Overexpression of Endothelial Nitric Oxide Synthase Attenuates Cardiac Hypertrophy Induced by Chronic Isoproterenol Infusion

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Endogenous nitric oxide (NO) inhibits the contractile response to β-adrenergic stimulation, but its effect on cardiac hypertrophy mediated by β-adrenoceptors remains unclear. The present study was designed to determine whether overproduction of endothelial NO synthase (eNOS) could inhibit cardiac hypertrophy induced by chronic isoproterenol (ISO) infusion (30 mg/kg per day) using eNOS overexpressing (eNOS-Tg) mice and wild-type (WT) mice. In a separate group, WT mice were treated with ISO and hydralazine to decrease blood pressure to the same levels in eNOS-Tg mice. The eNOS expression, NOS activity, and cGMP levels in the heart were remarkably higher in eNOS-Tg mice than in WT mice. ISO increased both heart weight and the heart/body weight ratio, which were significantly attenuated in eNOS-Tg mice compared with WT or hydralazine-treated WT mice. Histological examination revealed that the extent of fibrosis was not significantly different among the 3 groups, and that the increase in myocyte size was more than 10% lower in eNOS-Tg than in the other groups. In addition, up-regulated expression of atrial natriuretic peptide mRNA associated with cardiac hypertrophy was significantly inhibited in eNOS-Tg mice during ISO infusion. These results indicate that endogenous NO might act as a negative modulator for the hypertrophic response to β-adrenergic stimulation. (Circ J 2002; 66: 851–856)

Key Words: Adrenergic agonists; Hypertrophy; Myocytes; Natriuretic peptide; Nitric oxide

Cardiac hypertrophy is induced under various environments such as hypertension, valvular disease, volume overload, and neurohumoral activation. Several molecular mechanisms of cardiac hypertrophy have been proposed, and the accumulating evidence suggests that the counterbalance between hypertrophic stimuli and its negative regulators is important in the pathological status of cardiac hypertrophy. Although mechanical stress and neurohumoral factors, including catecholamines, are well known as positive modulators of cardiac hypertrophy, the negative intrinsic modulators of cardiac hypertrophy remain poorly understood.

All 3 isoforms of neuronal, inducible, and endothelial nitric oxide synthase (NOS) are present in the heart and are involved in various pathological conditions such as myocarditis and myocardial infarction. The NO produced by these isoforms is shown to inhibit cell growth in several cell types, including vascular smooth muscle cells. In cardiac myocytes, the bradykinin–NO pathway was reported to play an important role in the prevention of cardiac hypertrophy caused by angiotensin-converting enzyme inhibitors. In addition, administration of the NO precursor L-arginine attenuated cardiac hypertrophy in spontaneously hypertensive rats by increasing myocardial production of NO. On the other hand, there are contradictory reports showing that interleukin-1β induction of NO or pharmacologically relevant doses of nitroglycerin had no influence on the growth of cardiac myocytes induced by adrenergic stimulation. Thus, the inhibitory effects of NO on the growth of cardiac myocytes are controversial, and it remains unclear whether endogenous NO, particularly the endothelium-derived NO, can inhibit cardiac hypertrophy in vivo.

Adrenergic stimulation induces cardiac hypertrophy by either the β- or β-adrenergic pathway, and stimulation of the β-adrenoceptors by isoproterenol (ISO) is a well-known animal model of induced cardiac hypertrophy without systolic hypertension. In addition, β-adrenergic agonists not only stimulate the positive chronotropic and inotropic responses, but also induce the up-regulation of proto-oncogenes such as c-fos, c-jun, and c-myc. NO produced by endothelial NOS (eNOS) inhibits the inotropic response to adrenergic stimulation, especially to β-adrenergic stimulation, but the effects of eNOS-derived NO in cardiac hypertrophy induced by β-adrenergic stimulation are entirely unknown.

Recently we generated transgenic (eNOS-Tg) mice overexpressing eNOS in the endothelium under the control of preproendothelin-1 promoter. In these heterozygous mice, morphological and functional abnormalities are not observed in the heart in their life time. We used these eNOS-Tg mice in the present study to examine whether overexpression of eNOS could attenuate or inhibit cardiac hypertrophy induced by chronic ISO infusion. In addition, we assessed the effect of overexpressed eNOS on the up-
regulated expression of atrial natriuretic peptide (ANP) mRNA accompanying the cardiac hypertrophy.

