Endothelin Antagonism on Aldosterone-Induced Oxidative Stress and Vascular Remodeling

Qian Pu, Mario Fritsch Neves, Agostino Virdis, Rhian M. Touyz, Ernesto L. Schiffrin

Abstract—Endothelin A (ET\(_A\)) receptor blockade has prevented vascular remodeling in aldosterone and salt-induced hypertension. To evaluate effects of the ET\(_A\) receptor antagonist, BMS 182874, compared with the aldosterone antagonist, spironolactone, on vascular remodeling in aldosterone-infused rats not exposed to a high salt diet, Sprague-Dawley rats were infused subcutaneously with aldosterone (0.75 \(\mu g/h\)) and treated with BMS 182874 (40 mg \(\times\) \(\mu g\) \(\cdot\) \(kg\) \(^{-1}\) \(\cdot\) \(d\) \(^{-1}\)), spironolactone, or hydralazine (both 25 mg \(\times\) \(kg\) \(^{-1}\) \(\cdot\) \(d\) \(^{-1}\)) while receiving a normal salt diet for 6 weeks. Aldosterone increased systolic BP \((P<0.01)\), plasma endothelin \((3.33 \pm 0.32 \text{ versus } 1.85 \pm 0.40 \text{ pmol/L in control, } P<0.05)\), systemic oxidative stress as shown by plasma thiobarbituric acid–reacting substances and vascular nicotinamide adenine dinucleotide phosphate (NADPH) activity. Aldosterone increased small artery media thickness \((17.7 \pm 0.9 \text{ versus } 13.6 \pm 0.8 \mu m \text{ in control, } P<0.05)\) and media/lumen ratio \((7.6 \pm 0.4 \text{ versus } 5.5 \pm 0.4\% \text{ in control, } P<0.05)\), with growth index of 21% indicating hypertrophic remodeling. Laser confocal microscopy showed increased collagen and fibronectin deposition and intercellular adhesion molecule-1 (ICAM-1) content in the vessel wall of aldosterone-infused rats. The 3 treatments lowered BP, although hydralazine was slightly less effective. BMS 182874 and spironolactone decreased oxidative stress, normalized the hypertrophic remodeling, decreased collagen and fibronectin deposition, and reduced ICAM-1 abundance in the vascular wall of aldosterone-infused rats, whereas hydralazine only reduced NADPH activity in aorta but did not affect the remaining parameters. Vascular remodeling of small arteries occurs in aldosterone-infused rats exposed to a normal salt diet and may be mediated in part by ET-1 via stimulation of ET\(_A\) receptors. Endothelin blockade may exert beneficial effects on vascular remodeling, fibrosis, oxidative stress, and adhesion molecule expression in aldosterone-induced hypertension. (Hypertension. 2003;42:49-55.)

Key Words: mineralocorticoids \(\searrow\) receptors, endothelin \(\searrow\) arteries \(\searrow\) resistance \(\searrow\) extracellular matrix \(\searrow\) free radicals

Aldosterone, which is involved in the regulation of body sodium and water homeostasis, has also been shown to induce cardiac remodeling and fibrosis,\(^{1}\)\(^{-2}\) vascular hypertrophy,\(^{3}\)\(^{-5}\) and impaired endothelial function.\(^{6}\) Endothelin-1 (ET-1), a potent vasoconstrictor peptide produced in the vessel wall among other tissues, mainly by vascular endothelial cells,\(^{7}\) plays a central role in vascular remodeling in some forms of experimental and, perhaps, human hypertension.\(^{8}\) Activation of vascular ET-1 production is associated with hypertrophic remodeling of resistance arteries.\(^{9}\)\(^{-10}\) The local production of ET-1 may induce inflammation,\(^{11}\)\(^{-12}\) which is increasingly recognized as participating in the pathogenesis of cardiovascular disease. There are several known relationships between aldosterone and ET-1. Patients with primary aldosteronism have increased circulating ET levels.\(^{13}\) ET-1 inhibits basal or stimulated renin synthesis in vitro and in vivo\(^{14}\)\(^{-15}\) and stimulates aldosterone secretion.\(^{16}\)\(^{-17}\) The effect of aldosterone on rats may depend on salt status.\(^{1}\) ET\(_A\) receptor blockade prevents vascular remodeling in aldosterone and salt-induced hypertension.\(^{3}\) Furthermore, oxidative stress may increase ET-1 synthesis in endothelial and human vascular smooth muscle cells (VSMCs),\(^{18}\) and aldosterone may activate production of reactive oxygen species.\(^{6}\)

