Selective Aldosterone Blockade Prevents Angiotensin II/Salt-Induced Vascular Inflammation in the Rat Heart

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We studied the role of aldosterone (aldo) in myocardial injury in a model of angiotensin (Ang) II-hypertension. Wistar rats were given 1% NaCl (salt) to drink and randomized into one of the following groups (n = 10; treatment, 21 d): 1) vehicle control (VEH); 2) Ang II infusion (25 ng/min, sc); 3) Ang II infusion plus the selective aldo blocker, eplerenone (epl, 100 mg/kg-d, orally); 4) Ang II infusion in adrenalectomized (ADX) rats; and 5) Ang II infusion in ADX rats with aldo treatment (20 μg/kg-d, sc). ADX rats received also dexamethasone (12 μg/kg-d, sc). Systolic blood pressure increased with time in all treatment groups except the VEH group (VEH, 138 ± 6; Ang II/NaCl, 203 ± 12; Ang II/NaCl/epl, 196 ± 10; Ang II/NaCl/ADX, 181 ± 7; Ang II/NaCl/ADX/aldo, 236 ± 8 mm Hg). Despite similar levels of hypertension, epl and ADX attenuated the increase in heart weight/body weight induced by Ang II. Histological examination of the hearts evidenced myocardial and vascular injury in the Ang II/salt (7 of 10 hearts with damage, P < 0.05 vs. VEH) and Ang II/salt/ADX/aldo groups (10 of 10 hearts with damage, P < 0.05). Injury included arterial fibrinoid necrosis, perivascular inflammation (primarily macrophages), and focal infarctions. Vascular lesions were associated with expression of the inflammatory mediators cyclooxygenase 2 (COX-2) and osteopontin in the media of coronary arteries. Myocardial injury, COX-2, and osteopontin expression were markedly attenuated by epl treatment (1 of 10 hearts with damage, P < 0.05 vs. Ang II/salt) and adrenalectomy (2 of 10 hearts with damage, P < 0.05 vs. Ang II/salt). Our data indicate that aldo plays a major role in Ang II-induced vascular inflammation in the heart and implicate COX-2 and osteopontin as potential mediators of the damage.

A D M I N I S T R A T I O N OF E X O G E N O U S angiotensin (Ang) II to rodents leads to hypertension and myocardial injury. This was first described by Gavras et al. (1), who reported the presence of widespread focal myocardial infarctions in rabbits receiving iv infusions of Ang II, and later by Giacomelli et al. (2), who demonstrated induction of significant coronary injury associated with the myocardial damage in rats, involving primarily small intramyocardial arteries and arterioles. More recently, coronary injury in Ang II hypertension has been shown to have an early perivascular inflammatory component involving primarily macrophages (3).

Recently, a role for aldosterone (aldo) in Ang II-induced myocardial injury was demonstrated. Indeed, administration of a selective aldo blocker, eplerenone (epl), or aldo ablation with adrenalectomy markedly attenuated Ang II-induced myocardial necrosis in nitric-oxide-deficient rats (4). Similarly, myocardial protection with spironolactone, a non-selective aldo blocker, was recently reported in a genetic model of Ang II hypertension (5). However, the mechanisms by which aldo contributes to coronary injury in Ang II hypertension are largely unknown.

Classic effects of aldo involve its actions on the renal and intestinal epithelium, leading to sodium reabsorption and potassium excretion (6). These effects were not carefully evaluated on previous experiments, and the possibility that aldo antagonism was protective via a diuretic or natriuretic effect has not been examined.

Another potential mechanism relates to the potential proinflammatory effects of Ang II and aldo. Significant myocardial inflammation develops in Ang II hypertension (3), which is responsive to aldo blockade (5). Similarly, we have recently documented vascular inflammatory damage in the heart of aldo/salt, uninephrectomized rats, a hypertensive model characterized by the low levels of circulating Ang II (7). In these studies, the proinflammatory molecules cyclooxygenase 2 (COX-2) and osteopontin were identified as potential mediators of the coronary damage induced by aldo.

