Fibrosis, Matrix Metalloproteinases, and Inflammation in the Heart of DOCA-Salt Hypertensive Rats: Role of ETα Receptors

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Abstract—In deoxycorticosterone acetate (DOCA)-salt hypertension, the endothelin-1 system is activated and plays a role in cardiac fibrosis. Remodeling of extracellular matrix (ECM) may lead to interstitial fibrosis, which may contribute to heart failure. Imbalance in synthesis and degradation of the ECM by matrix metalloproteinases (MMPs) as well as inflammation may play a role in matrix protein deposition and cardiac remodeling in hypertension. We measured expression of the extracellular matrix protein fibronectin, the activity of the gelatinases MMP-2 and MMP-9, the proinflammatory transcription factor NFκB, and the adhesion molecules, vascular cell adhesion molecule (VCAM)-1 and platelet-endothelial cell adhesion molecule (PECAM)-1 in hearts of DOCA-salt hypertensive (DS) rats treated or not with the endothelin ETα antagonist BMS 182874 (BMS). Unilaterally nephrectomized rats (UniNx) were compared with DS rats treated or not with BMS 40 mg/kg/d. Fibronectin deposition was detectable at the first week, and remained elevated thereafter. This increase was abrogated by administration of the ETα antagonist. Enzymatic activity of gelatinases was increased (P<0.01) in DS compared with control during the first and second week. BMS blocked the increase of MMP-2 and MMP-9 activity at week 1 (P<0.05); MMP activity remained lower than in DS at week 2. NF-κB binding activity in DS was higher (P<0.05) than it was in controls during the second week, and was reduced by BMS. The adhesion molecules VCAM-1 and PECAM-1, and the antiapoptotic molecule xIAP were upregulated in the left ventricle of the heart of DS rats and downregulated in the rats treated with the ETα antagonist. In conclusion, cardiac extracellular remodeling in rats with endothelin-dependent hypertension was associated with increased fibronectin, MMP activity, and upregulation of inflammatory mediators, all of which were reduced by ETα antagonism.

Key Words: hypertension, mineralocorticoid ■ myocardium ■ endothelin ■ fibronectin ■ apoptosis

A number of studies have demonstrated that there is an endothelin (ET)-dependent component in blood pressure elevation and in vascular, cardiac, and renal complications in mineralocorticoid hypertension, including that in the deoxycorticosterone acetate (DOCA)-salt hypertensive rat.1-5 We previously showed that collagen deposition was dramatically increased in the left ventricle of DOCA-salt rats.3 Although development of left ventricular hypertrophy was unaffected by treatment with the ETα-selective endothelin receptor antagonist A-127722, cardiac collagen deposition was significantly reduced. Collagen deposition was associated with increased transforming growth factor (TGF)β1 expression, a well-known profibrotic factor, which was also abrogated by the ETα antagonist. Collagen deposition is not the only mechanism involved in remodeling of the heart in hypertension and under the action of hormones such as ET-1, angiotensin II, and aldosterone. Matrix metalloproteinase (MMP) expression is increased in the heart in experimental animals and in patients with heart failure.6,7 Indeed, MMPs have a major role in extracellular matrix (ECM) protein turnover.8 The enzymes capable of degrading fibrillar helices are interstitial collagens or MMP-1 and MMP-8. The resulting fragments unfold their triple helix conformation. So-formed single alpha-chains (gelatins) can be further degraded into oligopeptides by less specific proteinases such as gelatinases (72 kDa MMP-2 and 92 kDa MMP-9).9

During inflammatory responses, one of the mediators that is activated is the nuclear factor NFκB, which activates numerous genes that include adhesion molecules involved in recruitment of circulating leukocytes to sites of inflammation. Among these adhesion molecules are intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1).10,11 Other mechanisms including apoptosis may be activated during inflammatory response in tissues such as the heart. Apoptosis has been documented both in the myocardium in a number of clinically important states, including hypoxia12 and myocardial infarction,13 and in the hearts of patients with end-stage heart failure.14,15 Cellular...
factors including NF-xB have been shown to interact with key antiapoptotic genes such as Bcl-216 and inhibitor of apoptosis proteins (IAPs).17,18

In this study we asked the following questions: Is fibronec-
tin increased together with collagen in the left ventricle of DOCA-salt hypertensive rats, and, if so, is this increase dependent on ETα receptor activation? Will this be associated with ETα-dependent upregulation of MMP activity, and increased expression of mediators of inflammation like NF-xB and adhesion molecules such as ICAM-1, VCAM-1, and platelet-endothelial cell adhesion molecule-1 (PECAM-1) in the heart? Because apoptosis may be activated in cardiovascular tissues in DOCA-salt hypertensive rats,19 we proposed that there would be a reactive (compensatory) increase in xIAP expression in the left ventricle of these rats.

