was used for endothelial cells. For the detection of cardiomyocytes, smooth muscle cells, and fibroblasts, the samples were incubated at 4°C with biotin-conjugated anti-mouse IgG horse antibody (Vectorstain ABC-PO kit). For the detection of factor VIII, biotin-conjugated anti-goat IgG donkey antibody (Chemicon) was used. After the cells were rinsed three times with PBS, they were incubated with 0.3% H_2O_2 in methanol for 20 min at room temperature to inhibit endogenous peroxidase activity. The cells were rinsed with PBS and incubated with peroxidase-labeled avidin-biotin complex (Vectorstain, ABC-PO kit) for 2 h at room temperature. The positive cells were detected by 3,3-diaminobenzidine, a peroxidase substrate (Dojindo, Kumamoto, Japan), and counterstained with hematoxylin. The prepared cells were mounted as aqueous mounts (Mountquick Aqueous, Daido-sangyo, Tokyo, Japan), and the ratios of positive cells to whole cells were determined under a microscope.

Drugs. Perindoprilat was obtained from Servier (Coubevoie, France). Phenylephrine, bradykinin, L-NNA, and methylene blue were purchased from Sigma, and HOE-140 was from Peptide Institute (Osaka, Japan). The RNA isolation kit was purchased from Stratagene, and the Gene Amp RNA PCR Core kit was from Perkin Elmer (USA). 2,3-Diaminonaphthalene and NO-18 were from Dojindo.

Statistical analysis. All values are expressed as means ± SE. Statistical comparison was performed with analysis of variance followed by a Tukey-Kramer test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Effects of bradykinin on phenylephrine-induced hypertrophy and on nitrite content. Treatment with phenylephrine (10 μM) significantly increased protein/DNA content, an index of cardiomyocyte hypertrophy, compared with the control (Figs. 1, 2, 3, and 5). The

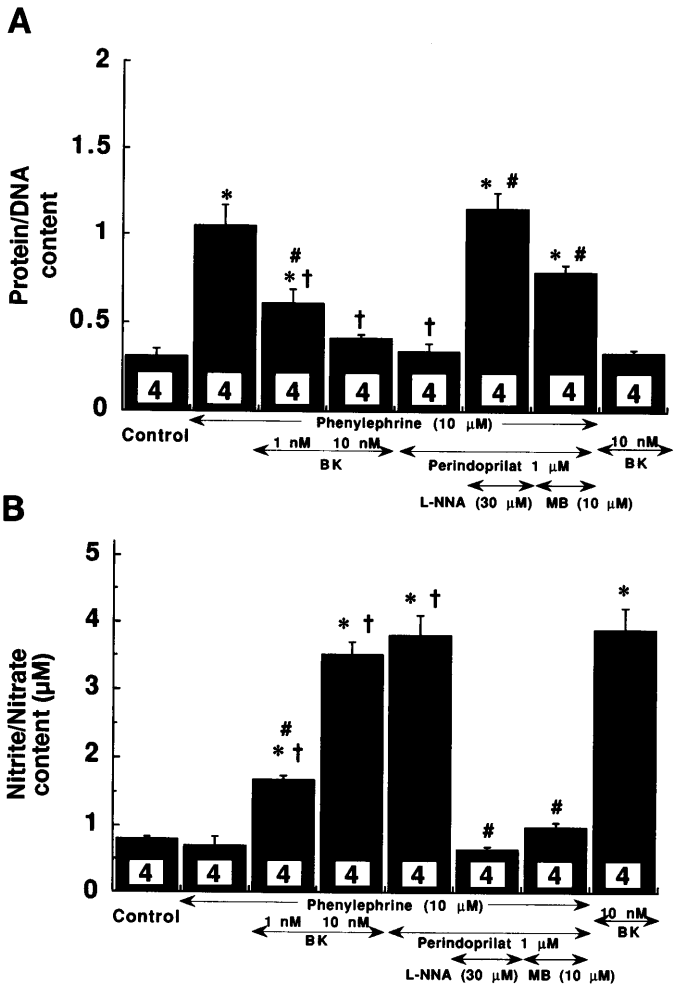

Fig. 1. Effects of bradykinin on phenylephrine-induced cardiomyocyte hypertrophy (*A*) and nitrite/nitrate content (*B*) in medium. Data are means ± SE for *n* no. of experiments (shown within columns). *$P < 0.05$ vs. control; †$P < 0.05$ vs. group treated with phenylephrine; #$P < 0.05$ vs. group treated with phenylephrine + perindoprilat. BK, bradykinin; L-NNA, N^{ω}-nitro-L-arginine; MB, methylene blue.

