Effects of Adrenomedullin on Cardiac Oxidative Stress and Collagen Accumulation in Aldosterone-Dependent Malignant Hypertensive Rats


Departments of Pharmacology (M.R., A.N., G.-X.Z., Y.-Y.F., S.K., Y.A.) and Cardio-renal and Cerebrovascular Medicine (M.R., P.G., N.H., K.O., M.K.) and Life Science Research Center (Y.N., Y.F.), Kagawa University Medical School, Kagawa, Japan.

Received March 23, 2006; accepted June 13, 2006

ABSTRACT

We examined the effects of adrenomedullin on cardiac oxidative stress and collagen accumulation in aldosterone-dependent malignant hypertensive rats. Spontaneously hypertensive rats (SHRs) were treated with one of the following combinations for 4 weeks: tap water and vehicle (0.5% ethanol, subcutaneously (s.c.), n = 5), 1% NaCl in drinking water and vehicle (n = 8), 1% NaCl and aldosterone (0.75 µg/h s.c., n = 8), and 1% NaCl, aldosterone, and adrenomedullin (1.3 µg/kg/h s.c., n = 8). Systolic blood pressure (SBP) and left ventricular (LV) weight were higher in aldosterone-treated SHRs than vehicle- or vehicle/1% NaCl-treated SHRs. Higher TBARS levels, NADPH oxidase activity, and mRNA changes were associated with increases in LV tissue levels of p22phox, gp91phox, fibronectin, collagen types I and III, as well as collagen content. Treatment with adrenomedullin did not alter SBP or LV weight but attenuated aldosterone-induced increases in TBARS levels, NADPH oxidase activity, and mRNA levels of p22phox, gp91phox, fibronectin, collagen types I and III, as well as collagen content in LV tissues. These data suggest that NADPH oxidase-mediated reactive oxygen species production is involved in the pathogenesis of cardiac collagen accumulation in aldosterone-dependent malignant hypertensive rats and that the cardioprotective effects of adrenomedullin are mediated through the suppression of this pathway.

Excessive collagen accumulation is a potential cause of abnormal tissue stiffness and dysfunction during the development of cardiac hypertrophy (Kim et al., 1995; Brilla, 2000). A growing body of evidence suggests that aldosterone plays an important role in extracellular matrix and collagen synthesis in myocardium (Young et al., 1994; Brilla, 2000; Zannad et al., 2000; Pitt et al., 2003; Bos et al., 2004). In patients with essential hypertension (Duprez et al., 1993; Soylu et al., 2004) and primary aldosteronism (Rossi et al., 1997; Nishimura et al., 1999), pathological patterns of left ventricular (LV) geometry are associated with elevated levels of plasma aldosterone. Furthermore, more severe and more frequent LV hypertrophy and remodeling were observed in patients with primary aldosteronism than in essential hypertensive patients (Rossi et al., 1997, 2002). Chronic infusion of aldosterone to rats led to LV hypertrophy with collagen accumulation, independent of its hemodynamic effects (Brilla and Weber, 1992; Brilla et al., 1993; Brilla, 2000). In patients with severe heart failure, administration of a mineralocorticoid receptor antagonist, spironolactone, to patients receiving standard therapy including an angiotensin-converting enzyme inhibitor markedly reduced serum levels of procollagen type III amino-terminal peptide, a specific marker for cardiac collagen synthesis (Zannad et al., 2000). Other studies have shown that monotherapy with a mineralocorticoid receptor antagonist significantly decreases LV mass in patients with essential hypertension (Pitt et al., 2003).

