Stress upregulates arterial matrix metalloproteinase expression and activity via endothelin A receptor activation

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Submitted 12 February 2003; accepted in final form 1 July 2003

Ergul, Adviye, Vera Portik-Dobos, Ararat D. Giulumian, Mariela M. Molero, and Leslie C. Fuchs. Stress upregulates arterial matrix metalloproteinase expression and activity via endothelin A receptor activation. Am J Physiol Heart Circ Physiol 285: H2225–H2232, 2003. First published July 3, 2003; 10.1152/ajpheart.00133.2003.—Degradation of the extracellular matrix proteins by matrix metalloproteinases (MMP) is an important regulatory step in the vascular remodeling process. Recent studies demonstrated that ETα receptors regulate cardiac MMP activity and fibrosis in DOCA-salt hypertension. However, little is known about endothelin (ET)-1 regulation of vascular MMP activity in hypertension. Thus early changes in ET-1-mediated regulation of MMP activity were measured in borderline hypertensive rats that develop impaired vasorelaxation and hypertension with chronic exposure to stress. Experiments were performed after 10 days of exposure to the behavioral stressor, air-jet stress, but before the onset of stress-induced hypertension. Study groups were 1) control (n = 8); 2) air-jet stress for 10 days (n = 8); 3) control plus ETα antagonist ABT-627 (n = 4), and 4) air-jet stress plus ETα antagonist (n = 4). MMP activity in the thoracic aorta was assessed by gelatin zymography. MMP protein and tissue ET-1 levels were evaluated by immunohistochemistry, and ET receptor density was determined by immunoblotting. Exposure to stress caused a twofold increase in plasma ET-1 levels (P < 0.05), and there was increased ET-1 staining at the tissue level. Total MMP activity and expression of MMP-2 and MMP-9 were increased in the stress group. ETα receptor antagonism prevented the increase in MMP expression and activation in the stress group. These results provide evidence that the MMP system is activated before the development of hypertension and ET-1 mediates these early changes in vascular remodeling.

endothelin-1; ABT-627

ENDOTHELIN-1 (ET-1) contributes to blood pressure elevation as well as cardiac, vascular, and renal complications in several experimental models, including DOCA-salt hypertensive rat, DOCA-salt-treated spontaneously hypertensive rats (SHRs), Dahl salt-sensitive rats, and aldosterone-infused rats (18, 21, 24, 25). In DOCA-salt hypertensive rats, enhanced myocardial remodeling and fibrosis associated with increased fibrinogen, matrix metalloproteinase (MMP) activity, and proinflammatory mediators occur in the left ventricle and ETα receptor antagonism inhibits these changes (1, 2). In clinical hypertension, ET-1 is increased in African-American hypertensive patients who present with increased incidence of cardiovascular complications, including left ventricle hypertrophy and stroke (7, 8). Although the exact mechanism is not clear, it has been proposed that African-Americans experience chronic sympathetic system activation due to more recurrent exposure to social and environmental stress, which contribute to the increased incidence of hypertension and related complications in this patient population (9). A recent study (33) demonstrated that African-American adolescents with family history of essential hypertension manifest greater increases in plasma ET-1 and total peripheral resistance in response to the mental stress of playing a video game. These results suggest that the ET system is activated in response to stress before the development of hypertension, but early effects of ET-1 on vascular and myocardial structure remain to be determined.

The borderline hypertensive rat (BHR) is the first-generation offspring of a female SHR and a male Wistar-Kyoto rat and mimics a number of features common in the development of hypertension in African-Americans (29). First, the BHR becomes hypertensive with repeated exposure to behavioral stress (29). Second, high-salt diet causes the development of hypertension (3). Third, exposure to stress for 10 days alters vascular contraction and relaxation in BHRs (11, 13). Enhanced sympathetic nervous system activity and peripheral vascular structural changes leading to increased vascular resistance are believed to contribute to stress-induced hypertension. Whether there are changes in the vascular structure in early phases of stress is unknown. Furthermore, the effect of stress on ET-1 activation in this model is not well defined.

