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Increased Expression of Nitric Oxide Synthases in Left and Right Ventricular Hypertrophy

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The present study was aimed to explore pathophysiological implications of nitric oxide in the development of left and right ventricular hypertrophy. To induce selective left and right ventricular hypertrophy, rats were made two-kidney, one clip (2K1C) hypertensive and treated with monocrotaline (MCT), respectively. Six weeks later, the hearts were taken and their ventricular tissue mRNA and protein expression of endothelial constitutive isoform of nitric oxide synthase (NOS) were determined by reverse transcription-polymerase chain reaction and Western blot analysis, respectively. In 2K1C hypertensive rats, the expression of NOS mRNA was increased in parallel with its proteins in the left ventricle, but not in the right ventricle. In MCT-treated rats, the expression of NOS mRNA and proteins were proportionally increased in the right ventricle, but not in the left ventricle. These results suggest that the expression of NOS is specifically increased in association with the ventricular hypertrophy, which may be a mechanism counteracting the hypertrophy.

Key Words: Heart hypertrophy, Nitric oxide synthase

INTRODUCTION

The heart has only a limited response to stress or injury. Adaptive responses include hypertrophy and dilatation, whereas sublethal cellular injury is characterized by various degenerative changes. The ventricular hypertrophy may develop in various pathophysiological situations, usually associated with increased ventricular workload such as in hypertension (Swynghedauw, 1999). The adaptive changes associated with the ventricular hypertrophy are important in the modulation of cardiac performance and the eventual development of heart failure.

Various vasoactive substances and growth factors have been suggested to be involved in the development of cardiac hypertrophy. They may directly promote the growth of myocardial cells or indirectly via inducing the proliferation of fibroblasts and deposition of extracellular matrix (Weber, 1997; Wollert & Drexler, 1999). Factors counteracting the hypertrophy may also exist. Among others, nitric oxide (NO) has been known to inhibit the mitogenesis and proliferation of smooth muscle cells (Garg & Hassid, 1989; Nakaki et al, 1990). The synthesis of NO is catalyzed by NO synthases (NOS), which then can be inhibited by analogues of the substrate to NOS, L-arginine. A long-term blockade of NO synthesis with L-arginine analogues results in perivascular fibrosis and myocardial hypertrophy (Numaguchi et al, 1995). It is thus hypothesized that endogenous NO plays a role in modifying the ventricular hypertrophy.

Furthermore, previous studies that examined the role of humoral factors have mostly focused on the left ventricular (LV) hypertrophy, whereas little has been known on the right ventricular (RV) hypertrophy. The present study was aimed to examine the pathophysiological implications of endogenous NO system in LV and RV hypertrophy. The ventricular expression of endothelial constitutive isoform of NOS was determined in rat models of selective LV and RV hypertrophy.

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METHODS

Animals

Male Sprague-Dawley rats were used. The experimental protocol was in conformity to the *Institutional Guidelines for the Care and Use of Laboratory Animals*. Chronic systemic hypertension is usually associated with LV hypertrophy. Therefore, to induce selective LV hypertrophy, two-kidney, one clip (2K1C) renovascular hypertension was made in rats (150~200 g) by constricting the left renal artery with a silver clip having an internal gap of 0.25 mm, while leaving the right renal artery untouched. Control rats were operated the same way except that no clipping was made. On the other hand, monocrotaline (MCT), a pyrrolizidine alkaloid, has been used to produce pulmonary vascular damage leading to pulmonary hypertension, and eventually RV hypertrophy and failure (Honda et al, 1992; Brunner, 1999). Therefore, to induce selective RV hypertrophy, rats (250~300 g) were subcutaneously injected with MCT (Sigma; 60 $mg \cdot kg^{-1}$), dissolved in phosphate-buffered saline, pH adjusted to 7.4 with 0.5 *N* HCl. Corresponding control rats were injected with the vehicle only. They were used 6 weeks later.

On the experimental day, the heart was taken and divided into RV and LV, in which the ventricular septum was included in LV. After weighing, RV and LV were rapidly frozen in liquid nitrogen, and kept at $-70^{\circ}C$ until assayed.

RNA extraction & RT-PCR

Total RNA was extracted as previously described (Jo et al, 1996). The extract was stored at $-70^{\circ}C$ as a suspension in 70% ethanol. RNA was spectrophotometrically quantified by measuring the optical density at 280 nm.

