Superoxide Anions Contribute to Impaired Regulation of Blood Pressure by Nitric Oxide During the Development of Cardiomyopathy

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ABSTRACT
Basal release of endothelium-derived nitric oxide (NO) has been shown to modulate vascular tone and arterial pressure, and may be altered in disease states. The present study was designed to evaluate the role of nitric oxide synthase (NOS) in the maintenance of mean arterial pressure (MAP) and heart rate (HR) in early and advanced stages of cardiomyopathy. MAP and HR were measured via a carotid arterial cannula in conscious, unrestrained male Golden Syrian and Syrian cardiomyopathic hamsters. Studies were performed in young hamsters (age, 60–90 days) at the early phase and old hamsters (age, 300–350 days) at the advanced phase of cardiomyopathy. N-Nitro-L-arginine (LNA; 0.3–30 μmol/kg i.a.), an inhibitor of NOS activity, produced a dose-dependent increase in MAP in YC (young control) and OC (old control) hamsters. The LNA-induced increase in MAP was significantly impaired in YM (young cardiomyopathic) and was abolished in OM (old cardiomyopathic) hamsters compared with control hamsters. Bradycardia in response to LNA was similar in all groups. The effects of LNA on MAP and HR were reversed by L-arginine (200 mg/kg i.a.). Phenylephrine (0.3–300 μg/kg i.a.), an α-adrenoceptor agonist, produced a dose-dependent increase in MAP which was similar in C and M hamsters at both ages, which indicated that impaired pressor responses to LNA were not caused by a nonspecific alteration in vascular responsiveness of M hamsters. Additionally, L-arginine (100 or 300 mg/kg i.a.), the precursor to NO and sodium nitroprusside (0.3–300 μg/kg i.a.), an NO donor, produced similar effects on MAP and HR in all groups of hamsters. Endothelial NOS protein levels in aorta isolated from each group of hamsters were similar. In the presence of tiron (1000 mg/kg), a superoxide anion scavenger, the effects of LNA on MAP were significantly restored in OM compared with OC hamsters. These results indicate that the role of NO in regulation of MAP is reduced during the development of cardiomyopathy. This effect is not the result of a deficiency of L-arginine, a reduced sensitivity to exogenous NO or a decrease in vascular endothelial NOS protein in cardiomyopathic hamsters. However, scavenging of NO by superoxide anions may contribute to the diminished role of NO in regulation of blood pressure in the advanced stage of cardiomyopathy.

Current treatment of cardiomyopathy, a degenerative condition of the myocardium frequently associated with heart failure, has done little to enhance patient survival (Factor and Sonnenblick, 1985). Decreased myocardial contractility and altered regulation of peripheral circulation are important contributors to the symptoms and prognosis of the disease process (Riegger, 1991). The increased systemic vascular resistance occurring in chronic congestive heart failure is only partly explained by increases in sympathetic nervous system activity. Abnormalities of the vascular endothelium, through the release of vasodilatory and vasopressor substances, may also contribute to increased vasomotor tone (Palmer et al., 1987; Luscher and Vanhoutte, 1990). A major vasodilatory agent produced by endothelial cells is NO, synthesized from the amino acid, l-arginine, by NOS (Furchgott, 1983; Ignarro, 1989). The endothelium releases NO under basal conditions as a result of shear stress exerted by the circulating blood. This basal release of NO is critically involved in maintaining normal vascular tone and blood pressure (Bassenge, 1989). Inhibition of NOS activity with an L-arginine analog, such as by LNA, results in vasocostriction of coronary, renal, mesenteric and hindquarter vascular beds associated with an increase in blood pressure in rats (Gardiner et al., 1990; Jones and Brody, 1992).