The aim of this study was to examine remodeling of resistance arteries in aldosterone-infused rats in the absence of salt loading and mechanisms involved therein. We hypothesized that these aldosterone-induced effects are mediated through ET-1–sensitive pathways that involve oxidative stress. The latter may contribute to vascular remodeling, fibrosis, and inflammation, even if rats are not challenged with a high salt diet. We also compared the effect of blockade of ET\(_A\) receptors with the effect of the mineralocorticoid receptor antagonist spironolactone and, to account for BP lowering, tested a vasodilator, hydralazine.

Methods

Animal Experiments

The study protocol was approved by the Clinical Research Institute Animal Care Committee and conducted according to recommenda-
Production and Study of Small Arteries

Rats were killed by decapitation after 6 weeks of aldosterone infusion. A third-order branch of the mesenteric vasculature was isolated and mounted on a pressurized myograph as described previously.30 Endothelium-dependent and -independent relaxation were assessed in norepinephrine (10−3 mol/L)-precontracted resistance arteries with acetylcholine and sodium nitroprusside, respectively. Lumen and media dimensions were measured with intraluminal pressure maintained at 45 mm Hg after being deactivated with 10 mmol/L EGTA.20

NADPH Oxidase Activity

Activity of NADPH oxidase was measured in aortic segments and mesenteric arteries by lucigenin chemiluminescence (5 mol/L)21, using NADPH (100 µmol/L) as substrate. Luminescence was measured every 1.8 seconds for 3 minutes in a luminometer (AutoLumat LB 953, Berthold). Background NADPH oxidase activity was measured at the beginning of each experiment. Activity of NADPH oxidase, after adding NADPH as substrate, was calculated by subtracting background activity. Specificity of the lucigenin signal for O2− generation was tested by adding diphenyleine iodium, a flavoprotein inhibitor, and tempol, a superoxide dismutase mimetic, which abolished the NADPH-induced response. Basal superoxide production without NADPH was barely detectable.

Laser Confocal Immunofluorescence Microscopy of Mesenteric Arteries

Vessels were fixed for laser confocal microscopy as previously described.22 Briefly, arteries were incubated with anti-collagen type I/III antibody (1:20, Calbiochem) and anti-fibronectin antibody (1:20, Calbiochem), and anti-intracellular adhesion molecule-1 (ICAM-1) antibody (mouse monoclonal IgG2a, 1:50, Santa Cruz Biotechnology, Inc) for 16 hours at 4°C. The vessel was washed and incubated for 1 hour at room temperature with fluorescein-conjugated F(ab′)2 fragments of anti-mouse IgG (20 µg/mL), anti-rabbit IgG (40 µg/mL) (both from Chemicon International), and 200 µg/mL Alexa Fluor 647 Anti-mouse IgG (Molecular Probes, Inc) for 1 hour at room temperature, respectively. Rhodamine phalloidin (10 µmol/L, Sigma) was added for the final 30 minutes of incubation to stain α-actin. After rinsing, the artery was mounted in 1:1 glycerol/PBS (pH 7.4) on a glass coverslip and studied with a laser scanning microscope (LSM) 510 system (Zeiss). An argon laser (488 nm) and two helium neon lasers (543 nm and 633 nm) were used with the following settings: stack size 1024×1024 pixels, pixel time 2.24 µs. Fluorescein isothiocyanate (FITC) was excited at 488 nm and captured as green; rhodamine, at 543 nm and captured as red; and Alexa Fluor 647, at 633 nm and captured as green after color-code transformation. The specimen was scanned in a point-by-point, line-by-line system. Using the Z-sectioning optical device, we obtained a stack of slice images (25 to 30 sections of 1.5- to 2.5-µm slices). In negative control samples without adding the first antibody, there was little or no autofluorescence on the vessel. Collagen and fibronectin in the vascular media were quantified by imaging (Northern Eclipse program, EMPIX Imaging Inc).