The nucleotide sequences of the primers were prepared as previously described (Tao et al, 1985; Ujiie et al, 1994). GAPDH primers were: sense primer, 5'-ATCAAATGGGGTGATGCTGGTGCTG-3', and antisense primer, 5'-CAGGTTTCTCCAGGC-GGCATGTCAG-3', allowing the amplification of 505 bp fragments. ecNOS primers were: sense primer, 5'-TACGGAGCAGCAAATCCAC-3', and antisense primer, 5'-CAGGCTGCAGTCCTTTGATC-3', allowing the amplification of 819 bp fragments. Total RNA (20 μg) was primed with oligo (dT) primers, and the first strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Promega; Madison, WI, USA) in a 50 μL of reaction volume for 90 min at $37^{\circ}C$. PCR cycles were performed in a DNA thermal cycler (PTC-100, M.J. Research; Watertown, MA, USA) with the following profile: for GAPDH (25 cycles), denaturation for 45 sec at $94^{\circ}C$, annealing for 45 sec at $56^{\circ}C$, and extension for 1 min at $72^{\circ}C$; and for ecNOS (26 cycles), denaturation for 45 sec at $94^{\circ}C$, annealing for 45 sec at $62^{\circ}C$, and extension for 1.3 min at $72^{\circ}C$. PCR cycles and the amounts of template were determined according to the relative abundance of mRNAs. At the end of PCR, one-tenth reaction mixture was separated on 1% of agarose gel containing 0.5 μg/mL of ethidium bromide. Polaroid film was scanned using Epson (GT-9500) scanner with a resolution of 72 DPI. The resulting image was analyzed using Image Analysis Program (NIH, Bethesda, MD, USA). The scale of each band was expressed by multiplying the mean density and the total area of the band. The resulting scale was then used to quantify each band.

Western blot analysis

The tissue protein preparation was used for blotting NOS. An equivalent amount of total tissue protein (100 μg) was loaded on each lane. The protein sample was electrophoretically size-separated with a discontinuous system, consisting of 7.5% polyacrylamide resolving gel and 5% polyacrylamide stacking gel. High-range molecular weight markers (BioRad; Hercules, CA, USA) were used as size standard. After the separation, the protein was transferred to a nitrocellulose membrane at 20 V overnight. The membrane was washed in Tris-based saline buffer (pH 7.4), containing 1% Tween-20 (TBST) and blocked with 5% non-fat milk in TBST for 1 hr. It was then incubated with 1 : 2,000 dilutions of monoclonal mouse anti-ecNOS antibodies (Transduction Laboratories; Lexington, KY, USA) in 2% non-fat milk/TBST for 1 hr at room temperature. It was further incubated with a horseradish peroxidase-labeled goat anti-mouse IgG (1 : 1,000) in 2% non-fat milk in TBST for 2 hr. The bound antibody was detected by enhanced chemiluminescence on X-ray film (Amersham; Little Chalfont, Buckinghamshire, England).

Table 1. Body weight, and left and right ventricular weights in 2K1C and MCT-treated rats

	2K1C		MCT	
	Control	Exp	Control	Exp
BW (g)	305±9	298±4	457±9	402±22†
LV/BW (mg/g)	2.43±0.11	3.19±0.30*	2.24±0.02	2.37±0.12
RV/BW (mg/g)	0.63±0.06	0.62±0.08	0.57±0.03	1.24±0.11†

Values are means±SEM. Abbreviations: BW, body weight; LV, left ventricle; RV, right ventricle; 2K1C, two-kidney, one clip; MCT, monocrotaline. *$p<0.05$, †0.01; *vs.* control (n=6 each).

Statistical analysis

All data are presented as mean±SEM. To compare the data between the control and experimental groups, unpaired *t*-test was used. Differences were considered significant if $P<0.05$.

RESULTS

Ventricular weights

Table 1 shows body weights, and LV and RV weights in 2K1C and MCT-treated rats at 6 weeks. 2K1C rats showed a significantly higher LV weights than the control. Weight ratios of LV/BW were significantly increased in the experimental group, whereas RV/BW ratios were not altered. On the other hand, MCT-treated rats showed a significantly lower body weight, and higher RV weights than the control. Ratios of RV/BW were increased, while those of LV/BW were not significantly altered.