Recent studies indicate the potential participation of reac-
tive oxygen species (ROS) in the pathophysiology of aldosterone-induced cardiovascular tissue injury (Sun et al., 2002; Iglarz et al., 2004; Park et al., 2004). In aldosterone/salt- or deoxycorticosterone acetate/salt-treated hypertensive rats, cardiovascular NADPH oxidase activity and ROS production were markedly augmented (Beswick et al., 2001; Iglarz et al., 2004; Park et al., 2004). In these animals, treatment with tempol, a superoxide dismutase mimetic, or apocynin, an NADPH oxidase inhibitor, significantly attenuated cardiovascular fibrosis (Iglarz et al., 2004; Park et al., 2004). More recently, it has also been indicated that adrenomedullin protects cardiovascular injury by reducing NADPH oxidase-mediated ROS production (Shimosawa et al., 2002; Kato et al., 2003; Kawai et al., 2004; Yoshimoto et al., 2004, 2005). Adrenomedullin is a vasodilator peptide originally isolated from human pheochromocytoma cells (Kitamura et al., 1993) and is widely distributed in various tissues and organs, including the heart (Kitamura et al., 2002; Eto et al., 2003; Zhao et al., 2006). However, to the best of our knowledge, there is no evidence that clearly demonstrates any beneficial effects of adrenomedullin on cardiac oxidative stress and remodeling that could occur during the development of aldosterone-dependent hypertension.

In the present study, we aimed to investigate whether treatment with adrenomedullin was cardioprotective by reducing NADPH oxidase-mediated ROS production during the development of aldosterone-dependent malignant hypertension. Therefore, we examined the effects of chronic treatment with adrenomedullin on the expression and activity of NADPH oxidase and on ROS and collagen levels in LV tissues of aldosterone-infused spontaneously hypertensive rats (SHRs), presenting a new model of malignant hypertension with secondary hyperaldosteronism.

**Materials and Methods**

**Materials.** Aldosterone was purchased from Wako Co. (Osaka, Japan). Adrenomedullin (52 human), diphenyleioidonium, lucigenin, and NADPH were obtained from Sigma Chemical Co. (St. Louis, MO).

**Animals and Experimental Groups.** All experimental procedures were performed according to the guidelines for the care and use of animals as established by Kagawa University Medical School. Experiments were performed on 10-week-old male SHRs and age-matched Wister-Kyoto (WKY) rats (SLC, Shizuoka, Japan). SHRs and WKY rats were housed in separate cages and maintained at room temperature under a 12-h light/dark cycle. At the beginning of the experiments, SHRs were randomly treated for 4 weeks with one of the following combinations: group 1, tap water and vehicle (0.5% ethanol, s.c., n = 5); group 2, 1% NaCl in the drinking solution and vehicle (n = 8); group 3, 1% NaCl and aldosterone (0.75 μg/h, s.c., n = 8); and group 4, 1% NaCl, aldosterone and adrenomedullin (1.3 μg/kg/h, s.c., n = 8). WKY rats were treated with tap water and vehicle (n = 5) or 1% NaCl in the drinking solution and vehicle (n = 8). The doses of aldosterone and adrenomedullin were determined according to the results of previous studies on rats (Mori et al., 2002; Nishikimi et al., 2002, 2004; Park et al., 2004).

Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and osmotic minipumps (Alzet, Cupertino, CA) were implanted s.c. at the dorsum of the neck to infuse vehicle, aldosterone, or adrenomedullin. Systolic blood pressure (SBP) was measured weekly by tail-cuff plethysmography (BP-98A; Softron Co., Tokyo, Japan). After 4 weeks of treatment, 24-h urine samples were collected using metabolic cages 1 day before harvesting. Animals were decapitated, and trunk blood was collected into chilled tubes containing EDTA. LV tissues were removed, immediately snap-frozen in liquid nitrogen, and then stored at −80°C.

**Real-Time Reverse-Transcriptase Polymerase Chain Reaction.** mRNA expression of p22phox, gp91phox, fibronectin, and collagen types I and III were analyzed by real-time polymerase chain reaction using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) (Nishiyama et al., 2004; Nagai et al., 2005a,b). All data were expressed as relative differences in vehicle-, aldosterone/1% NaCl-, and aldosterone and adrenomedullin/1% NaCl-treated SHRs and vehicle- or vehicle/1% NaCl-treated WKY rats compared with vehicle/1% NaCl-treated SHRs after normalization to the expression of glyceraldehyde-3-phosphate dehydrogenase. The primers for glyceraldehyde-3-phosphate dehydrogenase, p22phox, gp91phox, fibronectin, and collagen types I and III were synthesized as described previously (Nishiyama et al., 2004; Nagai et al., 2005a,b).