ABBREVIATIONS: eNOS, endothelial nitric oxide synthase; HR, heart rate; HW/BW, heart weight/body weight; MAP, mean arterial pressure; LNA, N-nitro-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; SNP, sodium nitroprusside; OC, old control hamster; OM, old cardiomyopathic hamster; PE, phenylephrine; S.E.M., standard error of the mean; YC, young control hamster; YM, young cardiomyopathic hamster; TSB, Tris-saline buffer.
The Syrian cardiomyopathic hamster (Bio 14.6), has proved to be a useful genetic model of congestive cardiomyopathy which is transmitted by an autosomal recessive gene expressed in 100% of affected lines (Gertz, 1972; Stroheck et al., 1979). Cardiomyopathic hamsters exhibit lower MAP and cardiac output, and higher left ventricular end-diastolic pressure and peripheral resistance than age-matched control hamsters (Trippodo et al., 1993). Additionally, endothelium-dependent vascular relaxation is impaired in coronary and cheek pouch arterioles of cardiomyopathic hamsters (Mayan and Rubenstein, 1992; Fuchs, 1996). Several similarities exist between the cardiomyopathy which occurs in hamsters compared with humans. In addition to a similar hemodynamic profile, increased levels of circulating catecholamines, decreased myocardial norepinephrine and increased myocardial dopamine, as well as greater fibrosis in the epicardium than in the endocardium also occur in both humans and hamsters with cardiomyopathy (Gertz, 1972). Additionally, the acute myocarditis observed in humans who later developed cardiomyopathy has histological similarities to the myocardial changes observed during the development of cardiomyopathy in hamsters (Strain et al., 1983).

In the early stages, the cardiomyopathic hamster is known to develop myocardial necrosis, fibrosis and calcification, whereas in the advanced stage, congestive heart failure is evident (Jasmine and Proschek, 1982). During the development of congestive heart failure, a compensatory increase in peripheral resistance is thought to maintain arterial pressure in the presence of reduced cardiac output. However, this compensatory response may further impair left ventricular function by enhancing afterload and increasing myocardial work. Indeed, clinical trials have shown that vasodilatory agents can improve survival and the rate of progression of congestive heart failure (Cohn et al., 1986; SAVE Investigators, 1992; SOLVD Investigators, 1992). Because NOS is known to mediate basal vascular tone, and has not been studied in cardiomyopathic hamsters, the present study was designed to evaluate the role of NOS in maintenance of blood pressure in early and advanced stages of cardiomyopathy.

Methods

Male Golden Syrian and Syrian cardiomyopathic hamsters (Bio 14.6; Biobreeders, Inc., Fitchburg, MA), ages 60 to 90 days (early stage of cardiomyopathy) and 300 to 350 days (advanced stage of cardiomyopathy) were anesthetized with pentobarbital (50 mg/kg i.p.). Under aseptic conditions, the right carotid artery was cannulated with polyethylene tubing (PE-50 attached to PE-10) for measurement of arterial pressure and HR. A PE-10 cannula was also advanced via the right femoral artery to the aorta for administration of drugs. The opposite ends of the cannulas were exteriorized at the nape of the neck and flushed with heparinized saline (100 U/ml). After a 24-hr recovery period, arterial pressure and HR were obtained from unrestrained hamsters in their home cage. Heart rate was derived with a cardiotachometer that was triggered from the arterial pressure pulse. Arterial pressure and HR were monitored by use of a Grass recorder.

Protocol. Several experiments were performed in separate groups of hamsters to assess the role of NOS in regulation of arterial pressure and HR. First, NOS activity was inhibited with LNA. LNA was administered at doses of 0.3 to 30 μmol/kg i.a. To evaluate sensitivity to exogenous NO, nitroprusside was administered at doses of 0.3 to 300 μg/kg i.a. To assess the responsiveness to L-arginine, the precursor to NO, L-arginine (100 and 300 mg/kg i.a.) was administered. As a control for selectivity, D-arginine was also administered at similar doses. Finally, tiron (1,000 mg/kg i.a.) was used to inhibit superoxide anion production. At this dose, tiron has been shown previously to reduce superoxide anion levels in vivo (Leffler et al., 1993). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), and were dissolved in saline, followed by adjustment of pH to 7.4 with NaHCO3, if necessary. All agents were administered in a volume of 100 μl, or less, as a bolus over a period of ~30 sec. The maximum change in arterial pressure and HR from baseline in response to each dose was measured and dose–response curves were plotted for each agent.