In the present study, we tested the hypothesis that aldo may be a major contributor to Ang II-induced myocardial injury through mechanisms that involve myocardial or vascular inflammation. Specifically, we examined whether aldo blockade by either adrenalectomy or pharmacological antagonism with epl would prevent Ang II-induced coronary inflammation and injury and whether aldo treatment would restore damage in adrenalectomized (ADX) rats. To better understand the mechanisms by which aldo contributes to coronary vascular injury, we determined the effects of treatments on urinary electrolyte and volume excretion. Furthermore, we examined the level of myocardial expression of COX-2 and osteopontin, as potential mediators of the vascular and myocardial injury in this model.

Materials and Methods

Treatment groups and experimental protocol

Male Wistar rats (200 g, Harlan Sprague Dawley, Inc., Indianapolis, IN) were used for this study in accordance with institutional guidelines for the humane treatment of animals. Animals had free access to Purina Lab Chow 5001 (Ralston Purina Co., St. Louis, MO) and tap water until initiation of the experiment. Beginning 1 d before initiation of treatment...
and continuing throughout the experiment, animals were handled and weighed daily and maintained in separate metabolic cages. On initiation of the study, animals were randomized into one of the following treatment groups and given 1% NaCl to drink (n = 10/group): 1) vehicle (NaCl); 2) Ang II/salt given Ang II infusion (25 ng/min, sc); 3) Ang II/salt/ADX group treated with Ang II and epl (100 mg/kg, orally, twice daily); 4) Ang II/salt/ADX, in which ADX rats were given Ang II and dexamethasone (12 μg/kg, sc); and 5) Ang II/salt/ADX/aldo group, in which ADX rats were given aldo (20 μg/kg, sc via minipump), Ang II, and dexamethasone.

Ang II (American Peptide Co.) in saline, d-aldosterone (Sigma, St. Louis, MO) in 2% ethanol, and vehicle (saline) were administered via Alzet miniosmotic pumps (model 2004; Alza Corp., Mountain View, CA). Administration of epl (Pharmacia Corp.), in 0.5% methylcellullose (Sigma), was by oral gavage twice daily (50 mg/kg at 0700 and 1700 h). Dexamethasone (Sigma) was administered by sc injection once per day in the ADX groups.

Animals were anesthetized with sodium pentobarbital (The Butler Co., Columbus, OH; 30–50 mg/kg, ip). Minipumps were implanted sc at the nape of the neck. Bilateral adrenalectomy was performed using a dorsolumbar approach, making separate incisions on each side.

Euthanasia

After 3 wk of treatment, animals were weighed and anesthetized with pentobarbital (The Butler Co., 70 mg/kg). Blood (7–10 ml) was collected from the abdominal aorta into lithium heparin tubes and no additive (Vacutainer, Becton Dickinson and Co., Franklin Lakes, NJ). Samples were centrifuged at 2500 rpm for 10 min at room temperature. Aliquots of plasma and serum were frozen and stored at –80°C. The heart was then removed, weighed, and perfused-fixed at 85 mm Hg with 10% phosphate-buffered formalin (Sigma), via the aortic root, for 15 min. The heart was then removed, weighed, and perfused-fixed at 85 mm Hg with 10% phosphate-buffered formalin (Sigma), via the aortic root, for 15 min.

Measurements and assays

Systolic blood pressure was measured by tail cuff plethysmography (model 179 Blood Pressure Analyzer, IITC Life Science, Woodland Hills, CA). Rats were warmed to 37°C in a rat restrainer and allowed to rest quietly. After 10 min, 5–8 measurements of blood pressure were taken and averaged for each animal. Blood pressure was measured on d1, 7, 14, and 20. Daily fluid intake, food intake, and urine output were measured gravimetrically throughout the experiment. Daily urinary Na+ and K+ concentrations were measured using a 911 Automatic Analyzer (Roche Molecular Biochemicals, Indianapolis, IN). Urinary protein excretion at the end of the experiment was measured with a Bio-Rad Laboratories, Inc. Protein assay kit. Serum Na+ and K+ concentrations were measured with a 911 Automatic Analyzer. Plasma and serum samples were evaluated by commercially available RIAs and protocols for plasma renin activity (PRA) (NEN Life Science Products, Beverly, MA), plasma aldosterone concentration (Diagnostic Products Corp., Los Angeles, CA), and serum corticosterone concentration (Diagnostic Products Corp.).