The present study was designed to determine behavioral stress-induced changes in vascular extracellular matrix proteins before the development of stress-influenced hypertension in the BHR. This study was performed using a behavioral stressor, air-jet stress, and a pharmacologic agent, the ETα antagonist ABT-627. The purpose of this study was to determine whether ETα receptor antagonism inhibits these changes in ET-1 expression and MMP activity in a model of stress-induced hypertension. The results presented here will help to further understand the role of ET system in stress-induced hypertension.

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duced hypertension in the BHR model. Because matrix MMPs regulate the turnover of structural proteins such as collagen and elastin in the extracellular matrix, this study focused on the expression and activity of vascular MMPs in response to stress. The second goal of the study was to investigate systemic and local ET-1 levels in response to stress and determine whether ET-1 contributes to changes in MMP expression and activation via the ET_\text{A} receptor.

**METHODS AND MATERIALS**

**Blood pressure measurements and tissue collection.** Female SHR and male Wistar-Kyoto rats were purchased from Taconic Farms (Germantown, NY) and bred at the Medical College of Georgia to obtain the first-generation offspring BHRs. Animal care and experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No 85-23, revised 1996). Male BHR were divided into two groups: control (n = 8) or exposed to air-jet stress (5 s) for 10 days; stressed group (n = 8). Animals were subjected to a random block of treatments in sound-insulated chambers. Chronic stress consisted of pulses of compressed air directed toward the face from a 1/8-in. opening at the front of the restrainer. Animals were subjected to a random duration of pulses (5–120 s) and interpulse intervals (5–120 s) for 2 h/day for 10 days. The ET_\text{A} receptor antagonist ABT-627 was administered to additional control and stressed animals (n = 4 per group) in the drinking water (5 mg/kg^-1·day^-1) for 10 days. This dose has been shown to block ET receptors (26, 34). After the exposure to air-jet stress on day 10, rats were anesthetized with ketamine (50 mg/kg im) and acepromazine (15 mg/kg im). Under aseptic conditions, a cannula was placed in the femoral artery and was exteriorized at the nape of the neck. The cannula was flushed with heparinized saline (100 U/ml) after a 1-day recovery period, arterial pressure was measured with a Grass recorder in unrestrained rats in their home cage. On day 11, rats were anesthetized with pentobarbital sodium (50 mg/kg ip), heparin (500 U) was administered in the left ventricle, and aortas were removed. After being rinsed with saline, aortic samples were snap frozen in liquid nitrogen for zymography and immunoblotting. A segment of the aorta was fixed in 10% formalin.

**MMP activity.** MMP activity was determined with gelatin zymography of aortic homogenates. Frozen specimens were homogenized in extraction buffer (1:10, wt/vol) containing 0.15 M NaCl, 20 mM ZnCl_2, 1.5 mM NaN_3, 10 mM cacodylic acid, and 0.01% Triton X-100. After centrifugation at 4°C for 10 min at a speed of 800 _g_, the supernatant was concentrated by using a Centriplus concentrator. Samples were centrifuged at 3,000 _g_ for 4.5 h at 4°C, and the protein content was measured by using protein assay (Bio-Rad; Richmond, CA). Samples were stored at –80°C in small aliquots. On the day of the experiment, samples were loaded on 10% gelatin zymography gels (Bio-Rad) and separated under nonreducing conditions. The gels were then rinsed twice in 2.5% Triton X-100 and incubated overnight (16 h) in substrate buffer containing 50 mM Tris-HCl, 5 mM CaCl_2, and 0.1% SDS. The membranes were then incubated overnight with the primary antibody as recommended by the manufacturer (Research Diagnostics; Flanders, NJ). Bands were visualized by using an enhanced chemiluminescence detection kit from Amersham Life Sciences (Arlington Height, IL).