Methods

Materials

Pentobarbital sodium was purchased from Abbot Laboratories (North Chicago, IL, USA). The ISOGEN RNA extraction kit and cGMP enzyme immunoassay kit were purchased from Nippongene Co (Tokyo, Japan) and Amersham Pharmacia Biotech, Ltd (Buckinghamshire, England), respectively. The rabbit polyclonal anti-eNOS antibody was obtained from Transduction Laboratories (Lexington, KY, USA) and all other chemicals were purchased from Nippongene Co (Tokyo, Japan) and Sigma Chemical Co (St Louis, MO, USA).

Animals

Mice were derived from the same genetic background as described previously. Male eNOS-Tg (n=68) and their wild-type littermates (WT, n=116) mice, weighing 25–30 g, were used in the present study. ISO in 0.9% saline was continuously administered to the mice at a rate of 30 mg/kg per day via subcutaneously implanted osmotic mini pumps (Alzet) as previously reported. Because our preliminary study revealed that the cardiac fibrosis induced by continuous ISO infusion became prominent from the 7th day, we assessed the following data for up to 7 days to exclude the influence of cardiac fibrosis on the hypertrophic responses to ISO. In eNOS-Tg mice, systemic blood pressure (BP) was approximately 16 mmHg lower than that in WT mice as described previously. To exclude the influence of different BP between the 2 genotypes on cardiac hypertrophy, an additional experiment was performed in which hydralazine was administered to the WT mice in their drinking water (180 μg/ml) concomitantly with the ISO to decrease BP to the same levels as in the eNOS-Tg mice. In this additional group, only hemodynamic and morphological analyses were carried out. All animal experiments were conducted according to the Guidelines for Animal Experimentation at Kobe University Graduate School of Medicine.

Hemodynamic Measurements

Systemic BP and heart rate (HR) were measured in the conscious and unrestrained state according to the method described previously. Briefly, after mice were anesthetized with pentobarbital sodium (80 mg/kg ip), one end of a catheter (SP-8 tubing connected to SP-31 tubing; Natsume manufactory, Osaka, Japan) was placed in the right femoral artery and the other end ran through a subcutaneous tunnel to exit at the back of the neck. The end of the catheter was connected to a pressure transducer, and continuous measurements of BP and HR were recorded on a Macintosh computer with Maclab system (Bioresearch center, Nagoya, Japan) at least 4 h after recovery from anesthesia.

Tissue Preparation

After the mice were killed by overdose of pentobarbital, the hearts were excised, rinsed with phosphate-buffered saline, blotted dry and weighed. The ventricles were separated from the atria, and the right ventricle was isolated by dissection along its septal insertion. Specimens for analyses of mRNA, protein, and cyclic GMP (cGMP) content were snap-frozen in liquid nitrogen and stored at –80°C until use. RNA Preparation and Analysis

Total RNA was extracted from the heart by ISOGEN RNA extraction kit. Total cardiac RNA (20 mg/lane) was separated on a 1% agarose-formaldehyde gel, transferred to nylon membranes, ultraviolet cross-linked, and hybridized using mouse ANP cDNA (courtesy of Dr Y. Saito, Nara Medical University) and rat glyceraldehyde-3-phosphate dehydrogenase cDNA. After blots were washed, the hybridized filters were analyzed with a bio-imaging analyzer (BAS 2000, Fuji, Tokyo, Japan).

Protein Analysis

Frozen hearts were homogenized in a buffer of 50 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EGTA, 1 mmol/L dithiothreitol (DTT), 1 mmol/L peptatin A, 2 mmol/L leupeptin, and 1 mmol/L (p-amidinophenyl) methanesulfonyl fluoride. Homogenates were ultracentrifuged at 100,000 G for 60 min at 4°C and the supernatants were collected as a cytosolic fraction. The pellets were solubilized with the buffer containing 10% glycerol and 20 mmol/L 3-(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate and ultracentrifuged to extract the particulate fraction. Protein samples (150 μg) from a particular fraction were separated on a 7.5% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Blots were blocked with 5% non-fat dry milk, incubated with a rabbit polyclonal anti-bovine eNOS antibody. This antibody has been proven to cross-react with mouse and bovine eNOS, but does not recognize inducible NOS. Immunoreactive bands were visualized with horseradish peroxidase-conjugated anti-rabbit IgG using an ECL detection kit.

NOS enzymatic activity was determined by the conversion of [PH]-L-arginine to [PH]-L-citrulline with saturating concentrations of substrate and cofactors as described previously. Enzyme activity was expressed as citrulline production in femtomol per milligram protein per minute.