Materials and Methods

The antibody against fibronecetin was from Calbiochem-Novabach Corporation. Antibodies against VCAM-1, ICAM-1, PECAM-1, and p65 subunit of NF-xB, as well as the secondary antibodies, anti-rabbit, anti-mouse, and anti-goat were bought from Santa-Cruz. The anti-xIAP was from Transduction Laboratories. The oligonucleotide containing the NF-xB binding site was from Promega Corporation.

Animal Experiments

The study was approved by the Animal Care Committee of the Clinical Research Institute of Montreal and conducted in accordance with recommendations of the Canadian Council of Animal Care. DOCA-salt hypertension was induced by the method of Ormsbee and Ryan.20 Male Sprague-Dawley rats (Charles River, St. Constant, QC, Canada) weighing 200 g were unilaterally nephrectomized under ketamine (50 mg/kg)-xylazine (5 mg/kg) anesthesia. Silicone rubber impregnated with DOCA (Sigma Chemical Co) (200 mg per rat) was implanted subcutaneously, and rats were offered 1% saline to drink. The ETA endothelin receptor antagonist, BMS182874 (BMS), was administered to DOCA-salt rats in the drinking water (40 mg/kg/d) for 1, 2, or 4 weeks. After warming the rats, systolic blood pressure (BP) was measured weekly by the tail-cuff method while the rats were under slight restraint. Readings were recorded on a model speciﬁed.

In Situ Zymography

Fresh frozen sections (5 μm) were incubated with 0.5 mg/mL gelatin-Orange Green in developing buffer, pH 7.4 (Tris Base 50 mmol/L, HCl 40 mmol/mL, NaCl 200 mmol/mL, CaCl2-2H2O 5 mmol/L, Brij 35 [wt/vol] 0.29, phenylmethylsulfonylfluoride [PMSF] 50 mmol/L) at 37°C for 3 hours. Slides were washed 3 times with PBS pH 7.4 to remove unbound gelatin. Gelatinase activity resulted in the loss of quenching; therefore an increase in activity was visualized as a linear increase in fluorescence. The presence of PMSF in the developing buffer ensures increased fluorescence was not due to a serine protease. Also, because MMP activity depends on the presence of calcium, this requirement for lytic activity was assessed with the addition of 50 mmol/L ethylenediaminetetraacetic acid (EDTA) in the developing buffer. DNA staining was performed with the fluorescent dye DAPI (4',6-diamidine-2'-phenylindole dihydrochloride) from Molecular Probes. Heart sections were incubated with 1 μg/mL DAPI in methanol for 30 minutes at 37°C and then washed 3 times with PBS, pH 7.4. A microscope equipped with a mercury lamp and epifluorescence optics was used to visualize fluorescence. The green fluorescence was quantified as percent area of fluorescence over total area, using an image analysis system (Northern Eclipse 5.0, EMPIX Imaging Inc).

Western Blot Analysis

Protein was extracted from frozen tissue in lysis buffer containing PBS (pH 7.4), 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L sodium orthovanadate, 1 mmol/L PMSF, 1% Nonidet P-40, and aprotinin, leupeptin, and pepstatin (1 μg/mL each). Protein concentration was determined using the BioRad protein assay (BioRad Laboratories Inc.). Samples were electrophoresed in reduced conditions in a 10% SDS-polyacrylamide gel at 60 V for 2 hours and transferred to a polyvinylidine difluoride membrane at 100 V for 1 hour. Membranes were incubated overnight at 4°C, with the specific antibodies at dilutions indicated in the Table. Horseradish peroxidase-conjugated IgG was used as a secondary antibody for 1 hour at room temperature. Bands were visualized by chemiluminescence kit (Roche Molecular Biochemicals) and quantified by densitometry.