**NADPH Oxidase Activity.** NADPH oxidase-derived superoxide anion (O2−) generation was measured using lucigenin-enhanced chemiluminescence, as described previously (Zalba et al., 2000; Kim et al., 2002; Nakano et al., 2005). In brief, LV tissues (80–100 mg) were placed in chilled phosphate-buffered saline containing protease inhibitor and homogenized on ice. Protein concentration of homogenates was measured using the Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA). After centrifuging, the supernatant was transferred into a glass test tube containing lucigenin (final concentration 5 μM in Krebs-HEPES buffer). Chemiluminescence was then recorded every 30 s for 10 min with a luminescence reader (BLR-301, Aloka, Tokyo, Japan), and the readings in the last 5 min were averaged. After measurement of background lucigenin chemiluminescence, NADPH was added to a final concentration of 100 μM. Thereafter, chemiluminescence was recorded another 10 min, and the readings in the last 5 min were averaged. To verify if the lucigenin signal reflects O2− generation, an NADPH oxidase inhibitor, diphenylene iodonium (Hancock and Jones, 1987), was added at the end of measurements (final concentration, 10 μM). In all samples, diphenylene iodonium reduced NADPH-induced increases in chemiluminescence to background levels (data not shown). The differences between the values obtained before and after adding the NADPH were calculated, and the activity of NADPH oxidase was expressed as counts per minute per milligram of protein.

**Other Analytical Procedures.** The degree of lipid peroxidation in plasma and LV tissues was determined using biochemical assays of the thiobarbituric acid reactive substances (TBARS), as described previously (Nishiyama et al., 2004; Rahman et al., 2004; Nagai et al., 2005b). Collagen content in the LV tissues was determined on the basis of hydroxyproline concentration (Nishiyama et al., 2004; Nagai et al., 2005b). The value of collagen content was expressed as microgram per milligram of dry tissue weight. Sodium and potassium concentrations in plasma and urine were measured using flame photometry (Hitachi 750; Hitachi, Tokyo, Japan) (Nishiyama et al., 2006).

**Statistical Analysis.** Values are presented as mean ± S.E. Statistical comparisons of differences were performed using one- or two-way analyses of variance combined with Fisher’s post hoc test. P < 0.05 was considered statistically significant.

**Results**

**Blood Pressure and LV Weight.** The changes in SBP are shown in Fig. 1. Baseline SBP was significantly higher in SHRs than WKY rats. Treatment with vehicle or vehicle/1% NaCl did not alter SBP in WKY rats (133 ± 1 and 127 ± 2 mm Hg, respectively, at week 4). Furthermore, SBP was similar between vehicle- and vehicle/1% NaCl-treated SHRs (207 ± 4 and 201 ± 5 mm Hg, respectively, at week 4). However, aldosterone/1% NaCl treatment further increased...
SBP in SHRs (220 ± 8 mm Hg at week 4). In aldosterone/1% NaCl-treated SHRs, SBP was not altered by chronic treatment with adrenomedullin (219 ± 4 mm Hg at week 4). These data are consistent with those of previous studies (Mori et al., 2002; Nishikimi et al., 2002), showing that chronic s.c. infusion of adrenomedullin did not alter SBP in hypertensive rats.

As shown in Table 1, body weight (BW) was similar between vehicle- or vehicle/1% NaCl-treated SHRs and WKY rats, respectively. Four weeks of infusion of aldosterone or aldosterone plus adrenomedullin to 1% NaCl-treated SHRs prevented the rise in BW that was seen in vehicle- or vehicle/1% NaCl-treated SHRs and WKY rats. On the other hand, aldosterone/1% NaCl treatment significantly increased the LV and LV weights/BW ratio. Concurrent administration of adrenomedullin did not significantly alter the aldosterone-induced increases in LV and LV weights to the BW ratio (Table 1).