NOS expression

Fig. 1 and Fig. 2 show the expression of NOS mRNA and proteins, respectively, in RV and LV in 2K1C rats. Anti-ecNOS monoclonal antibodies were hybridized with proteins of approximately 140 kDa. The abundance of NOS mRNA was significantly increased in LV, but not in RV. Accordingly, the expression of NOS proteins was 1.5 to 2-fold increased in LV, but not in RV.

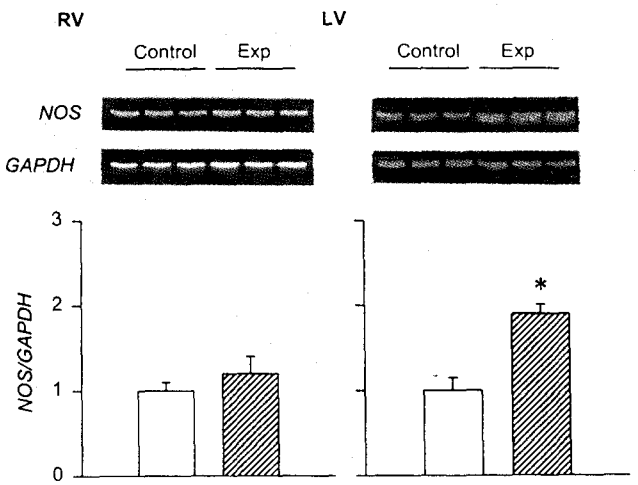

Fig. 1. Expression of NOS mRNA in the right and left ventricles (RV & LV) in 2K1C rats. Fluorographs show representative ethidium bromide-stained agarose gels containing RT-PCR products, and histograms show densitometric data (n=6 each). *$P<0.05$, *vs.* control.

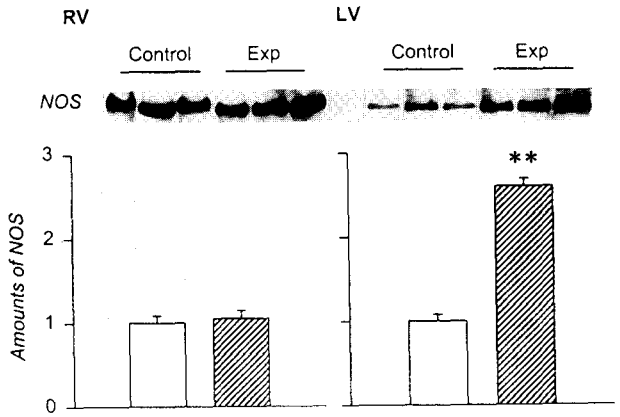

Fig. 2. Expression of NOS proteins in the right and left ventricles in 2K1C rats. Autoradiograms represent typical NOS protein expression, and histograms show densitometric data (n=4 each). **$P<0.01$, *vs.* control.

In MCT-treated rats, the abundance of NOS mRNA was significantly increased in RV, but not in LV (Fig. 3). At the same time, the expression of NOS proteins was increased 2 to 3-fold in RV, but not in LV (Fig. 4).

DISCUSSION

In the present study, specific LV and RV hyper-

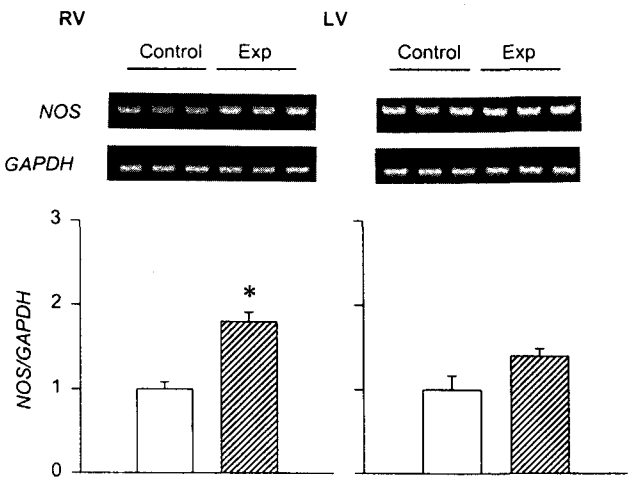

Fig. 3. Expression of NOS mRNA in the right and left ventricles in MCT-treated rats. Fluorographs show representative ethidium bromide-stained agarose gels containing RT-PCR products, and histograms show densitometric data (n=6 each). $*P<0.05$, *vs.* control.