Measurement of Immunoreactive ET in Plasma

Plasma immunoreactive ET was measured by radioimmunoassay after passage though a C18 Sep-Pak cartridge as previously described.23 Intra-assay and interassay variation was below 15%.

Measurement of Thiobarbituric Acid–Reacting Substances in Plasma

Blood collected on EDTA was centrifuged and the plasma stored at −20°C overnight. Plasma thiobarbituric acid–reacting substances (TBARS) were measured colorimetrically.24 TBARS values were expressed in µmol/mL malondialdehyde (MDA) equivalents. Briefly, plasma was mixed with 2% butylated hydroxytoluene and quinoline reagent (26 mmol/L thiobarbituric acid and 918 mmol/L trichloroacetic acid) and boiled for 15 minutes. The reaction mixture was cooled and centrifuged at 3000g for 10 minutes. The soluble phase was measured with a spectrophotometer at 535 nm. MDA standards (Sigma) were diluted in the range of 0 to 4 µmol/mL.

Data Analysis

Data are presented as mean±SEM. Groups were compared by ANOVA, using repeated-measurements testing and Newman-Keuls test where appropriate. P<0.05 was considered significant. Growth index was calculated as the difference between the cross-sectional area of the media from aldosterone-infused and control rats divided by that of control rats.

Results

Systolic Blood Pressure, Heart Weight, and Plasma ET levels

Aldosterone-infused rats exhibited significantly elevated systolic BP after 1 week (Figure 1). Systolic BP was significantly lower in aldosterone-infused rats treated with BMS 182874, spironolactone, or hydralazine. The response to hydralazine however seemed slower than to the other agents, and BP was not different from that of untreated aldosterone-infused rats during the first week. After the first week, BP in the hydralazine group tended to remain higher (although not significantly different) than in response to BMS 182874 or spironolactone. Heart weight normalized for body weight was greater in aldosterone-infused rats. Spironolactone signifi-
cantly decreased relative heart weight, whereas BMS182874 or hydralazine had no effect on relative heart weight (Table). There was no significant difference in body weight between groups. Plasma endothelin was increased in aldosterone-infused rats compared with control rats. Spironolactone tended to reduce plasma endothelin levels without achieving statistical significance. Neither BMS nor hydralazine altered plasma endothelin (Table).

**Measurement of Oxidative Stress: Vascular NADPH Oxidase Activity and Plasma TBARs (MDA)**

NADPH oxidase activity, a major source of superoxide anion in the vasculature, was significantly \( P < 0.01 \) increased in mesenteric arteries and aorta of aldosterone-infused rats compared with controls (Figures 2A and 2B). BMS 182874 and spironolactone decreased NADPH oxidase activity in mesenteric arteries and aorta. Hydralazine decreased the NADPH oxidase activity only in aorta but not in mesenteric arteries.

Plasma TBARs (measured as MDA) levels were significantly higher \( P < 0.01 \) in aldosterone-infused rats compared with controls. BMS 182874 or spironolactone significantly decreased \( P < 0.01 \) plasma MDA (Figure 2C), whereas hydralazine had no effect.

**Endothelial Function of Mesenteric Resistance Arteries**

Mesenteric small artery relaxation in response to acetylcholine and to sodium nitroprusside (SNP) was unchanged after aldosterone infusion in this particular set of experiments. Neither BMS 182874 nor spironolactone or hydralazine had any effect on endothelium-dependent or -independent relaxation (data not shown).