Tissue processing and staining

The equatorial regions of the heart were routinely processed into paraffin blocks. Five-micrometer sections were processed for hematoxylin and eosin staining. These slides were used to assess myocardial injury.

Immunohistochemistry

Five-micrometer sections were deparaffinized in xylene (two 5- to 10-min incubations) and rehydrated by 3-min incubations in ethanol as follows: two incubations in 100% ethanol, followed by two incubations in 95% alcohol and one incubation in 70% alcohol. Once hydrated, sections were rinsed in tap water for 1 min and distilled water for 1 min. Endogenous peroxide activity was blocked by placing slides in 3% H2O2 for 15 min, followed by a 5-min rinse in distilled water. Slides were processed for antigen retrieval using citric acid, pH6.0. Slides were heated to boiling, cooled for 20 min at 25°C, and rinsed in distilled water. Slides were stained using an autostainer (DAKO Corp., Carpinteria, CA). Before staining, slides were rinsed and incubated in blocking buffer (described in the Vectastain ABC kit; Vector Laboratories, Inc. (Burlingame, CA) and containing 10 ml Tris/NaCl blocking buffer and three drops of normal serum, corresponding to secondary antibody) for 20 min.

Primary antibodies used for staining were osteopontin, diluted at 1:100 (mouse monoclonal, MPIHb10, University of Iowa), ED-1 (MAB1435, Chemicon International, Temecula, CA), and COX-2, diluted at 1:300 (mouse polyclonal, affinity-purified, 160126, Cayman Chemical, Ann Arbor, MI). Slides were incubated with primary antibodies for 60 min, followed by biotinylated antibodies at a final concentration of 5 μl/ml for 30 min at 25°C. Staining was visualized with the Vectastain ABC-AP kit (Vector Laboratories, Inc.) and diaminobenzidine staining (DAKO Corp.). Slides were rinsed in water and counter-stained with hematoxylin for approximately 30 sec. Isotype-matched IgG (Sigma) was used as a negative control for the primary antibodies.

Statistical analysis

Data were analyzed using a one-way ANOVA on the rank transformed values. The analysis for the end-point measurements was done on the end-point values, and daily measurements were done on the baseline values and change from baseline. The planned comparisons between the vehicle/salt and Ang II/salt means with the rest of the treatment groups was examined by the least-significant-differences mean comparison procedure and considered significant if the one-tailed P value was less than 0.05. Semiquantitative myocardial and vascular injury data were examined by Fisher’s exact test. Data were analyzed using SAS statistical software package (SAS PC, version 6.12; SAS Institute, Inc., Cary, NC). Data are reported as mean ± se of the mean (sem).

Results

Systolic blood pressure

The effects of treatments on systolic blood pressure are shown in Table 1. Ang II/salt treatment increased blood pressure significantly vs. vehicle/salt. The increase in blood pressure produced by Ang II/salt treatment was not atten...
uated by epl treatment. ADX tended to reduce blood pressure, although this reduction was not statistically significant. The combination of Ang II and aldo in ADX animals severely elevated blood pressure, to levels significantly higher than adrenal-intact, Ang II-infused rats.

Cardiac hypertrophy and myocardial injury

Cardiac hypertrophy, as assessed by heart-weight-to-body-weight ratio, was increased by Ang II/salt vs. vehicle/salt treatment (Table 1). Both epl administration and adrenalectomy significantly reduced total heart weight and heart-weight-to-body-weight ratio, compared with the Ang II/salt group; aldo treatment in ADX animals significantly increased total heart weight and heart-weight-to-body-weight ratio vs. aldo-deficient ADX rats.

Semiquantitative histopathologic evaluation of the hearts revealed minor vascular changes in 1 of the 10 control rats receiving vehicle/salt treatment. Significant vascular and myocardial damage were evident in 7 of the 10 Ang II/salt-treated animals (Table 1). Damage included perivascular leukocyte infiltration with focal vascular lesions characterized by fibrinoid necrosis of the media and occasional myocardial infarctions (Fig. 1A). Infiltrating cells were primarily mononuclear cells that stained positive for an ED-1 monoclonal antibody, suggesting they were macrophages (Fig. 1B). Adrenalectomy and epl treatment largely attenuated the myocardial and vascular injury associated with Ang II/salt treatment (Table 1). A representative photomicrograph of an animal receiving epl is shown in Fig. 1C. Treatment with aldo completely restored vascular and myocardial injury with hearts from all animals in this group exhibiting damage (Table 1, Fig. 1D).