Electrophoretic Mobility Shift Assay for Measurement of NF-xB Activity

Nuclear protein was extracted as described previously.21 Briefly, frozen tissues were homogenized and resuspended in 1 mL 50 mmol/L Tris (pH 7.4) containing 1 mmol/L orthovanadate, 1 μg/μL pepstatin, and 1 μg/μL aprotinin. The suspension was centrifuged at 2500g for 4 minutes at 4°C. The pellet was resuspended in 1 mL lysis buffer containing 20 mmol/L HEPES (pH 7.9), 350 mmol/L NaCl, 20% glycerol, 1 mmol/L MgCl2, 0.5 mmol/L EDTA, 0.1 mmol/L EGTA, 1% Nonidet P-40 and protease inhibitors (pepstatin [1 μg/μL], aprotinin [1 μg/μL], leupeptin [1 μg/μL]), PMSF [1 mmol/L], orthovanadate [1 mmol/L]), incubated on ice for 30 minutes, and centrifuged at 16 200g for 10 minutes at 4°C. The supernatant was aliquoted and frozen at −80°C until use. Protein concentration was assayed by BioRad reagent. Twenty micrograms of nuclear protein were incubated in 1× gel shift binding buffer (50 mmol/L Tris, 250 mmol/L NaCl, 20% glycerol, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 5 mmol/L MgCl2, 0.25 mg/mL poly dIdC) with 0.5 ng of 32P-dATP end-labeled double-stranded oligonucleotide containing the NF-xB (5'-AGTTGAGGGGACTTTCC-CAGGC-3') binding site for 30 minutes at room temperature. In competition assays, 50 ng of unlabeled oligonucleotide was used whereas supershift assays used an anti-p65 antibody that does not interfere with the oligonucleotide-protein binding site. The DNA-protein complexes were analyzed on a 4% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer, dried, and autoradiographed.

Analysis of Data

Results were analyzed by ANOVA and a Newman-Keuls post hoc test and were considered statistically significant if P<0.05.

Results

BP rose with DOCA-salt treatment to 225±9.4 mm Hg compared with control (112±2.6 mm Hg, P<0.01). BP of
DOCA-salt rats treated was significantly lower with the ET$_\alpha$ selective antagonist BMS (137±3.4 mm Hg, $P<0.01$ versus DOCA-salt), similar to what we previously reported with other ET$_\alpha$ selective antagonists.\(^3\) In that report,\(^3\) increased deposition of collagen in the left ventricle of the heart was also shown to occur throughout the period studied. Here we show that, in all times studied (1, 2, and 4 weeks), the DOCA-salt rat left ventricle exhibited significant increases in fibronectin expression (Figure 1), which were abrogated by treatment with ET$_\alpha$ antagonist.

Gelatinase activity (probably representing activity of MMP-2 and MMP-9) was examined by in situ zymography (Figure 2). Gelatinase activity was inhibited by the metal chelator EDTA, but not by the serine protease inhibitor PMSF, which confirmed that lysis of gelatin was due to MMP activity. The top of Figure 2 shows representative gelatinase activity on heart sections of control, DOCA-salt, and ET$_\alpha$ antagonist-treated rats. Gelatinase activity was significantly increased in hearts of DOCA-salt rats at 1, 2, and 4 weeks, compared with the other 2 groups (Figure 2, bottom), and reduced by ET$_\alpha$ antagonist treatment.

NF-$\kappa$B binding was studied by EMSA and specificity demonstrated by supershift with antibody against NF-$\kappa$B p65 subunit and by competition with excess unlabeled specific NF-$\kappa$B binding oligonucleotides (Figure 3). Bottom panels of Figure 3 show that NF-$\kappa$B DNA binding was increased in DOCA-salt rat hearts compared with control during the second week ($P<0.05$), and tended to remain elevated on the fourth week (not achieving significance, however). Treatment with the ET$_\alpha$ antagonist reduced NF-$\kappa$B activity.

VCAM-1 expression (Figure 4A) was significantly increased in hearts of DOCA-salt rats at week 1 ($P<0.01$ versus control), and week 2 ($P<0.05$ versus control), and was decreased by the ET$_\alpha$ antagonist ($P<0.01$ during week 1, $P<0.05$ at week 2). At week 4, VCAM-1 expression was not significantly different between groups. ICAM-1 expression was similar in all groups (not shown). PECAM-1 expression (Figure 4B) was increased in hearts of DOCA-salt rats from week 1 to 4 ($P<0.05$ versus control group). PECAM-1 expression in heart was significantly decreased by the ET$_\alpha$ antagonist at week 1 and 4 ($P<0.05$), and at week 2 there was a trend to decrease, which did not reach statistical significance, in the treated group.