Immunofluorescence

To examine the distribution of eNOS in the heart, immunofluorescence labeling was performed by the labeled streptavidin biotin method. In brief, frozen sections were fixed in acetone, blocked with carrier protein, and incubated with a polyclonal anti-eNOS antibody. The sections were then incubated with biotinylated goat anti-rabbit IgG, and subsequently with fluorescein isothiocyanate-labeled streptavidin. Specificity of staining was assessed by substitution of non-immune serum for primary antibody. The fluorescent eNOS was examined by a laser scanning confocal imaging system (Nippon Bio-Rad Laboratories, Tokyo, Japan).

Measurements of cGMP Concentrations in the Heart

According to the method of a previous study, the frozen hearts that had been excised from WT mice (n=8) and eNOS-Tg mice (n=10) were homogenized in 6% trichloracetic acid, and centrifuged at 2,000 G. After the TCA in the supernatant fraction was extracted with diethyleter and lyophilized, cGMP was measured with an enzyme immunoassay kit following the manufacturer’s instructions. The concentration of resuspended, acetylated cGMP was expressed as femtomoles per milligram protein.

Morphological Examination (Histological Analysis and Image Analysis)

To evaluate the myocyte cross-sectional area and fibrotic...
changes, morphometric analysis was performed. The hearts of 5 mice from each group were fixed with 10% formalin, embedded in paraffin, and sectioned at 4 mm thickness. In hematoxylin-eosin stained sections of the lateral mid-free wall of the left ventricle, those containing clear capillary profiles were selected and the area of myocytes that had been cut transversely and showed nuclei were measured using the NIH Image software. Approximately 100 cells were counted per each animal. In addition, the fibrotic lesions were measured in Azan-stained heart sections, and the ratio of fibrosis to total myocardial area was calculated using the same method. All morphometric measurements were performed by 2 independent researchers operating in a blinded manner. Interobserver difference in the measured area was less than 5%.

Statistical Analysis
Data are expressed as mean ± SEM. Comparisons of the difference among groups were performed using two-way ANOVA followed by Bonferroni’s multiple-comparison t test. A value of p<0.05 was considered statistically significant.

Results

Hemodynamic Evaluation
Before ISO infusion, the mean BP in eNOS-Tg mice was 16 mmHg lower than that in WT mice, as reported previously (Fig 1A). Although chronic ISO infusion showed a tendency to increase BP in all groups, the BP in eNOS-Tg mice was continuously lower than that in WT mice. The concomitant administration of hydralazine decreased BP in WT mice to a similar level of eNOS-Tg mice throughout the experiment. ISO infusion slightly increased HR in all groups, but there was no significant difference among the groups (Fig 1B).

Expression of eNOS, NOS Activity and cGMP Concentration in the Heart
As shown Fig 2A, the concentration of eNOS protein was markedly increased in eNOS-Tg mice compared with WT mice. n=6 for each data group. (B) Ca2+-dependent NOS activity in the heart was significantly higher in eNOS-Tg mice than in WT mice. n=6 for each data group. (C) Cardiac cGMP contents in the heart from eNOS-Tg mice were also significantly higher than those in WT mice. n=8–10 for each data group. Immunofluorescence labeling of eNOS revealed the intense immuno-reactivity of eNOS in the endothelium of coronary vessels in eNOS-Tg mice. Bar=20 mm. *p<0.01 vs WT mice.
Increased cardiac fibrosis was not obvious.

### Attenuation of Cardiac Hypertrophy in Response to ISO in eNOS-Tg Mice

Before ISO infusion, neither body weight nor heart weight differed among the 3 groups (body weight: 26.4±0.8 g in WT mice, 26.9±0.7 g in WT mice treated with hydralazine, and 26.0±0.6 g in eNOS-Tg mice, p = NS; heart weight: 106.5±3.1 mg in WT mice, 108.3±2.5 mg in WT mice treated with hydralazine, 106.8±3.1 mg in eNOS-Tg, p = NS). Cardiac hypertrophy was apparent on the 3rd day, maximal on the 5th day, and continued to the end of the treatment (Fig 3A). On the 5th day, the increase in heart weight reached a maximum (141.2±4.0 mg in WT mice, 142.2±4.0 mg in WT mice treated with hydralazine, and 132.1±3.6 mg in eNOS-Tg mice); however, the increases in both heart weight and body weight ratio in the eNOS-Tg mice were significantly attenuated compared with the WT mice (Fig 3A). Despite the similar BP between eNOS-Tg and hydralazine-treated WT mice, these parameters were significantly reduced in the eNOS-Tg mice.