**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>SHR/Tap Water (n = 5)</th>
<th>SHR/1% NaCl + Vehicle (n = 8)</th>
<th>SHR/1% NaCl + Aldosterone (n = 8)</th>
<th>SHR/1% NaCl + Aldosterone + Adrenomedullin (n = 8)</th>
<th>WKY/Tap Water (n = 5)</th>
<th>WKY/1% NaCl + Vehicle (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial BW (g)</td>
<td>244 ± 6</td>
<td>243 ± 2</td>
<td>246 ± 3</td>
<td>241 ± 4</td>
<td>284 ± 2*</td>
<td>270 ± 5*</td>
</tr>
<tr>
<td>Final BW (g)</td>
<td>302 ± 7</td>
<td>310 ± 7</td>
<td>263 ± 6*</td>
<td>244 ± 8*</td>
<td>352 ± 4*</td>
<td>334 ± 6*</td>
</tr>
<tr>
<td>LVW (g)</td>
<td>0.88 ± 0.02*</td>
<td>0.98 ± 0.02</td>
<td>1.17 ± 0.03*</td>
<td>1.15 ± 0.04*</td>
<td>0.71 ± 0.02*</td>
<td>0.68 ± 0.02*</td>
</tr>
<tr>
<td>LVW/BW (mg/g)</td>
<td>2.93 ± 0.09*</td>
<td>3.25 ± 0.07</td>
<td>4.42 ± 0.17*</td>
<td>4.78 ± 0.20*</td>
<td>2.04 ± 0.06*</td>
<td>2.05 ± 0.02*</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. vehicle/1% NaCl-treated SHR.
Fibronectin and Collagen. Fibronectin and collagen types I and III mRNA levels in LV tissues were similar among vehicle- or vehicle/1% NaCl-treated SHRs and WKY rats (Fig. 5, A–C). However, aldosterone/1% NaCl-treated SHRs showed significantly higher mRNA levels of fibronectin and collagen types I and III than those of vehicle/1% NaCl-treated SHRs (by 2.2- /H11006 0.1-, 2.2- /H11006 0.2-, and 3.1- /H11006 0.2-fold, for each). In aldosterone/1% NaCl-treated SHRs, treatment with adrenomedullin prevented increases in mRNA levels of fibronectin and collagen types I and III.

As shown in Fig. 5D, collagen contents in LV tissues were similar among vehicle- or vehicle/1% NaCl-treated SHRs and WKY rats (17 ± 1, 17 ± 2, 15 ± 1, and 16 ± 1 μg/mg, for each). However, aldosterone/1% NaCl-treated SHRs showed significantly higher collagen contents than those of vehicle/1% NaCl-treated SHRs (22 ± 2 μg/mg). In aldosterone/1% NaCl-treated SHRs, treatment with adrenomedullin markedly attenuated aldosterone-induced increases in collagen contents (18 ± 1 μg/mg).

Discussion

In this study, we examined the effects of adrenomedullin on cardiac oxidative stress and collagen accumulation in aldosterone/salt-treated SHRs, a new model of human malignant hypertension with secondary hyperaldosteronism. Our results showed that chronic administration of aldosterone/salt led to the development of LV hypertrophy and increased expression of fibronectin and collagen types I and III, as well as collagen content in SHRs. The present study also provided evidence that aldosterone/salt-induced increases in LV ROS levels are associated with increases in NADPH oxidase activity and p22phox and gp91phox expression. These data indicate that NADPH oxidase-dependent ROS production is involved in cardiac collagen synthesis in aldosterone-dependent malignant hypertensive rats. We also observed that chronic treatment with adrenomedullin prevented the augmentation of p22phox and gp91phox expression, NADPH oxidase activity, and TBARS levels in LV tissues of aldosterone-induced malignant hypertensive rats. In addition, treatment with adrenomedullin resulted in the marked attenuation of fibronectin and collagen types I and III expression and collagen contents without affecting blood pressure. These results suggest that exogenously administered adrenomedullin elicits cardioprotective effects via the inhibition of NADPH oxidase-dependent ROS production and collagen accumulation in aldosterone-dependent malignant hypertension.