Figure 1. Fibronectin expression in left ventricle of DOCA-salt hypertensive rats and effect of ET$_\alpha$ antagonism. Results are means±SEM (n=3). * $P<0.05$, † $P<0.01$ versus other groups. UniNx=unilaterally nephrectomized controls, ET$_\alpha$A=ET$_\alpha$ receptor antagonist. Top panel shows representative Western blot of results at 1 week.

Figure 2. Top, In situ zymography to evaluate gelatinase activity on sections of left ventricle. A, Oregon Green 488 conjugated-gelatin. B, Costaining with DAPI to show nuclei. C, Disappearance of lysis with EDTA confirmed that lysis was due to MMPs and not a serine protease. Bottom, Quantification of fluorescence from degraded gelatin. Results are means±SEM (n=3). * $P<0.05$ versus DOCA-salt+ET$_\alpha$A antagonist, † $P<0.01$ versus UniNx control.
IAP expression was 1.6-fold higher in hearts of DOCA-salt rats after the first week (Figure 5). This response was abrogated in rats treated with the ET A antagonist. No significant differences between groups were observed at weeks 2 and 4.

**Discussion**

The present study demonstrates molecular events associated with fibrosis and remodeling in the heart of rats with DOCA-salt hypertension. In this model of mineralocorticoid hypertension, activation of inflammatory mediators and matrix metalloproteinases as well as antiapoptotic molecules could be major components of cardiac remodeling in response to salt and mineralocorticoids, and could play a critical role leading to cardiac fibrosis. Since cardiac fibrosis that occurs in response to salt and mineralocorticoids is mediated at least partly by ET-1,3 we hypothesized that ET A receptor antagonism would abrogate these pathophysiologic responses. Our results demonstrate that inflammatory mediators (NF-κB), adhesion molecules VCAM-1 and PECAM-1, MMPs, and the antiapoptotic molecule xIAP are upregulated in the heart of DOCA-salt rats, and that this can be prevented by an ET A receptor antagonist. The mechanisms whereby mineralocorticoids together with salt may upregulate the endothelin system1–5 remain elusive; the possible role of vasopressin has been suggested.22,23 Mineralocorticoids could potentiate the action of vasopressin on ET-1 expression in the heart, or alternatively, mineralocorticoids could directly or indirectly stimulate vasopressin secretion, which could in turn stimulate ET-1 expression.22,23

Collagen deposition, such as that we documented in hearts of DOCA-salt rats,4 and more recently in rats treated with aldosterone and salt,5 has a particular time-course, with increase in procollagen I mRNA expression found early, and procollagen III mRNA expression significantly different only at week 4. The collagen III/collagen I ratio could play an important role in raising cardiac stiffness, because a lower ratio results in a stiffer, less compliant ventricle. Here we showed that fibronectin, another fibrillar component of extracellular matrix with influence on mechanical properties of tissues, is increased early on in the left ventricle of DOCA-salt rats, and that this increase was prevented by the ET A antagonist. Fibronectin is a glycoprotein that forms a bridge between cells and the interstitial collagen network. The early and sustained increase of fibronectin deposition in the heart could precede collagen I deposition and contribute to extracellular-cell attachment remodeling on which collagen deposition becomes embedded.

Breakdown of mature extracellular matrix proteins involves many MMPs and interplay with their inhibitors, the
tissue inhibitors of metalloproteinases (TIMPs). Increased gelatinase activity in hearts of DOCA-salt hypertensive rats may be part of the remodeling process of the extracellular matrix that contributes to distortion of cardiomyocyte architecture and organization. New collagen and fibronectin deposition may contribute to worsening of cardiac stiffness and contractility. The reversal of gelatinase activity by ET\(_A\) antagonism may contribute to prevent cardiac remodeling. Inhibition of ET\(_A\) receptors prevented MMP activation in postmyocardial infarction in the rat. The authors speculated that ET\(_A\) antagonists may act on cardiac fibroblasts. Increased gelatinase activity in DOCA-salt heart sections could be due to MMP-2, which, unlike MMP-9, is synthesized by many cell types including fibroblasts, and may be stimulated by TGF\(\beta\). TGF\(\beta\) expression in the left ventricle increases early in DOCA-salt hypertension. TGF\(\beta\) may play a role in extracellular matrix remodeling by stimulating collagen synthesis and increasing turnover and degradation of collagen.