Western blot analysis. Control and cardiomyopathic hamsters of the same age used above were anesthetized with pentobarbital (50 mg/kg i.p.) and the aorta was removed and immediately frozen in liquid nitrogen. In these same hamsters, hearts were removed for determination of HW/BW ratios. Aortas were pulverized with a pre-frozen mortar and pestle, and protein concentration was determined with the Bradford assay. Tissue samples (50 μg) were separated on 7.5% denaturing sodium dodecyl sulfate polyacrylamide gels. Proteins were blotted with Trans-blot dry blotter onto nitrocellulose. Blots were blocked at 4°C with 6% nonfat dry milk in TSB and then washed in TSB and incubated with the primary antibody, anti-endothelial NOS (anti-eNOS, Amersham, Arlington Heights, IL) and 5% non-fat dry milk in TSB for 2 hr at room temperature. Blots were incubated with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin antibody (1:3,000) for 1 hr. Finally, the specific proteins were detected by enhanced chemiluminescence (ECL,Amersham) and autoradiography. Blots were stripped and reprobed with anti-tubulin antibody to confirm equal loading.

Data analysis. All data are reported as mean ± S.E.M. Statistical differences were determined by analysis of variance for repeated measures followed by the Student’s modified t test with Bonferroni correction for multiple comparisons. Differences were considered significant at P < .05.

Results

Base-line MAP, HR and HW/BW obtained from YC, YM, OC and OM hamsters are shown in table 1. MAP was similar in YC (n = 23) and YM (n = 25) hamsters, but was significantly reduced in the advanced phase of cardiomyopathy in OM (n = 28) compared with OC (n = 34) hamsters. HW/BW was significantly higher in YM (n = 6) than in YC (n = 6) hamsters, which indicates mild hypertrophy. Hypertrophy was further enhanced in OM (n = 6) compared with OC (n = 6) hamsters.

A summary of the response to inhibition of NOS with LNA is shown in figure 1, A and B. LNA produced a dose-dependent increase in MAP in YC (n = 6) and OC (n = 8) hamsters. After each dose of LNA, the maximum increase in MAP was reached within 10 min, followed by a stabilization of MAP at that level. Inhibition of NOS resulted in a significantly higher increase in MAP in OC than in OM (n = 6) hamsters over the entire dose-response curve. At the highest LNA dose there was also a significant difference between YC and YM (n = 7) hamsters. Administration of vehicle produced no effect on MAP or HR. LNA produced a dose-dependant bra-

**TABLE 1**

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<td>MAP (mm Hg)</td>
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<td>HR (beats/min)</td>
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<td>HW/BW (mg/g)</td>
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* P < .05 vs. respective control.
dyscardia, which was similar in all groups (fig. 1B). The effects of LNA on MAP and HR were significantly reversed by L-arginine (200 mg/kg i.a.), which was given after completion of the dose-response curve to LNA. The percent changes in MAP were 39 ± 4 vs. 10 ± 4 in YC, 11 ± 6 vs. −5 ± 6 in YM, 43 ± 5 vs. 10 ± 5 in OC and 1 ± 3 vs. −4 ± 2 in OM before and after L-arginine, respectively. The percent changes in HR were −30 ± 9 vs. −7 ± 4 in YC, −34 ± 3 vs. −8 ± 3 in YM, −25 ± 5 vs. −4 ± 2 in OC and −28 ± 7 vs. −8 ± 2 in OM before and after L-arginine, respectively. These results indicate that the actions of LNA were caused by competitive inhibition of NOS.

To determine whether a nonspecific alteration in responsiveness to pressor agents occurs during development of cardiomyopathy, dose-response curves to PE, which produces vasoconstriction through activation of alpha adrenoceptors, were performed in YC (n = 6), YM (n = 6), OC (n = 6) and OM (n = 5) hamsters (fig. 2, A and B). PE produced a dose-dependent increase in MAP that was similar in all groups, which indicates that the impaired responsiveness in cardiomyopathic hamsters is selective to inhibition of NOS activity. Reflex bradycardia was also observed in response to PE. A significantly lower degree of bradycardia was observed in the OM hamsters than in other groups.

To assess sensitivity to NO, dose-response curves to SNP, an exogenous NO donor that produces vasodilation, were performed in YC (n = 5), YM (n = 6), OC (n = 6) and OM (n = 5) hamsters as indicated in figure 3, A and B. SNP produced similar, dose-dependent decreases in MAP and reflex tachycardia in all groups. The possibility that reduced levels of L-arginine, the precursor to NO, could decrease NOS activity was evaluated by administering L-arginine at doses of 100
and 300 mg/kg i.a. L-Arginine (100 mg/kg i.a.) produced decreases of 9 ± 3%, 12 ± 4%, 7 ± 2% and 9 ± 2% in YC, YM, OC and OM hamsters, respectively, whereas L-arginine (300 mg/kg i.a.) produced decreases of 15 ± 4%, 15 ± 2%, 15 ± 2% and 14 ± 4% in YC, YM, OC and OM hamsters, respectively (n = 6/group). The same doses of D-arginine had no effect on MAP. Aortic levels of eNOS were measured in YC, YM, OC and OM hamsters (n = 3/group). A typical Western blot is shown in figure 4 which indicates that eNOS protein levels are similar in all groups. Tubulin levels were also similar in all groups, which indicates similar protein loading.