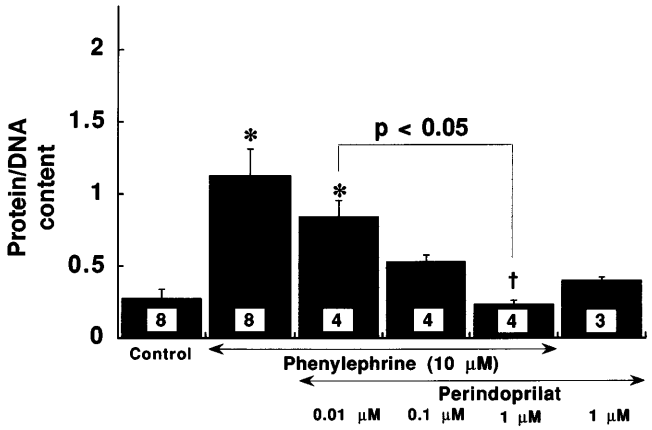

Fig. 2. Effects of perindoprilat on phenylephrine-induced cardiomyocyte hypertrophy. Data are means ± SE for *n* no. of experiments (shown within columns). $*P < 0.05$ vs. control, $\dagger P < 0.05$ vs. group treated with phenylephrine.

increase in protein/DNA content induced by phenylephrine (10 μM) was inhibited by bradykinin (1 and 10 nM; Fig. 1). Bradykinin (10 nM) alone failed to change the protein/DNA content in cardiomyocytes (Fig. 1).

Although phenylephrine did not change the nitrite/nitrate content released into the culture medium, bradykinin significantly enhanced the content compared with the control and treatment with phenylephrine (Fig. 1).

Antihypertrophic effects of perindoprilat. The increase in protein/DNA content induced by phenylephrine (10 μM) was inhibited by perindoprilat (0.01–1 μM) in a concentration-dependent manner (Fig. 2). In particular, the highest concentration of perindoprilat in the present study completely inhibited the induction of hypertrophy (Fig. 2). Perindoprilat alone did not change the protein/DNA content in the cardiomyocytes (Fig. 2).

Involvement of bradykinin and NO in antihypertrophic effects of perindoprilat. The inhibitory effect of perindoprilat (1 μM) on the hypertrophy of cardiomyocytes induced by phenylephrine (10 μM) was abolished by HOE-140 (1 μM; kinin B_2 antagonist) and L-NNA (30 μM; NOS inhibitor), respectively (Figs. 1 and 3). HOE-140 and L-NNA themselves did not change basal protein/DNA content (data not shown). In addition, incubation with methylene blue (10 μM), an inhibitor of guanylate cyclase, also hampered the antihypertrophic effects of perindoprilat in the heart cells (Fig. 1).

Treatment with perindoprilat significantly increased the nitrite/nitrate content in the medium, and this change was also inhibited by HOE-140, L-NNA, and methylene blue, respectively (Figs. 1 and 3). There was a significant correlation between the protein/DNA content of the cultured cells and the nitrite/nitrate content in the medium (Fig. 4).

Effects of NO donor. The increase in protein/DNA content induced by phenylephrine (10 μM) was inhibited by NOC-18 (3, 30 μM), an NO donor. The antihypertrophic effects of NOC-18 were as potent as those of perindoprilat (Fig. 5).

RT-PCR for bradykinin B_2 receptors. When 10 μl of the RT-PCR products were electrophoresed in 2% agarose gel, the expected size (314 bp) of the band was detected (Fig. 6). There was no difference in the RT-PCR products between control cells and the cells treated with phenylephrine.