COX-2 and osteopontin immunohistochemistry

Immunohistochemistry staining for COX-2 and osteopontin are shown in Figs. 2 and 3. There was no staining of COX-2
or osteopontin in hearts from vehicle/salt animals (not shown). Hearts from animals treated with Ang II/salt exhibited COX-2 and osteopontin staining that was primarily localized to medial cells of affected, and some unaffected, coronary arteries but was also present in macrophages in the perivascular space and areas of myocardial necrosis (Figs. 2A and 3A). There was no significant expression of COX-2 or osteopontin in cardiomyocytes. Adrenalectomy or epl treatment markedly blunted the Ang II/salt-induced staining for COX-2 (Fig. 2, B and C) and osteopontin (Fig. 3, B and C). In hearts from animals in the Ang II/salt/ADX/aldo group, staining for COX-2 and osteopontin was seen, similar to the Ang II/salt group (Figs. 2D and 3D).

**Plasma, serum, and urine parameters**

Ang II/salt treatment did not significantly increase plasma aldo concentrations (Table 2). However, significant increases in plasma aldo levels were observed in Ang II-infused animals receiving epl. Plasma aldo levels in ADX animals were below the limits of detection, and aldo treatment in ADX animals significantly elevated plasma aldo levels. There were no differences in serum corticosterone concentrations among the adrenal-intact groups (Table 2). Adrenalectomy reduced serum corticosterone levels below limits of detection in both aldo-deficient and aldo-replaced groups. PRA was significantly reduced by infusion of the exogenous Ang II (seven values below the limit of detection, Table 2); epl treatment modestly, but significantly, attenuated this decrease. Adrenalectomy significantly increased PRA, and aldo treatment in ADX animals markedly decreased PRA (eight values below the limit of detection).

Serum Na⁺ was not affected by Ang II/salt or epl treatment (Table 2). Serum Na⁺ was reduced in the Ang II/salt/ADX group and increased by aldo treatment in the Ang
II/salt/ADX/aldo group. Ang II/salt treatment reduced serum K\(^+\) vs. vehicle/salt. This effect was prevented by epl treatment. Adrenalectomy significantly increased serum K\(^+\); aldo treatment in ADX animals markedly reduced serum K\(^+\) to below vehicle/salt and Ang II/salt values.

Daily body weights and metabolic measurements

The effects of treatment on daily measures of body weight, food intake, and fluid intake are shown in Fig. 4. Body weights in the Ang II/salt treatment group were significantly lower than those from the vehicle/salt group (Fig. 4A; \(P < 0.05\)); epl treatment did not cause any changes in body weights throughout the study, with respect to vehicle/salt rats. Body weights for the ADX group were significantly lower than the adrenal-intact groups throughout the study; aldo treatment slightly increased body weight vs. the ADX, aldo-deficient group, although it remained significantly lower than that of the adrenal-intact animals (\(P < 0.01\) for both ADX groups vs. vehicle/salt).

Data for daily food intake is shown in Fig. 2B. Animals in all groups significantly reduced food intake immediately after surgery but recovered during the following days. Food intake remained similar in Ang II/salt- and vehicle/salt-treated rats throughout the study. Administration of epl also did not affect daily food intake, with respect to vehicle/salt controls. Food intake in ADX animals was significantly lower than in adrenal-intact animals throughout the experiment. In ADX animals, aldo treatment did not significantly ameliorate the decrease in food intake (\(P < 0.01\) for both ADX groups vs. vehicle/salt).

Ang II/salt treatment did not affect fluid intake vs. vehicle/salt animals until d 16 of Ang II infusion and thereafter,

Fig. 3. Immunohistochemistry for osteopontin. Photomicrographs of sections processed with a monoclonal antibody against osteopontin. Vehicle-treated animals did not express osteopontin in the heart (not shown). Ang II/salt treatment induced osteopontin expression in the media of coronary arteries (A). The expression of osteopontin in the heart was markedly attenuated by epl treatment (B) or adrenalectomy (C). Administration of aldo restored Ang II + 1%-NaCl-induced osteopontin expression (D).
when a significant increase in fluid intake was evident in this group, compared with vehicle/salt (Fig. 4A, P < 0.05). No significant differences in fluid intake were observed in Ang II-infused rats receiving epl, when compared with vehicle/salt controls. Fluid intake was significantly increased in ADX animals vs. vehicle/salt group after the first week of treatment and remained elevated until the end of the experiment (P < 0.05). Treatment with aldo, in ADX animals, further increased fluid intake to more than double that of the vehicle/salt group (P < 0.01).

