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

RICARDO ROCHA, CYNTHIA L. MARTIN-BERGER, POCHANG YANG, RACHEL SCHERRER, JOHN DELYANI, AND ELLEN MCMAHON

Pharmacia Corporation, Cardiovascular and Metabolic Diseases, Skokie, Illinois 60077 and St. Louis, Missouri 63167

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, 136 ± 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. (*Endocrinology* 143: 4828–4836, 2002)

ADMINISTRATION OF EXOGENOUS 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 nonselective 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 cyclooxigenase 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

Abbreviations: ADX, Adrenalectomized; aldo, aldosterone; Ang, angiotensin; COX-2, cyclooxygenase 2; epl, eplerenone; PRA, plasma renin activity; RAAS, renin angiotensin aldosterone; VEH, vehicle control.

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 control (NaCl); 2) Ang II/salt given Ang II infusion (25 ng/min, sc); 3) Ang II/salt/epl group treated with Ang II and epl (100 mg/kg·d, orally, twice daily); 4) Ang II/salt/ADX, in which ADX rats were given Ang II and dexamethasone (12 μg/kg·d, sc); and 5) Ang II/salt/ADX/aldo group, in which ADX rats were given aldo (20 μg/kg·d, sc via minipump), Ang II, and dexamethasone.

Ang II (American Peptide Co.) in saline, d-aldo (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% methylcellulose (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 tubes (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.

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 d –1, 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 aldo 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% H_2O_2 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, MPIIIb10, 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-

TABLE 1. Effect of treatment on blood pressure, cardiac hypertrophy and myocardial injury

Treatment groups	Systolic blood pressure (mm Hg)	Body weight (g)	Heart weight (mg)	Heart weight/ body weight (mg/g)	Myocardial and vascular injury (hearts with damage/total number)
Vehicle/salt	136 ± 6	348 ± 6	975 ± 24	2.8 ± 0.1	1/10
Angiotensin II/salt	203 ± 12[a]	332 ± 10	1078 ± 31[a]	3.3 ± 0.1[a]	7/10[a]
Angiotensin II/ salt/eplerenone	196 ± 10[a]	339 ± 6	1013 ± 36[b]	3.0 ± 0.1[a,b]	1/10[b]
Angiotensin II/ salt/ADX	181 ± 7[a]	270 ± 6[a,b]	795 ± 21[a,b]	2.9 ± 0.1[b]	2/10[b]
Angiotensin II/ salt/ADX/aldosterone	237 ± 8[a,b,c]	294 ± 10[a,b]	1211 ± 84[a,b,c]	4.1 ± 0.2[a,b,c]	10/10[a,c]

Values are expressed as means ± SEM (n = 10 per group).
[a] Significantly different from vehicle/salt, $P < 0.05$.
[b] Significantly different from ang II/salt, $P < 0.05$.
[c] Significantly different from ang II/salt/ADX, $P < 0.05$.