### Morphometry of the Ventricular Myocytes

Before ISO treatment, the myocyte cross-sectional area was not significantly different among the 3 groups (Fig 3B). ISO increased the myocyte size in each group from the 3rd day of infusion, and continued to do so until the end of the treatment. However, the increase in myocyte size was significantly attenuated in eNOS-Tg mice compared with the other groups during the treatment. Because cardiac fibrosis is induced by chronic ISO infusion,23 the extent of fibrosis was evaluated at each time point of ISO infusion. Before ISO treatment, the distribution of fibrosis was less than 1% of the total cardiac area in both genotypes, but by the 7th day, ISO had increased cardiac fibrosis in all groups, although the extent of the increases did not differ among the 3 groups (4.9±1.2% in WT mice, 5.2±1.3% in WT mice treated with hydralazine, and 5.6±1.2% in eNOS-Tg mice on the 7th day; p = NS).

### Expression of ANP mRNA in the Heart

The gene expression of ANP, which is associated with cardiac hypertrophy, was evaluated in WT and eNOS-Tg mice (Fig 4). Expression of ANP mRNA was not significantly different between the 2 genotypes before ISO infusion, but by the 3rd day ISO had elevated the ANP expression in WT mice and it remained at a high level until the end of the experiment. In contrast, eNOS-Tg mice showed a slight increase in the ANP expression on the 3rd day, but was significantly lower than the expression in WT mice.

### Discussion

In the present study, we demonstrated that the overexpression of eNOS attenuated the cardiac hypertrophy induced by chronic ISO infusion in genetically engineered mice. In these eNOS-Tg mice, the ISO-induced increases in heart weight and myocyte size were significantly reduced, compared with WT and hydralazine-treated WT mice.
and the transient up-regulation of ANP mRNA expression was significantly inhibited compared with WT mice.

The mechanisms of the hypertrophic response to β-adrenergic stimulation have been extensively investigated. The cardiac hypertrophy induced by ISO is mediated by both hypertrophy of the cardiac myocytes and replacement-type interstitial fibrosis. In most animals, ISO induces subendocardial fibrosis and increases the collagen volume of the heart23,24 but in the present study the ISO-induced fibrosis was less than 6% of the total cardiac area in both genotypes on the 7th day. It has been reported that chronic ISO infusion induces considerably less connective tissue in mice than in other animals25 and therefore a reduction in the size of cardiac myocytes rather than in the extent of cardiac fibrosis would be responsible for the attenuation of cardiac hypertrophy in eNOS-Tg mice.

This transgenic mouse, overexpressing eNOS mainly in vascular endothelial cells, shows systemic hypertension18. During ISO infusion, the BP in the eNOS-Tg mice was continuously less than that in the WT mice (Fig 1A). Because the different BP between WT and eNOS-Tg mice may have contributed to the cardiac hypertrophic response to ISO, the WT mice were separately given hydralazine to decrease arterial pressure to similar levels of the eNOS-Tg mice. However, these mice showed no significant reduction in heart weight, heart/body weight ratio or myocyte size (Fig 3). These findings indicate that the small difference in BP between the 2 genotypes is not responsible for the attenuation of cardiac hypertrophy in eNOS-Tg mice.

Participation of cGMP is highly likely as a mechanism of the attenuated cardiac hypertrophy induced by eNOS overexpression. There is increasing evidence that cGMP produced by guanylate cyclase is involved in the inhibitory effects of NO and ANP on myocyte hypertrophy.26,27 In addition to the NOS enzymes, ANP exerts its biological effects via guanylate cyclase coupled with the natriuretic peptide receptor that produces cGMP. In experimental animal models, mice deficient in natriuretic peptide receptor that produces cGMP. In experimental animal models, mice deficient in natriuretic peptide receptor A developed cardiac hypertrophy in the natural course28 and in Dahl salt-sensitive rats, gene transfer of ANP attenuated cardiac hypertrophy.29 These mechanisms have not been elucidated clearly, but the guanylate cyclase/cGMP pathway seems to be involved in the attenuation of cardiac hypertrophy. In our present study, cardiac cGMP content in the eNOS-Tg mice was significantly higher than in WT mice (Fig 2C), which suggests that the NO/guanylate cyclase/cGMP pathway is involved in the inhibitory effects of eNOS overexpression. To confirm its precise role in the attenuation of hypertrophy, the administration of NOS inhibitors can induce morphological alterations in heart weight, heart/body weight ratio or myocyte size (Fig 3). These findings indicate that the small difference in BP between the 2 genotypes is not responsible for the attenuation of cardiac hypertrophy in eNOS-Tg mice.

References
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