The effects of treatment on daily measurements of urine output and urinary electrolyte excretion are shown in Fig. 5. Marked increases in urine output, urinary sodium excretion, and the urinary sodium-to-potassium excretion ratio (urinary sodium/urinary potassium) occurred in all groups upon initiation of the high-salt diet on d 0. No significant differences in these parameters were observed between vehicle/salt- and Ang II/salt-treated rats until d 17 and thereafter, when significant differences were observed in Ang II/salt-treated animals (P < 0.05). Administration of epl did not induce significant increases in urine output, urinary sodium excretion, or urinary sodium/urinary potassium at any time point, when compared with Ang II-infused rats receiving vehicle. ADX rats demonstrated significantly higher urine output and urinary sodium/urinary potassium than did adrenal-intact rats, starting on d 5 (P < 0.05). However, total urinary sodium excretion was similar in this group to that in adrenal-intact, Ang II/salt-treated rats. Urate output, urinary sodium excretion, and urinary sodium/urinary potassium were markedly higher in aldo-infused, ADX rats than in animals in all other groups (P < 0.001). Urinary K⁺ excretion was not affected by Ang II/salt or Ang II/salt plus epl treatment (Fig. 5C). Modest decreases in urinary K⁺ excretion occurred with ADX (P < 0.05 vs. vehicle/salt) that were not significantly affected by the aldo infusion.

**Discussion**

The purpose of the present experiment was to determine some of the potential mechanisms by which aldo contributes to myocardial damage in Ang II/salt hypertensive rats. We found that administration of Ang II/salt treatment induced the development of hypertension, cardiac hypertrophy, and inflammatory damage in the coronary arteries, involving the proinflammatory mediators COX-2 and osteopontin. Coronary vascular injury and myocardial hypertrophy were attenuated by the selective aldo blocker epl and by adrenalectomy, which eliminated the presence of aldo. The protective effect of adrenalectomy was lost when ADX rats were infused with aldo. Thus, aldo seems to be an important mediator for the development of the vascular inflammatory injury in coronary arteries in Ang II/salt hypertensive rats.

The results of the present experiment are consistent with several studies examining the influence of mineralocorticoids on the vasculature of the heart. In mineralocorticoid/salt hypertensive rats, coronary vascular injury develops after 4 wk, leading to myocardial fibrosis (8). Vascular injury in this model is preceded by the development of a vascular inflammatory phenotype that involves the expression of COX-2 and the cytokines osteopontin and MCP-1 (7). These types of changes, induced by mineralocorticoids, were thought to require several weeks to develop. However, Fujisawa et al. (9) have identified mineralocorticoid/salt-induced vascular inflammatory changes in the heart as early as 4–8 d after initiation of treatment in saline-drinking, uninephrectomized rats. In rats double-transgenic for the human renin and angiotensinogen genes, mineralocorticoid blockade attenuated vascular injury as well as the expression of IL-6 and basic fibroblast growth factor and the activation of the inflammatory transcription factors nuclear factor-κB and activator protein-1 (5). Thus, there is accumulating evidence that aldo is involved in the development of inflammatory vascular damage in the heart. The results of the present study fully support this hypothesis.