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

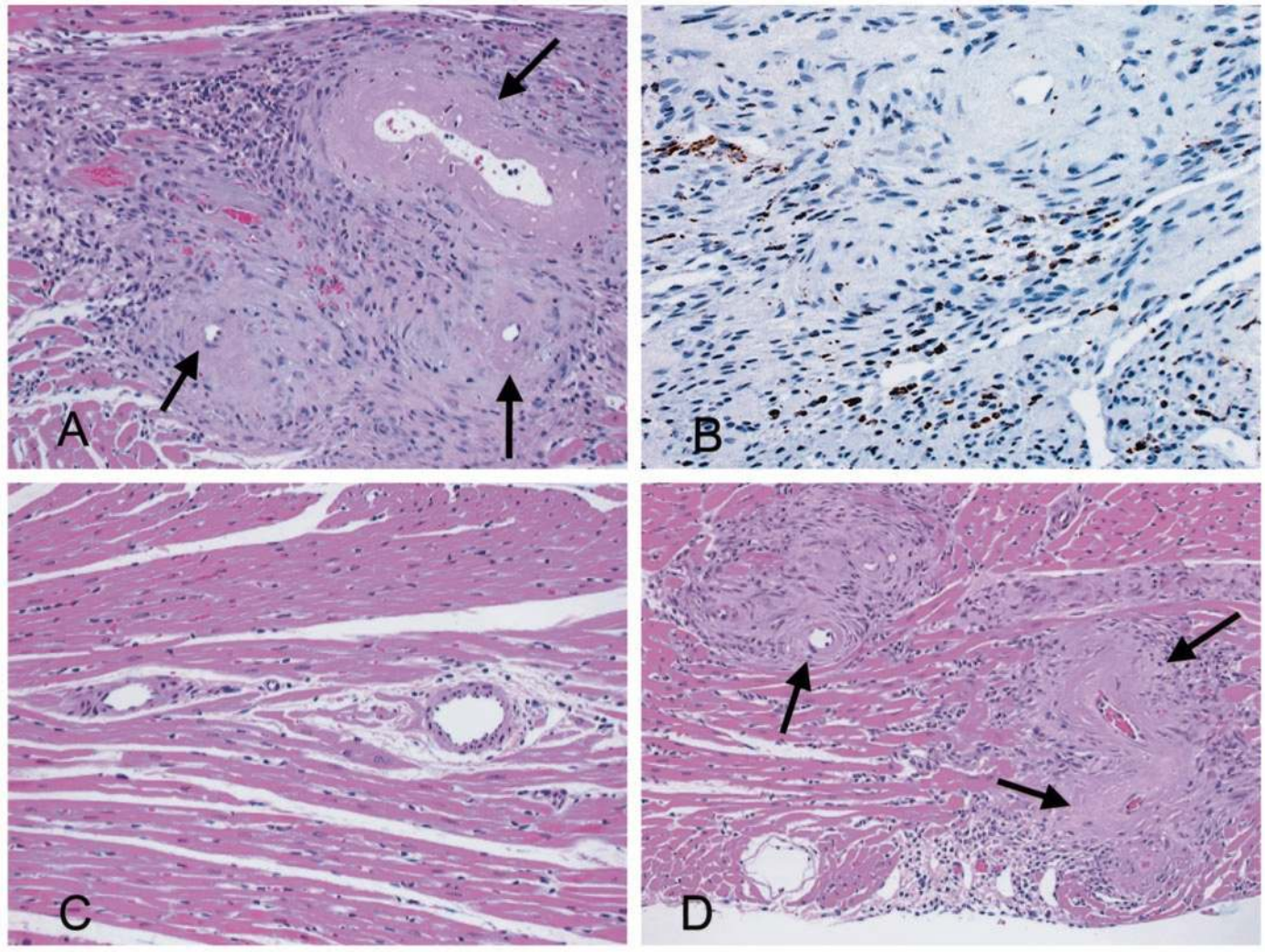

FIG. 1. Effect of treatment on myocardial and vascular damage. Photomicrographs of representative hematoxylin- and eosin-stained coronal sections of hearts from rats in the different experimental groups. Focal lesions of medial fibrinoid necrosis were observed in response to Ang II/salt treatment (*arrows*), associated with a prominent perivascular inflammatory response (A). Macrophages were frequently found associated with the coronary lesions and infiltrating the perivascular space (B). Adrenalectomy or epl treatment attenuated lesion development (C). Severe vascular inflammatory lesions were observed in all ADX animals with aldo treatment (D, *arrows*).