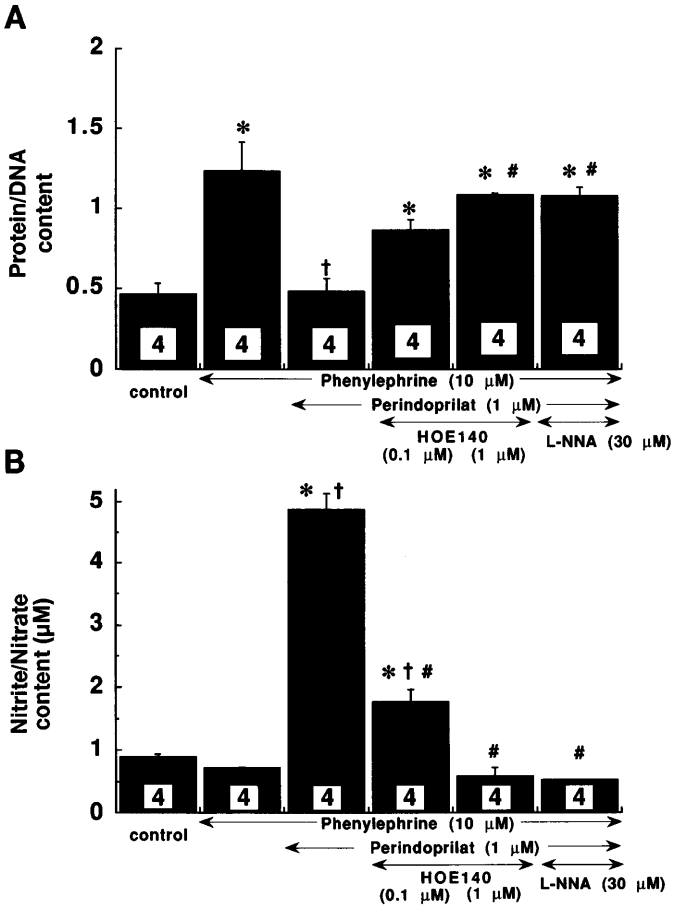

Fig. 3. Effects of HOE-140 and L-NNA on cardiomyocyte hypertrophy (*A*) and nitrite/nitrate content (*B*) in medium. Data are means ± SE for *n* no. of experiments (shown within columns). $*P < 0.05$ vs. control, $\dagger P < 0.05$ vs. group treated with phenylephrine, $^{\#}P < 0.05$ vs. group treated with phenylephrine + perindoprilat.

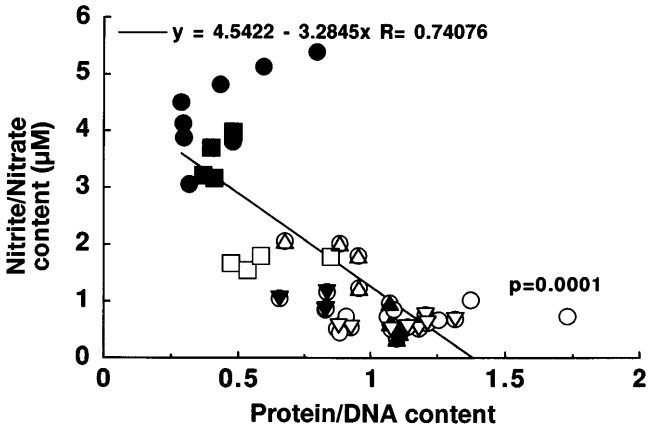

Fig. 4. Correlation between cardiomyocyte hypertrophy and nitrite/nitrate content in medium. ○, Phenylephrine (10 μM); ●, phenylephrine (10 μM) + perindoprilat (1 μM); □, phenylephrine (10 μM) + bradykinin (1 nM); ■, phenylephrine (10 μM) + bradykinin (10 nM); △, phenylephrine (10 μM) + perindoprilat (1 μM) + HOE-140 (0.1 μM); ▲, phenylephrine (10 μM) + perindoprilat (1 μM) + HOE-140 (1 μM); ▽, phenylephrine (10 μM) + perindoprilat (1 μM) + L-NNA (30 μM); ▼, phenylephrine (10 μM) + perindoprilat (1 μM) + MB (10 μM).