**Morphological Characteristics of Resistance Arteries**

Aldosterone infusion resulted in increased media thickness and media-to-lumen ratio (Figures 3B and 3C) \( P < 0.05 \) and a trend to an increase in media cross-sectional area of mesenteric arteries \( P < 0.04 \) only on a one-sided post hoc \( t \) test compared with control rats (Figure 3D). The growth index of small arteries from aldosterone-infused rats was 21\%, indicating moderate hypertrophic remodeling. BMS 182874 or spironolactone treatment prevented hypertrophic remodeling induced by aldosterone \( P < 0.05 \) for media width and media-to-lumen ratio; only on one-sided post hoc \( t \) test for media cross section after BMS 182874, \( P < 0.02 \), or spironolactone, \( P < 0.03 \), whereas hydralazine had no effect on the structure of small arteries despite its hypotensive action.

**Vascular Extracellular Matrix Deposition and Inflammatory Markers**

Aldosterone infusion increased collagen and fibronectin deposition as well as density in the media of mesenteric arteries compared with control rats (Figures 4 and 5), which was prevented by either BMS 182874 or spironolactone. ICAM-1 expression was increased in the endothelial cell layer and adventitia of small arteries in aldosterone-infused rats (Figure 6). The increase of ICAM-1 was prevented by BMS 182874 and by spironolactone, but hydralazine had no effect on either vascular extracellular matrix or inflammatory mediators.

### Table: Relative Heart Weight and Plasma Immunoreactive Endothelin Concentration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Aldosterone</th>
<th>Aldosterone + BMS</th>
<th>Aldosterone + Spironolactone</th>
<th>Aldosterone + Hydralazine</th>
</tr>
</thead>
<tbody>
<tr>
<td>HW/BW, mg/g</td>
<td>3.02 ± 0.12</td>
<td>3.56 ± 0.12*</td>
<td>2.96 ± 0.32*</td>
<td>2.40 ± 0.39†</td>
<td>3.34 ± 0.07*</td>
</tr>
<tr>
<td>BW, g</td>
<td>419 ± 12</td>
<td>423 ± 11</td>
<td>404 ± 11</td>
<td>414 ± 13</td>
<td>382 ± 6</td>
</tr>
<tr>
<td>Plasma endothelin, pmol/L</td>
<td>1.85 ± 0.20</td>
<td>3.33 ± 0.30‡</td>
<td>3.06 ± 0.39*</td>
<td>2.57 ± 0.20*</td>
<td>2.85 ± 0.16*</td>
</tr>
</tbody>
</table>

Results are presented as mean ± SEM. HW indicates heart weight; BW, body weight; BMS, BMS 182874.

*\( P < 0.05 \) vs control; ‡\( P < 0.01 \) vs control; †\( P < 0.05 \) vs aldosterone.

**Figure 2.** Bar graphs depict activity of NADPH oxidase in mesenteric arteries (A) and aorta (B), and plasma concentration of TBARS, a marker of oxidative stress (C) from normotensive control rats, untreated aldosterone-infused rats, and aldosterone-infused rats treated with BMS 182874, spironolactone, or hydralazine. Results are presented as mean ± SEM. *\( P < 0.01 \) vs normotensive control, †\( P < 0.01 \) vs aldosterone-infused rats.
Discussion

Aldosterone infusion raised systolic BP, increased plasma ET levels, induced oxidative stress (elevated plasma TBARs and enhanced vascular NADPH oxidase activity), and resulted in hypertrophic remodeling of small arteries. Vascular changes were associated with collagen and fibronectin deposition in the media and increased abundance of ICAM-1 in the vascular wall. The 3 antihypertensive treatments used prevented the rise in systolic BP, although the response to hydralazine was delayed and seemed weaker than to the other agents. BMS 182874 and spironolactone decreased oxidative stress, normalized resistance artery growth, prevented collagen and fibronectin deposition, and decreased ICAM-1 expression in the vascular wall of aldosterone-infused rats, whereas hydralazine failed to have any major effect on any of these parameters despite lowering BP. Spironolactone tended to decrease plasma endothelin levels. The present results suggest that, even in the absence of salt loading, ET-1 acting via ETA receptors plays an important role in small artery remodeling in response to aldosterone, an effect mediated in part via oxidative stress.