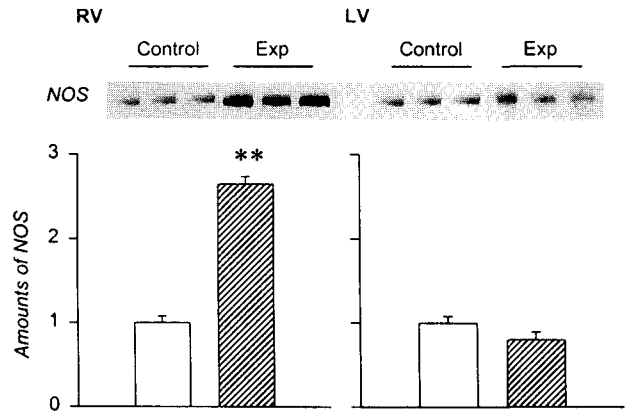

Fig. 4. Expression of NOS proteins the right and left ventricles in MCT-treated rats. Autoradiograms represent typical NOS protein expressionm, and histograms show densitometric data (n=4 each). $**P<0.01$, *vs.* control.

trophy were produced in 2K1C hypertensive and MCT-treated rats, respectively. Normalization of blood pressure in rats with aortic constriction by calcium antagonists or direct vasodilators failed to modify the cardiac hypertrophy (Linz et al, 1989). This finding suggests that the ventricular hypertrophy cannot solely be attributed to an adaptive process in response to an increased mechanical load. Instead, various vasoactive substances and growth factors, such as angiotensin II, aldosterone, endothelin-1, and TGF-β have been suggested to contribute to the cardiac hypertrophy and remodeling (Guarda et al, 1993; Ito et al, 1993; Sato & Funder, 1996; Weber, 1997).

An enhanced activity of cardiac renin-angiotensin system (RAS) has been demonstrated in the hypertrophied LV, in which the pressure-overload was induced by aortic stenosis (Schunkert et al, 1990; Weinberg et al, 1997) or abdominal aorta constriction (Baker et al, 1990). Hyperthyroidism-induced cardiac hypertrophy is also associated with increases of renin mRNA and protein, and angiotensin II in rats (Kobori et al, 1999). Conversely, angiotensin converting enzyme inhibitors and angiotensin II receptor antagonists caused a significant regression of LV hypertrophy in TGR(mRen2)27 transgenic rats (Ohta et al, 1996). These inhibitors and antagonists also prevented the development of cardiac hypertrophy induced by pressure overload or volume overload (Baker et al, 1990; Ruzicka & Leenen, 1995).

It has been also suggested that endothelin-1 is involved in the development of cardiac hypertrophy and fibrosis through increasing extracellular matrix synthesis (Guarda et al, 1993). However, the effects of endothelin may be related to an interaction of endothelin-1 with angiotensin II in the progression of ventricular hypertrophy (Miyauchi et al, 1993; Berthold et al, 1999). Angiotensin II stimulates the synthesis of endothelin-1 in many cell types including cardiomyocytes (Ito et al, 1993). Conversely, an endothelin-1 blockade prevents the cardiac hypertrophy induced by angiotensin II infusion (Herizi et al, 1998).

On the contrary, a reciprocal inhibition exists between NO and angiotensin II (Shultz et al, 1990; Ito et al, 1991) or endothelin (Miller et al, 1989; Boulanger & Luscher, 1990). NO may function as an inhibitor of smooth muscle cell mitogenesis and proliferation (Garg & Hassid, 1989; Wunsch et al, 1991; Villarreal & Dillmann, 1992). Furthermore, the vascular and myocardial remodeling was characterized by increased tissue activities of angiotensin converting enzyme in rats with long-term blockade of NO synthesis (Takemoto et al, 1997).

The present study showed that the expression of NOS was increased in association with LV and RV hypertrophy. Since both NOS mRNA and proteins were increased in parallel, the increased protein expression may be attributed to an increased transcription of NOS genes. Furthermore, the increased expression was specifically localized to the hypertrophied ventricle only: an increase in LV, but not in RV, in 2K1C rats; and in RV, but not in LV, in

MCT-treated rats. This finding is in line with the previous observation that the expression of NOS is markedly increased in proliferating compared with quiescent non-growing cells (Arnal et al, 1994). The resultant increase of NO synthesis may then be indicative of cell proliferation of the hypertrophied ventricle, and possibly play a role in counteracting the hypertrophy.

In summary, the expression of NOS was specifically increased in association with the ventricular hypertrophy, which may be a mechanism counter-regulating the hypertrophy.

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