**Immunohistochemistry.** Aortic segments were fixed in 10% formalin, and sections were mounted on slides. Immediately before immunostaining, sections were permeabilized with 1% Triton X-100, 0.1% Tween 20, and 0.02% NaN_3. The membranes were then incubated overnight with the primary antibody as recommended by the manufacturer. Bands were visualized by using an enhanced chemiluminescence detection kit from Amersham Life Sciences (Arlington Height, IL).

**Western blot analysis.** Vascular ET_\text{A} receptor density and MMP-2 and MMP-9 levels were determined by immunoblotting. Vascular extracts (20 µg) were separated on 10% SDS gels under nonreducing conditions by using a Tris-glycine running buffer (0.2 M Tris-base, 0.2 M glycine, pH 6.8, and 0.1% SDS). The separated samples were transferred to a nitrocellulose membrane in Tris-glycine transfer buffer supplemented with 20% methanol. The immunoblots were blocked for 1 h in blocking grade powdered goat milk (5%) diluted in 0.2 M Tris-base, 1.4 M NaCl, 0.1% Tween 20, and 0.02% NaN_3. The membranes were then incubated overnight with the primary antibody as recommended by the manufacturer. Bands were visualized by using an enhanced chemiluminescence detection kit from Amersham Life Sciences (Arlington Height, IL).
control and stress groups treated with \( \text{ET}_A \) antagonist ABT-627, a similar pattern of staining was observed (data not shown).

\( \text{ET}_A \) receptor density was determined by immunoblotting and two bands at 54 and 39 kDa corresponding to native and glycosylated forms of receptors, respectively, were detected. Densitometric analysis of both bands indicated a twofold increase in stressed animals compared with controls (Fig. 2). In ABT-627-treated animals, there was no difference between the control and stress groups.

**Vascular MMP activity.** Total MMP activity in thoracic aorta of control and stressed BHR as well as ABT-627-treated control and stressed rats was assessed by using gelatin zymography. A representative gelatin zymogram is shown in Fig. 3A. The main proteolytic activity in the untreated group corresponds to 72 kDa proMMP-2 and two active forms of MMP-2 at 67 and 55 kDa, whereas in the \( \text{ET}_A \) receptor antagonist-treated group, a 55-kDa band was not detected. In addition, there was very faint activity corresponding to proMMP-9, which did not differ among groups (data not shown). Densitometric analysis of all the lytic bands combined in each sample demonstrated that total MMP activity was increased in the stress BHR group (\( P < 0.05 \) vs. control) and the \( \text{ET}_A \) receptor antagonism prevented the increase in MMP activation especially at the 55-kDa level (\( P < 0.05 \) vs. untreated BHR) (Fig. 3B).

To determine whether and to what extent MMP activity correlates with protein levels and to evaluate the localization in the vessel wall, MMP-2 and MMP-9 were investigated by immunohistochemistry. Breast cancer tissue (for MMP-2 and MMP-9) was used as a positive control. As shown by representative images in...
Figs. 4 and 5, there was augmented staining for MMP-2 and to a lesser extent for MMP-9 in the stress group, and treatment with ABT-627 decreased staining in the stress group. Staining was observed both in the medial and adventitial layers. Medial staining was diminished in the absence of a primary antibody, indicating specific staining for MMP proteins, whereas adventitial staining was unchanged, suggesting non-specific staining due to lipids. The increase in MMP-2 and MMP-9 levels was also confirmed by immunoblotting of vascular homogenates. As shown in Figs. 4 and 5, both MMP-2 and MMP-9 were significantly elevated in the stress group, which was prevented by ABT-627.