Administration of deoxycorticosterone and salt to unilaterally nephrectomized rats is associated with severe BP elevation and activation of the vascular, cardiac, and renal endothelin systems. If no salt was administered and blood pressure rose less, the endothelin system was not activated and vascular remodeling was less pronounced. Administration of aldosterone plus salt upregulated vascular and cardiac

Figure 3. Bar graphs depict lumen diameter (A), media thickness (B), mediolumen ratio (C), and medial cross-sectional area (D) of relaxed mesenteric arteries from normotensive control rats, untreated aldosterone-infused rats, and aldosterone-infused rats treated with BMS 182874, spironolactone, or hydralazine. Results are presented as mean±SEM. *P<0.05 vs normotensive control, †P<0.05 vs aldosterone-infused rats.

Figure 4. Laser confocal microscopic images of collagen and fibronectin deposition in the media of small mesenteric arteries from normotensive control rats, untreated aldosterone-infused rats, and aldosterone-infused rats treated with BMS 182874, spironolactone, or hydralazine. Vessels were labeled with rhodamine phalloidin for smooth muscle-actin (red), anti-collagen I/III for collagen type I and III (green), and anti-fibronectin for fibronectin (green).
activation of tissue ET-1 production even in the presence of moderate BP rise and no salt loading.

Mechanisms whereby aldosterone infusion results in increased tissue production of ET-1 remain unclear. We previously showed a role for vasopressin, stimulated in the DOCA-salt hypertensive rat, partly mediating the upregulation of ET-1. Mineralocorticoid receptors have been found in endothelial and VSMCs in the aorta and pulmonary artery of rabbit and in cardiac myocytes and endothelial cells. It is possible that there is a direct effect of aldosterone on ET-1 production, but this has not yet been demonstrated.

Activity of vascular NADPH oxidase, a major source of superoxide anion in blood vessels, was increased, associated with increased lipid peroxidation measured by TBARs in plasma, suggesting enhanced production of reactive oxygen species. Oxidative stress may be involved in the upregulation of vascular ET-1 or could be a consequence of its increase. Reactive oxygen species react with nitric oxide (NO) to produce peroxynitrite (OONO·), with subsequent lipid peroxidation of arachidonic acid and formation of F2-isoprostanes. Free radical–generated F2-isoprostanes stimulate ET-1 expression on endothelial cells. In other studies, reactive oxygen species stimulated ET-1 generation by endothelial cells. In the present experiments, the ETα antagonist decreased oxidative stress, which may mean that ET-1 stimulated free radical production. Because BP elevation is accompanied by increased oxidative stress, this response to ETα antagonism could be related to BP lowering. Hydralazine administration, which lowered BP and may inhibit NADPH oxidase, only partially reduced enhanced NADPH oxidase activity in aorta without effect on mesenteric arteries and did not significantly reduce plasma TBARs. However, it must be acknowledged that, in this particular set of experiments, hydralazine seemed slightly less effective than the other agents, as already mentioned. Nevertheless, we believe that, taken together, these data suggest that BP lowering did not reduce oxidative stress and that ET-1 may have stimulated reactive oxygen species generation. However, a role of

**Figure 5.** Bar graphs depict quantification of collagen density in the media of the mesenteric arteries from normotensive control rats, untreated aldosterone-infused rats, and aldosterone-infused rats treated with BMS 182874, spironolactone, or hydralazine. Results are presented as mean±SEM. **P<0.01 vs normotensive control, ‡P<0.01 vs aldosterone-infused rats.

**Figure 6.** Laser confocal microscopic images of intercellular adhesion molecule (ICAM)-1 at the level of endothelium (top row) and adventitia (bottom row) in small mesenteric arteries from normotensive control rats, untreated aldosterone-infused rats, and aldosterone-infused rats treated with BMS 182874, spironolactone, or hydralazine. Vessels were labeled with rhodamine phalloidin for smooth muscle actin (red) and anti-ICAM-1 for ICAM-1 (green). Similar images were observed in vessels from at least 3 rats for each group.
aldosterone-induced oxidative stress on ET-1 production cannot be completely ruled out.