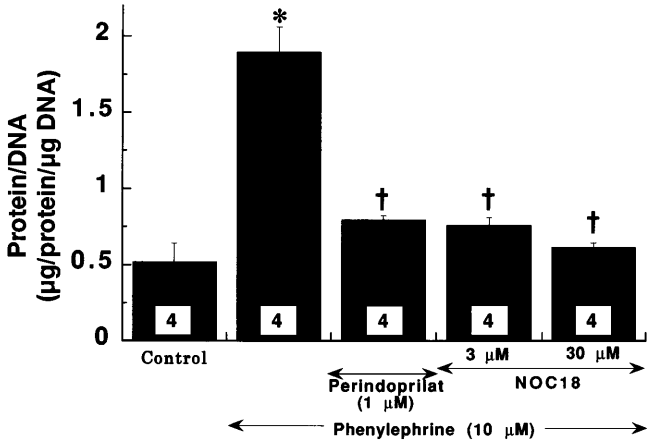

Fig. 5. Effects of NOC-18 (nitric oxide donor) on phenylephrine-induced cardiomyocyte hypertrophy. Data are means ± SE for *n* no. of experiments (shown within columns). $*P < 0.05$ vs. control, $\dagger P < 0.05$ vs. group treated with phenylephrine.

Histochemistry. The cells positive to anti-titin antibody, which is specific to cardiomyocytes, showed a weak contrast, but the all-positive cells were stained homogeneously in the dishes. In contrast, very few α-smooth muscle actin-positive cells were present in the dishes. Cellular fibronectin-positive cells were also detected in the dishes, and some of these cells were gathered. Thus some cells were identified as fibroblasts, although their numbers were <10% of total cells in the dishes. The cells positive to factor VIII-related antigen, indicating endothelial cells, were not seen in this experiment. Therefore, the ratio of cardiomyocytes to all cultured cells was not <90%.

DISCUSSION

Cultured heart cells are suitable tools for examining the mechanism of the progression of cardiac hypertrophy. However, the purity of cardiomyocytes in the culture dishes requires caution in interpreting the results of the experiments. In the present study, cells that are positive to many kinds of antibodies to various types of cells were counted, indicating that more than 90% of the cultured cells were identified as cardiomyocytes. The ratio of cardiomyocytes to whole cells in the present study is comparable to those of previous reports (20, 24).

In the present study, phenylephrine was used to induce the hypertrophy of heart cells because an earlier study (20) showed that norepinephrine induces cardiac hypertrophy via α_1-adrenergic receptors in the cultured cardiomyocytes. Indeed, phenylephrine increased the protein/DNA content, an index of the hypertrophy in heart cells, and bradykinin (1, 10 nM) significantly attenuated it. In porcine coronary arteries, bradykinin at 1 and 10 nM causes 80–100% relaxation to the contraction by prostaglandin $F_{2\alpha}$ (23). Thus bradykinin can work as both an antihypertrophic substance in the heart cells and an inducer of vascular relaxation at the same concentrations. The results of the present study are consistent with a previous study (10) showing that bradykinin prevents the progression of cardiac hypertrophy caused by stenosis of rat aorta. The kinin-kallikrein system and bradykinin B_2 receptors are expressed in rat hearts (12, 15). Indeed, bradykinin B_2 receptor mRNA was detected in cardiomyocytes by RT-PCR in the present study. In addition, bradykinin remarkably amplified the nitrite/nitrate content in the medium. Hence, it is apparent that the bradykinin-NO pathway works in the cultured heart cells and can modify the progression of cardiac hypertrophy. This physiological role of the bradykinin-NO pathway is also supported by the previous study (10), which showed that the prevention of cardiac hypertrophy by bradykinin is abolished by treatment with NOS inhibitor in the rat aortic-banding model.