DISCUSSION

Because the BHR model is sensitive to a number of environmental stimuli, it has been used extensively for studies focusing on behavioral stress (29). Prolonged exposure to stress ultimately results in elevated blood pressure that persists even after removal of the stress (29). Although 10 days of air-jet stress as used in this study does not alter the resting arterial blood pressure, changes in vascular reactivity can be detected in this period of time before the development of hypertension (10, 11). Therefore, 10-day exposure of BHRs to stress offers a good model to study changes in vascular function and structure before the development of hypertension allowing for determination of vascular changes independent of changes in blood pressure. With the use of this model, the present study sought to answer three important questions: 1) Is the ET system activated in early stages preceding the development of hypertension in a stress-induced model of hypertension? 2) Is the MMP system, critical for the regulation of vascular structure, altered in response to stress? and 3) Are changes in the MMP system mediated by ET-1? Our findings demonstrate for the first time that stress stimulates the production of ET-1 and increases MMP activity in the vasculature before changes in blood pressure. Furthermore, MMP activation can be prevented by the administration of an ETA receptor antagonist during the stress period, providing evidence that ET-1 is, in part, responsible for the stimulation of vascular MMP activity.

There is accumulating evidence that ET-1 is important in the pathogenesis of hypertension in several experimental models, including DOCA-salt hypertensive rats (19–21), Dahl salt-sensitive rats (5), as well as angiotensin II-infused rats (22). In addition to lowering blood pressure, ET receptor antagonists provide protective effects on target organs in these models (30). Recent studies reported that ETA receptor antagonism decreases collagen accumulation and improves MMP-2 activity in kidneys from stroke-prone SHRs (32) as well as decreasing fibronectin levels and MMP activity in the left ventricle of DOCA-salt hypertensive rats that display cardiac fibrosis (2). In clinical hypertension, there is a consensus that ET-1 levels are increased in patients with low-renin and salt-sensitive forms of hypertension, which is predominantly seen in the African-American population (6–8, 30). In this patient population, frequent exposure to behavioral and environmental stress plays an important role in the development of hypertension (9). Interestingly, Treiber and colleagues (33) reported that in young healthy African-American adolescents with a family history of hypertension, stress induces elevations in plasma ET-1 levels. The same group (4) also reported that in this
cohort, left ventricular mass is significantly greater even before the development of hypertension. On the basis of these past observations, we wished to investigate the ET system as well as vascular structure at different stages in a stress-induced model of hypertension. The present study provides evidence that both plasma and local vascular ET-1 levels are increased as early as 10 days after exposure to behavioral stress. In addition, with the use of an immunoblotting approach, we studied the expression of ETA receptors, and two specific bands corresponding to the native and glycosylated form of receptors were detected as previously reported (14). ETA receptors that mediate the contractile and proliferative response to ET-1 are upregulated in response to stress, and this increase in ETA expression is diminished in animals treated with ABT-627. The explanation for this intriguing result is threefold. First, ET-1 may stimulate ETA receptor expression directly, and in the presence of receptor antagonist, this stimulation is blocked. Elevated ET-1 levels have been reported to decrease ET-1 binding due to receptor desensitization (28, 31), but whether decreased binding is associated with alterations in protein levels is unclear. Second, ET-1 may regulate ETA expression indirectly via the activation of intermediate factors, and this possibility warrants future studies. Third, although it has not been reported in the literature, ABT-627 may have a nonspecific effect on ETA receptor expression. Although the reason(s) for restored ETA expression in the ABT-627-treated stress group remains to be determined, the novel finding of this study is that the ET system is activated before the development of hypertension in the stress-induced hypertension model.

In essential hypertension, peripheral vascular resistance is increased due to decreased lumen diameter, and abnormal vascular reactivity may originate from structural and functional changes of blood vessels (15, 16). Functional changes involve impaired vascular relaxation and increased sensitivity to vasoconstrictors.