In agreement with previous studies (Newaz and Nawal, 1998), plasma TBARS levels of SHRs were significantly higher than those of WKY rats. However, we did not find any differences in LV tissue TBARS levels, expression of NADPH oxidase components (p22phox and gp91phox), or NADPH oxidase activity between SHRs and WKY rats. Likewise, salt
treatment alone did not alter LV TBARS levels, NADPH oxidase activity, and p22phox and gp91phox expression in these animals, indicating that systemic but not cardiac oxidative stress is enhanced in SHRs and salt-treated SHRs. On the other hand, aldosterone/salt treatment significantly increased TBARS levels, NADPH oxidase activity, and expression of p22phox and gp91phox in LV tissues of SHRs. These data suggest that NADPH oxidase-mediated ROS production in LV tissues is enhanced during the development of aldosterone-dependent malignant hypertension. The present study also showed that augmentation of fibronectin and collagen type I and III gene expression as well as collagen content in
LV tissues is associated with increases in ROS levels in aldosterone/salt-treated malignant hypertensive rats, suggesting the potential contribution of ROS to the pathogenesis of aldosterone-dependent cardiac collagen synthesis. Organ-protective effects of antioxidants on hypertension and tissue injury have been demonstrated in a variety of animal models (Newaz and Nawal, 1998; Nakano et al., 2003; Nishiyama et al., 2004; Park at el., 2004). Recent studies have also indicated that adrenomedullin elicits antioxidant effects (Shimosawa et al., 2002; Yoshimoto et al., 2004, 2005). In rat aortic vascular smooth muscle and endothelial cells, adrenomedullin attenuates angiotensin II-stimulated increases in intracellular ROS and NADPH oxidase activity (Yoshimoto et al., 2004, 2005). Animal studies have also demonstrated that adrenomedullin gene delivery reduces cardiac O$_2^-$ levels and NADPH oxidase activity in ischemia reperfusion injury (Kato et al., 2003). Kawai et al. (2004) showed that in adrenomedullin knockout mice, severe femoral arterial intimal thickening induced by cuff placement is associated with the up-regulation of NADPH oxidase components and enhanced O$_2^-$ production. Collectively, these observations support the concept that adrenomedullin reduces NADPH oxidase-dependent ROS production. In the present study, we observed that treatment with adrenomedullin significantly attenuated increases in p22phox and gp91phox expression, NADPH oxidase activity, and TBARS levels in LV tissues of aldosterone/salt-treated SHR. These data indicate that adrenomedullin attenuates NADPH oxidase-mediated ROS production in aldosterone-dependent malignant hypertensive rats. In vitro studies have indicated that adrenomedullin inhibits collagen deposition by inhibiting NADPH oxidase-mediated O$_2^-$ generation (Yoshimoto et al., 2004, 2005). In the present study, treatment with adrenomedullin attenuated aldosterone-induced increases in LV ROS levels, NADPH oxidase expression and activity, as well as in collagen accumulation, independent of blood pressure changes. These data support the hypothesis that the cardioprotective effects of adrenomedullin are associated with the attenuation of NADPH oxidase-mediated ROS production in aldosterone-dependent malignant hypertension. To support this hypothesis further, it will be necessary to determine whether induction of oxidative stress negates cardioprotective effects of adrenomedullin.

In the present study, we aimed to examine the effects of exogenously administered adrenomedullin. Therefore, we did not clarify the role of endogenous adrenomedullin. In addition, the present in vivo experiments did not allow us to present any data regarding the precise mechanisms by which adrenomedullin attenuates NADPH oxidase-mediated ROS production. Since plasma sodium and potassium levels or their urinary excretion rates were not changed by adrenomedullin infusion, the cardioprotective effects of adrenomedullin cannot be explained by changes in electrolyte balance. Although blood pressure was not changed by adrenomedullin, it is possible that aldosterone-induced NADPH oxidase activation is attenuated by adrenomedullin-mediated improvement of tissue microcirculation. Alternatively, adrenomedullin may directly inhibit NADPH oxidase activity by some molecular mechanisms, as suggested by other investigators (Yoshimoto et al., 2004, 2005). Clearly, further studies are needed to address these issues.

In conclusion, the present results suggest that exogenously administered adrenomedullin elicits cardioprotective effects through the attenuation of NADPH oxidase-mediated ROS production and collagen accumulation in aldosterone-induced malignant hypertensive rats. Treatment with adrenomedullin therefore might be a potentially useful therapeutic strategy for preventing cardiac injury in aldosterone-dependent malignant hypertension.

References


Address correspondence to: Dr. Akira Nishiyama, Department of Pharmacology, Kagawa University Medical School, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan. E-mail: akira@kms.ac.jp