Superoxide anions are scavengers of NO and have been shown to be elevated in cardiomyopathic hamsters (Fukuchi et al., 1991). Because the greatest impairment in NO-mediated regulation of blood pressure was present in OM hamsters, these hamsters were pretreated with tiron, a scavenger of superoxide anions. Tiron was administered as a bolus dose of 1,000 mg/kg i.a. 20 min before performing a dose-response curve to LNA. A comparison of the effect of tiron pretreatment on the response to LNA in OC (n = 8) and OM (n = 6) hamsters is summarized in table 2. Although tiron alone did not lower MAP, pretreatment with tiron resulted in an increase in MAP in response to LNA (10 and 30 μmol/kg i.a.) in OM hamsters, which was not observed in the absence of tiron. At doses of 0.3, 1 or 3 μmol/kg, LNA did not significantly alter MAP in either untreated or tiron-pretreated OM hamsters. Tiron did not alter the effect of LNA on HR.

Discussion

Heart failure is characterized by systemic vasoconstriction that contributes to maintenance of arterial pressure in the
presence of reduced cardiac output. However, this compensatory mechanism may aggravate rather than relieve congestive heart failure by increasing cardiac work and oxygen consumption. The present study investigated the role of NO in regulating blood pressure during development of cardiomyopathy and heart failure. NO is formed endogenously from L-arginine and participates in the regulation of vascular tone (Palmer et al., 1987). L-Arginine analogs constrict isolated blood vessels and increase systemic arterial pressure of anesthetized rabbits and guinea pigs (Aisaka et al., 1989; Rees et al., 1997).

**Table 2**

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<th>MAP (% Δ)</th>
<th>LNA (10 μmol/kg)</th>
<th>LNA (30 μmol/kg)</th>
<th>Tiron + LNA (10 μmol/kg)</th>
<th>Tiron + LNA (30 μmol/kg)</th>
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<td>OC</td>
<td>34 ± 6</td>
<td>42 ± 6</td>
<td>45 ± 9</td>
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<td>OM</td>
<td>2 ± 3</td>
<td>1 ± 3</td>
<td>21 ± 8*</td>
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*P < 0.05 vs. LNA alone.
Inhibition of NOS has also been demonstrated to increase blood pressure of healthy humans (Haynes et al., 1993).

Several studies have demonstrated that vascular relaxation mediated by endothelium-derived NO is altered in disease states such as atherosclerosis, diabetes and hypertension (Shirasaki et al., 1988; Kamata et al., 1992; Najibi et al., 1994). Studies performed in a dog model of congestive heart failure have yielded conflicting results. One study demonstrated reduced production of NO, whereas another study showed enhanced production of NO in coronary arteries from dogs with heart failure (O'Murchu et al., 1994; Wang et al., 1994). Studies in a rat model of chronic heart failure suggested an alteration of endothelial cell production or release of endothelium-derived relaxing factor (Ontkean et al., 1991). In cardiomyopathic hamsters, acetylcholine was found to elicit vasoconstriction of cheek pouch arterioles, whereas vasodilation was observed in response to acetylcholine in control hamsters (Mayan and Rubenstein, 1992). A previous study performed in this laboratory demonstrated that NO-mediated relaxation was impaired in coronary arteries from cardiomyopathic compared with control hamsters (Fuchs, 1996). Conversely, endothelium-dependent relaxation was observed in coronary arteries in congestive heart failure, which supports a beneficial role of coronary endothelium to resist vasoconstriction during heart failure (Larosa et al., 1994).