**Fig. 4.** Increased MMP-2 expression in the medial layer of aortic rings from control and stress animals ($n = 3$ in each group). A: breast cancer tissue was used as positive control. B: nonspecific staining in the absence of primary antibody is shown. C–F: MMP-2 levels in vascular homogenates were determined by immunoblotting, and a representative blot is given under each group. G: staining intensity in immunohistochemistry slides was analyzed by Metamorph analysis software and plotted as average staining intensity (means ± SE). *$P < 0.05$ vs. control or treatment groups.
Altering in vascular structure arise from vascular remodeling and result in collagen deposition leading to decreased arterial compliance. Our group demonstrated previously that behavioral stress causes decreased vasorelaxation, which is linked to altered phosphorylation of small heat shock proteins that bind to actin cytoskeleton, and these changes in vascular function occur before the development of hypertension in the BHR model (10). However, whether vascular structure is influenced by stress early in the disease process remained unknown. Because MMPs regulate the turnover of extracellular matrix proteins that are critical for vascular structure, the present study investigated whether MMP activity is altered in response to stress. MMPs are a family of zinc-dependent enzymes, and several species are commonly expressed in the vasculature, including MMP-1, MMP-2, and MMP-9 (2, 12, 23, 27). All of these enzymes are secreted in zymogen forms (proMMPs), which are later activated by other proteinases to yield active MMPs (23). MMP-1, a 52- and 42-kDa protein in latent and active forms, respectively, can degrade fibrillar collagen, whereas MMP-2 and MMP-9 can process gelatin (denatured collagen) into smaller fragments. proMMP-2 is a 72-kDa protein with two active forms (~66 and 54 kDa) (17). Molecular mass of MMP-9 is 92 and 82 kDa for latent and active forms, respectively (12). To be able to evaluate both MMP and ET systems in each animal individually without pooling tissue from several animals, we chose to study aortas in the present study. Vascular MMP activity was assessed by gelatin zymography, which detects primarily MMP-2 and MMP-9 activity. Total vascular MMP activity was increased in the stress group compared with control, and this increase was predominantly in the 66- and 54-kDa forms. In ABT-627-treated animals, total MMP activity was restored to control levels. However, compared with untreated control and the stress group, the 56-kDa form was not

![Image of aortas](https://via.placeholder.com/150)

**Fig. 5.** Increased MMP-9 expression in the medial layer of aortic rings from control and stress animals (n = 3 in each group). A: breast cancer tissue was used as positive control. B: nonspecific staining in the absence of a primary antibody is shown. C–F: MMP-9 levels in vascular homogenates were determined by immunoblotting, and a representative blot is given under each group. G: staining intensity in immunohistochemistry slides was analyzed by Metamorph analysis software and plotted as average staining intensity (means ± SE). *P < 0.05 vs. control or treatment groups.
detected in animals that received ABT-627, and 72-kDa proMMP was more prominent. These findings suggest that ET-1 antagonism prevented the processing of latent enzyme to smaller molecular mass active form. Because there was no significant difference in blood pressure measurements among the study groups, effects of ABT-627 on MMP activation appear to be independent of blood pressure lowering. These results suggest that ET-1 plays an important role in the regulation of MMP activation in aorta. Whether similar changes occur in small resistance arteries remains to be determined. In addition, this study evaluated early changes in extracellular matrix proteins involved in the vascular remodeling process. After 10 days of stress, MMP activity was stimulated, but collagen type I levels were not altered (data not shown). Although this study did not assess lumen-to-wall ratio, we speculate that stress induces MMP activation with no significant change in wall thickness in 10 days.

In conclusion, the expression and activity of vascular MMP proteins that are important for the regulation of extracellular matrix proteins and remodeling processes are upregulated in response to behavioral stress. This increase is associated with parallel increases in plasma and local ET-1 levels, and ETA receptor antagonism restores the MMP activity in the stressed animals. These intriguing findings provide direct evidence that stress induces early changes in the vascular structure via the stimulation of the ET system.

We thank Abbott Laboratories and Dr. Jerry Wessale for the ABT-627 compound.

DISCLOSURES

This study was supported by grants from Pfizer, the American Heart Association, and the American Diabetes Association (to A. Ergul) and National Heart, Lung, and Blood Institute Grant HL-49924 to L. Fuchs.

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