The mechanism of the increase in NO release by bradykinin was not clarified well. Recent reports have shown that two subtypes of NOS, the inducible type (NOS II) and constitutive type (NOS III), are expressed in heart (1, 2). In particular, NO produced by NOS II

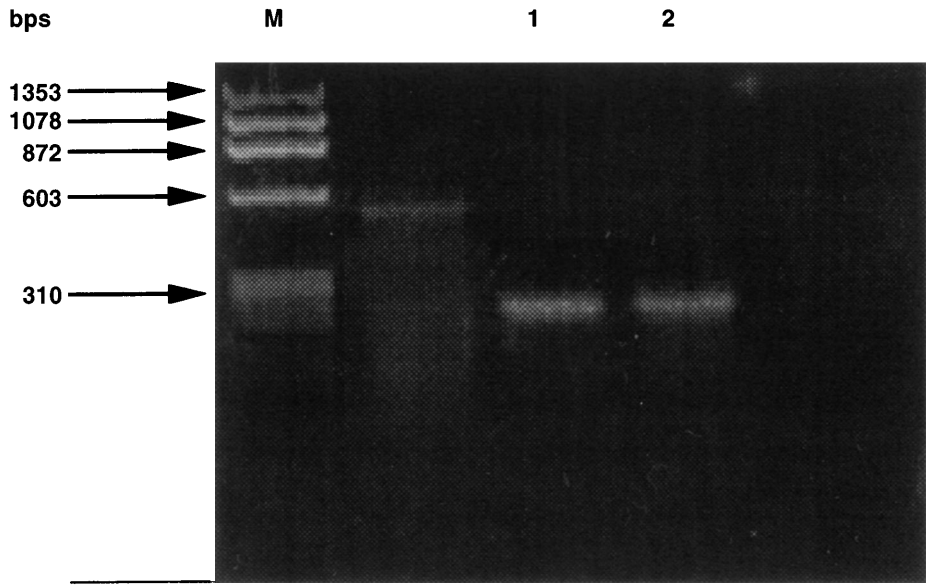

Fig. 6. Detection of bradykinin B_2-receptor mRNA in cardiomyocytes by reverse transcription-polymerase chain reaction. bps, base pairs; M, high-range molecular weight marker; 1, sample from heart cells treated with vehicle; 2, sample from heart cells treated with phenylephrine (10 μM).

inhibits the proliferation of smooth muscle cells (16). We detected the expression of both NOS II and NOS III in cardiomyocytes in our preliminary study (unpublished data). However, the present study has a limitation on further speculation concerning the type of NOS responsible for NO production induced by bradykinin.

Perindoprilat (1 μM) as well as bradykinin inhibited the phenylephrine-induced hypertrophy of cardiomyocytes and augmented the nitrite/nitrate content in the medium. In addition, these effects of perindoprilat were abolished by HOE-140, L-NNA, and methylene blue. NOC-18, an NO donor, also inhibited the cardiac hypertrophy. Thus the antihypertrophic effects of perindoprilat are likely a result of activation of the bradykinin-NO pathway. Although we did not determine whether the bradykinin content is enhanced by perindoprilat, a previous study has demonstrated that perindopril, a prodrug of perindoprilat, inhibits the degradation of bradykinin in rat heart (4). An endogenous kinin-kallikrein system in the cultured heart cells may produce bradykinin spontaneously.

In summary, the present study demonstrated that bradykinin reduces the phenylephrine-induced hypertrophy of cardiomyocytes by enhancement of NO release. The antihypertrophic effects of perindoprilat are likely explained, at least in part, by the bradykinin-NO pathway. In the therapeutics of congestive heart failure, ACE inhibitors may be more beneficial than angiotensin II-receptor antagonist in terms of activating the bradykinin-NO pathway.

Address for reprint requests: T. Shibano, New Product Research Laboratories II, Tokyo R & D Center, Daiichi Pharmaceutical, Ltd. 1–16, Kita-kasai 1-Chome, Edogawa-ku, Tokyo 134, Japan.

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