In studies of human congestive heart failure, impaired responses to both endothelium-dependent and -independent vasodilators were observed in lower limb blood flow (Lindsay et al., 1996). Conduit artery distensibility was found to be increased by acetylcholine in healthy patients, but not in patients with congestive heart failure, which implies that NO-mediated increases in distensibility are impaired in patients with congestive heart failure (Ramsey et al., 1995). Additionally, in humans with congestive heart failure endothelium-dependent vasodilatation of large arteries was impaired (Kaiser et al., 1989). These studies support the findings of the present study, which demonstrate an impaired regulation of blood pressure by NO partly mediated by superoxide anions in the advanced stage of cardiomyopathy of the genetic hamster model. It is interesting to note that this model of cardiomyopathy does not exhibit hypertension, because chronic inhibition of NOS activity is known to produce hypertension. This discrepancy cannot be explained by the results of this study, but it is possible that in the advanced phase of cardiomyopathy, cardiac output is so low that the increase in peripheral resistance does not result in hypertension. A significant bradycardia in response to LNA was also observed. Inhibition of NOS has also been shown to produce bradycardia in rats and humans (Hecker et al., 1990; Dananberg et al., 1993).

The mechanisms mediating the impaired response to LNA in cardiomyopathy were also evaluated in the present study. First, it was demonstrated that the responsiveness to other pressor agents, such as PE, remains intact, which indicates a selective effect of the NOS inhibitor. Interestingly, the PE-induced reflex bradycardia was significantly lower in OM hamsters than in other groups, which suggests a reduced sensitivity of aortic and carotid baroreceptors. This observation has also been described as an abnormality of autonomically mediated HR control in patients with cardiac dysfunction in head-up tilt responses (Goldstein et al., 1975; Kubo and Cody, 1983).

The results of this study suggest that the sensitivity to NO is not impaired and that vascular eNOS protein levels are not reduced in cardiomyopathy. Additionally, a deficiency of L-arginine does not appear to contribute to the reduced responsiveness to LNA, because the mild hypotension observed in response to L-arginine was similar in control and cardiomyopathic hamsters. However, reduced regulation of blood pressure by NO in cardiomyopathy may be partly mediated by scavenging of NO by superoxide anions. Shear stress induces continuous release of NO from endothelial cells, which is involved in maintaining vascular tone and blood pressure (Bassenge, 1989). In the presence of a high level of superoxide anions, the amount of circulating NO available for contributing to basal vascular tone would be reduced. Under these conditions, inhibition of NOS activity would have a reduced effect on vascular tone and blood pressure. Superoxide anions can be produced by endothelial cells (Rubanyi, 1988). Enhanced levels of free radicals have been observed in cardiomyopathic hamsters hearts compared with controls, whereas chronic treatment with an antioxidant reduced development of myocardial necrotic lesions in cardiomyopathic hamsters (Fukuchi et al., 1991). The myocardial lesions observed in cardiomyopathic hamsters have been shown to be similar to those observed during reperfusion injury, which has been attributed to generation of free radicals (Fukuchi et al., 1991; Nagano et al., 1994). We have also previously demonstrated that inhibition of superoxide anion production can restore endothelium-dependent coronary artery relaxation in cardiomyopathic hamsters (Fuchs, 1996). The studies above support the finding that tiron, an inhibitor of superoxide anion production, can partially restore the responsiveness to inhibition of NOS in the advanced stage of cardiomyopathy.

Because tiron did not completely restore the effect of LNA, other mechanisms may contribute to this response in cardiomyopathy. Abnormal intracellular calcium handling results in calcium overload in myocytes of cardiomyopathic hamsters (Factor and Sonnenblick, 1985). It is unknown if a calcium channel defect is also present in endothelial cells of the cardiomyopathic hamster. However, because NOS is a calcium-dependent enzyme, the function of NOS may be altered in cardiomyopathic hamsters. This hypothesis will require further evaluation. Another interesting possibility relates to the finding that accumulation of an endogenous inhibitor of NOS, N^G,N^G-dimethylarginine, occurs in humans in chronic renal failure (Vallance et al., 1992). This leads to the speculation that if this inhibitor was present in cardiomyopathic hamsters, exogenous inhibitors of NOS, such as LNA, would have a lessened effect. Although inhibition of NOS has been shown to increase blood pressure through vasoconstriction, the possibility that LNA is acting directly on the myocardium to alter contractile force cannot be ruled out because contractile force was not measured in this study. However, it is unlikely that this mechanism contributes to the hypertension produced by inhibition of NOS activity. In summary, this study has demonstrated a decreased regulation of blood pressure by NO that is partly mediated by enhanced superoxide anions in the advanced stage of cardiomyopathy.
References


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