BMS 182874 and spironolactone had similar effects on oxidative stress. In humans blockade of mineralocorticoid receptors with spironolactone improved endothelium-dependent relaxation by increasing NO bioavailability through reduction in vascular oxidative stress. Together with other data, this agrees with the notion that when oxidative stress and ET-1 are elevated, bioactive NO is reduced, and conversely, when they are low, NO is normal. Nitric oxide may downregulate the expression of ET-1. In agreement with our previous study in aldosterone and salt-induced hypertension, here endothelial function of mesenteric arteries in aldosterone-infused rats was not altered, suggesting that the increased ET-1 production and enhanced oxidative stress were regulated independently from NO. A possible explanation for normal endothelial function is that BP elevation was less severe compared with DOCA-salt- or Dahl-salt–sensitive rats in which endothelial dysfunction in association with increased oxidative stress are nearly always documented.

The presence of hypertrophic remodeling of small arteries in aldosterone-infused rats agrees with studies in primary aldosteronism patients, in whom increased media and media-to-lumen ratio of resistance arteries have been documented. Confocal microscopy showed an associated change in extracellular matrix components, consistent with enhanced collagen deposition associated with small artery remodeling in DOCA-salt–hypertensive rats. ET-1 activates the procollagen I promoter in renal vessels and glomeruli. The ETα antagonist results suggest that increased collagen and fibronectin in response to aldosterone was mediated via ET-1 and activation of ETα receptors.

Inflammation plays an important role in atherosclerosis, hypertension, and diabetes. In humans, this notion has been based primarily on the demonstration of elevated levels of serum C-reactive protein. Here, associated with vascular remodeling and fibrosis, there was increased expression of ICAM-1, which is known to participate in the inflammatory process. These results demonstrate for the first time that, in this model of hypertension, an inflammatory response is associated with vascular remodeling and enhanced oxidative stress. Adhesion molecules participate in recruitment of circulating leukocytes to sites of inflammation and may be activated by nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) during the inflammatory response. NF-κB activation may result from increased oxidative stress, which may be induced by ET-1.

There are conflicting reports on the effects of endothelin antagonists in experimental hypertension. In mineralocorticoid or salt-induced hypertensive models in which preproET-1 overexpression has been demonstrated in blood vessels, endothelin receptor antagonists decreased blood pressure and improved vascular remodeling beyond blood pressure lowering. In contrast, the spontaneously hypertensive rat (SHR) and high-renin rat models of hypertension, including transgenic (mREN2)27 rats, a model of monogenic renin-dependent hypertension, are characterized by absence of vascular preproET-1 overexpression, and endothelin receptor antagonists have no effect on left ventricular hypertrophy or vascular remodeling. Interestingly, in transgenic (mREN2)27 rats, aldosterone secretion was endothelin-dependent.

In summary, an ETα receptor antagonist normalized hypertrophic vascular remodeling, oxidative stress, extracellular matrix accumulation, and adhesion molecule expression, suggesting that increased production of ET-1 acting via ETα receptors may trigger elevated oxidative stress, followed by upregulation of NF-κB, adhesion molecules and chemokines, extracellular matrix accumulation (collagen and fibronectin deposition), and vascular growth. We believe that BP did not appear to have a role in the prevention of remodeling, fibrosis, oxidative stress, and adhesion molecule expression, because hydralazine was without effect despite its hypotensive action. A limitation of the study is that hydralazine was slightly less effective in this experimental paradigm than the other agents, not allowing us to rule out totally a role of BP.

Perspectives

Blockade of the endothelin system may exert beneficial effects on vascular remodeling in aldosterone-induced hypertension, and blockade of aldosterone effects may in part abrogate the pathophysiological participation of the endothelin system in cardiovascular disease.

Acknowledgments

This work was supported by grant 37917 to E.L.S. and a group grant to the Multidisciplinary Research Group on Hypertension at CIHR. Dr Pu was supported by a fellowship from the Heart and Stroke Scientific Research Corporation of Canada. The authors are grateful to Suzanne Diébold and André Turgeon for their excellent technical assistance.

References


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Hypertension. 2003;42:49-55; originally published online June 2, 2003;
doi: 10.1161/01.HYP.0000078357.92682.EC
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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