Inflammatory damage in coronary arteries was characterized by accumulation of ED-1-positive, mononuclear cells, probably macrophages. Cellular changes were accompanied by the vascular expression of the inducible inflammatory mediator COX-2 and the cytokine and adhesion molecule osteopontin. The role of these inflammatory mediators in Ang II/salt hypertension is relatively
unclear. Osteopontin is an acidic, high-capacity calcium-binding, phosphorylated protein found as a component of the extracellular matrix in mineralized tissues or as a circulating cytokine (10). Its expression is induced in blood vessels in response to hormonal stimulus such as Ang II (11, 12) or during vascular repair or regeneration (11, 13). It can activate macrophages and T-cells to migrate and produce other cytokines via αvβ3 or CD44 receptors (14, 15). In addition, osteopontin has the ability to induce vascular smooth muscle cell proliferation and migration via αvβ3 integrins, leading to abnormal vascular remodeling (16, 17). The role of COX-2 in vascular pathology is less well understood. COX-2 expression has been demonstrated in human atherosclerotic vessels, primarily in proliferating vascular smooth muscle cells and macrophages (18). Young et al. (19) have also demonstrated a role for COX-2 in vascular smooth muscle cells proliferation in studies where they showed that COX-2 was required for tumor necrosis factor α- and Ang II-induced vascular smooth muscle cells proliferation. To our knowledge, the present study is the first to report the expression of COX-2 in coronary arteries of Ang II/salt hypertensive rats. In addition, our results suggest that COX-2- and osteopontin-mediated vascular inflammation may be part of the mechanisms by which aldosterone participates in the development of coronary injury in Ang II/salt hypertensive rats.

The beneficial effects of epl or adrenalectomy were achieved independently of reductions in systolic blood pressure. These results are consistent with previous reports identifying a similar dissociation between blood pressure and end-organ damage in rats with abnormal activation of the renin angiotensin aldosterone (RAAS). Ang II/salt myocardial necrosis and renal arteriopathy were reduced by aldosterone blockade, without modifying blood pressure in nitric oxide-deficient rats (4). Antagonism of the RAAS prevented nephrosclerosis and stroke in stroke-prone SHR without reducing systolic blood pressure (20–22). Also, in uninephrectomized, aldosterone-deficient rats, lowering systolic blood pressure by administration of a mineralocorticoid receptor antagonist (RU 28318) into the cerebral ventricles prevented the development of hypertension but not myocardial fibrosis (23). Similarly, hypertension, but not myocardial fibrosis, was prevented with hydralazine in nitro-arginine methyl ester-treated rats, whereas Ang II type I receptor antagonism reduced both systolic blood pressure and cardiac injury (24). Thus, abnormal activation of the RAAS can mediate cardiovascular injury through mechanisms independent of blood pressure.

The beneficial effects of epl were also independent of changes in urine output or urinary sodium excretion. Indeed, daily urine output, urinary sodium excretion, or urinary sodium/urinary potassium were never higher in epl- than in vehicle-treated, Ang II/salt-treated rats. No differences in serum sodium were observed among adrenal-intact groups. Thus, in the present experiment, the protective effects of epl cannot be attributed to a diuretic or natriuretic effect of the aldosterone blocker. Urinary potassium excretion was also similar in adrenal intact animals. However, Ang II/salt treatment induced a significant reduction in serum potassium that was prevented by epl. Based on these observations, we cannot exclude the possibility that epl provided benefit, at least in part, by preventing the hypokalemic effects of Ang II/salt treatment. In the present study, adrenalectomy was associated with significant changes in fluid and electrolyte homeostasis, both in aldosterone-deficient and in aldosterone-replaced rats. Consequently, we cannot exclude that these changes may have contributed to the myocardial changes observed in these two groups.

FIG. 4. Effect of treatments on body weight, and food and fluid intake. Rats were housed in individual metabolic cages, 2 d before initiation of the treatments. Surgical implantation of minipumps and adrenalectomy was performed on d 0. A 1%-NaCl drinking solution was started in all animals immediately after surgery. Animals were killed on d 21. Values represent mean ± SEM.
In conclusion, Ang II/salt treatment increased systolic blood pressure and induced cardiac hypertrophy and coronary vascular injury in the rat. Injury was primarily inflammatory in nature and associated with the expression of COX-2 and osteopontin in coronary walls. Administration of epler reduced cardiac hypertrophy and attenuated myocardial and vascular injury and COX-2 and osteopontin expression, without affecting blood pressure, diuresis, or natriuresis. The effect of epler was similar to that of adrenalectomy. Treatment with aldosterone in ADX animals reversed the beneficial effect of adrenalectomy. These data indicate that aldosterone mediates, at least in part, the deleterious consequences of Ang II/salt treatment in the rat heart and support a beneficial effect of aldosterone blockade in the treatment of hypertensive myocardial disease.

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