NF-\(\kappa B\), increased by an ET\(_A\)-dependent pathway in the present experiment paradigm, regulates expression of genes involved in immune, inflammatory, and growth responses. Inflammatory changes induced by L-NAME may be abrogated by a NF-\(\kappa B\) decoy strategy. However, NF-\(\kappa B\) decoy oligodeoxynucleotides were unable to reduce gene expression of TGF\(\beta\), as well as perivascular and cardiac fibrosis. NF-\(\kappa B\) activation may result from increased oxidative stress, which may be induced by ET-1. A recent study showed reactive oxygen species-dependent NF-\(\kappa B\) activation in kidney of mineralocorticoid hypertensive rats. ET-1 may activate profibrotic factors and cardiac fibrosis through different pathways, involving TGF\(\beta\) on the one hand and inflammatory changes through NF-\(\kappa B\) on the other. Several TGF-\(\beta\)-responsive promoters have been identified, including fibronectin and collagen I promoters. This may underlie the finding that, whereas collagen and fibronectin are already upregulated at 1 week, NF-\(\kappa B\) binding activity rose only at 2 weeks of DOCA-salt treatment.

The endothelium becomes dysfunctional in the early stages of vascular diseases, and this involves, among other molecular and cellular processes, an inflammatory component mediated by adhesion molecules, targets of NF-\(\kappa B\). Transcriptional activation of those genes is tightly regulated by NF-\(\kappa B\). In the present study, VCAM-1 and PECAM-1 expression were increased in ET\(_A\) receptor-dependent fashion in DOCA-salt hypertensive rats, whereas ICAM-1 appeared unaffected. Upregulation of these adhesion molecules may not be simultaneous. In a model of experimental colitis in which VCAM-1 played a central role in leukocyte recruitment, immunoneutralization of ICAM-1 had no therapeutic effect. Interestingly, adhesion molecule upregulation occurred at a time when NF-\(\kappa B\) upregulation could not be detected.

NF-\(\kappa B\) may be a ubiquitous multifunctional signaling system that contributes to cell survival. It may also be proapoptotic in some cell types. We previously reported that the activation of apoptosis in cardiovascular tissues of DOCA-salt hypertensive rats may fine-tune cardiovascular growth. For this reason we examined the expression of the inhibitor of apoptosis xIAP and found it enhanced at an early stage in DOCA-salt rats, which resembles what we previously described for TGF\(\beta\). xIAP has recently been demonstrated to function as a cofactor of TGF\(\beta\). xIAP may thus potentiate TGF\(\beta\)-induced signaling. xIAP may also be involved in the activation of transcriptional mediators of TGF-\(\beta\) signaling.

In the present study, as in previous ones, ET\(_A\) antagonism was associated with moderate BP reduction in DOCA-salt hypertensive rats. Whether BP reduction may contribute to the present findings is not clarified by this study. Although the development of cardiac fibrosis and left ventricular hypertrophy concomitant with blood pressure rise in the DOCA-salt model may indicate a role for blood pressure elevation, cardiac fibrosis occurs in both ventricles whereas atrial natriuretic peptide gene expression is stimulated only in the left but not the right ventricle, and prevention of cardiac fibrosis by subhypotensive doses of spironolactone support a blood pressure-independent effect of mineralocorticoids in the induction of cardiac fibrosis. In this model cardiac fibrosis affects both the right and the left ventricle, which is more typically a hormonal than a hemodynamic effect. Thus, in contrast to cardiac hypertrophy that can be attributed to BP elevation, cardiac fibrosis and inflammation may be attributed to mineralocorticoid action.

In conclusion, these findings together with previous data suggest that in the early phase of fibrosis in the heart of DOCA-salt hypertensive rats, ET-1, via the ET\(_A\) receptor, activates TGF\(\beta\), NF-\(\kappa B\) activity, and xIAP expression. Inflammation in response to upregulation of NF-\(\kappa B\) activity is associated with increased PECAM-1 and VCAM-1. Antagonism of ET\(_A\) receptors may provide a new therapeutic strategy for targeting hormonally mediated changes occurring in the heart in some forms of cardiovascular disease with increased mineralocorticoid activity, in order to prevent inflammation, fibrosis, and extracellular matrix remodeling.

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References


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