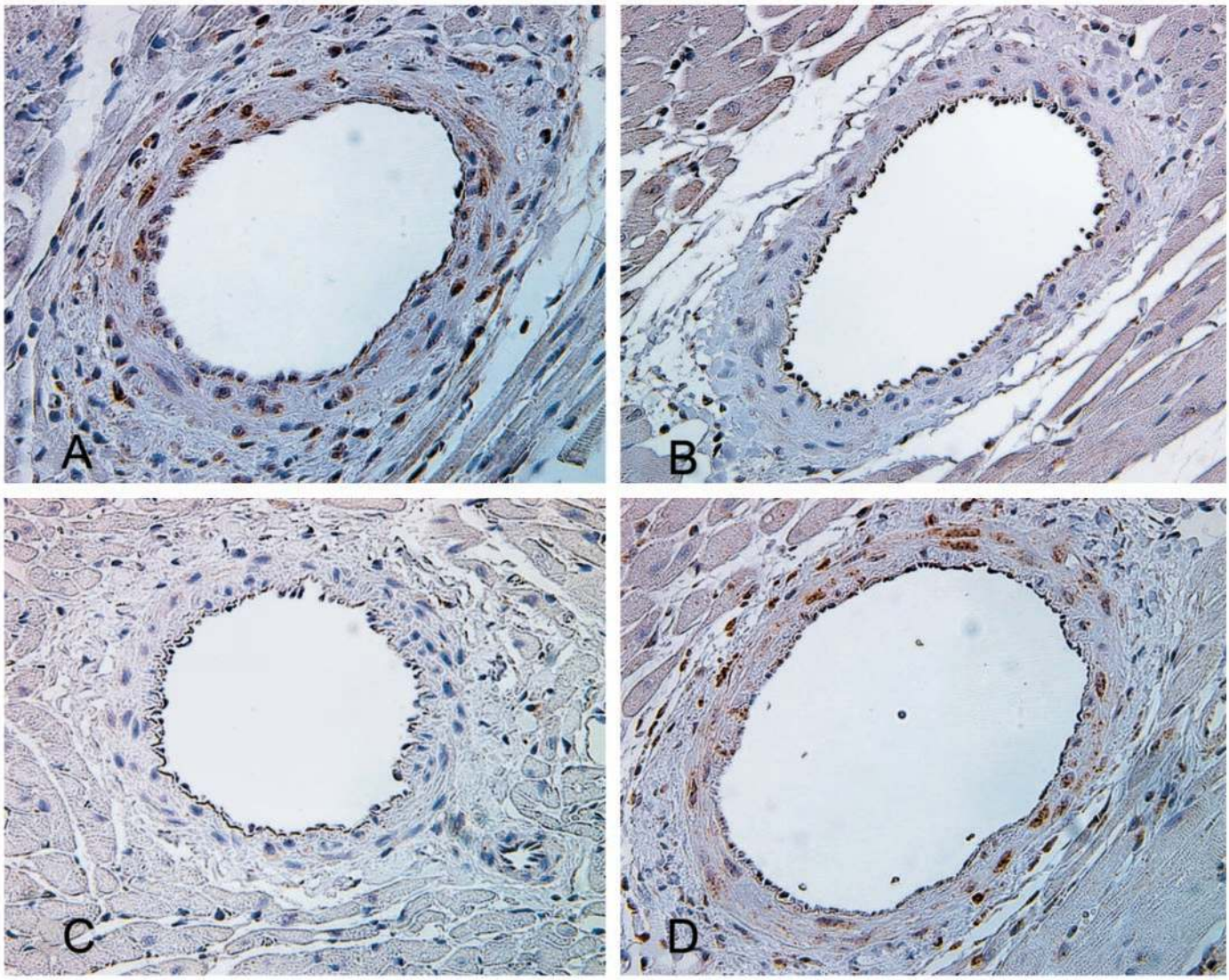

FIG. 2. Immunohistochemistry for COX-2. In vehicle animals, COX-2 immunostaining was only detected in occasional resident macrophages (not shown). Ang II/salt treatment induced COX-2 expression in the media of coronary arteries (A). COX-2 expression was markedly reduced by epl treatment (B) and adrenalectomy (C). Administration of aldo in ADX animals restored Ang II/salt-induced COX-2 expression (D).

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

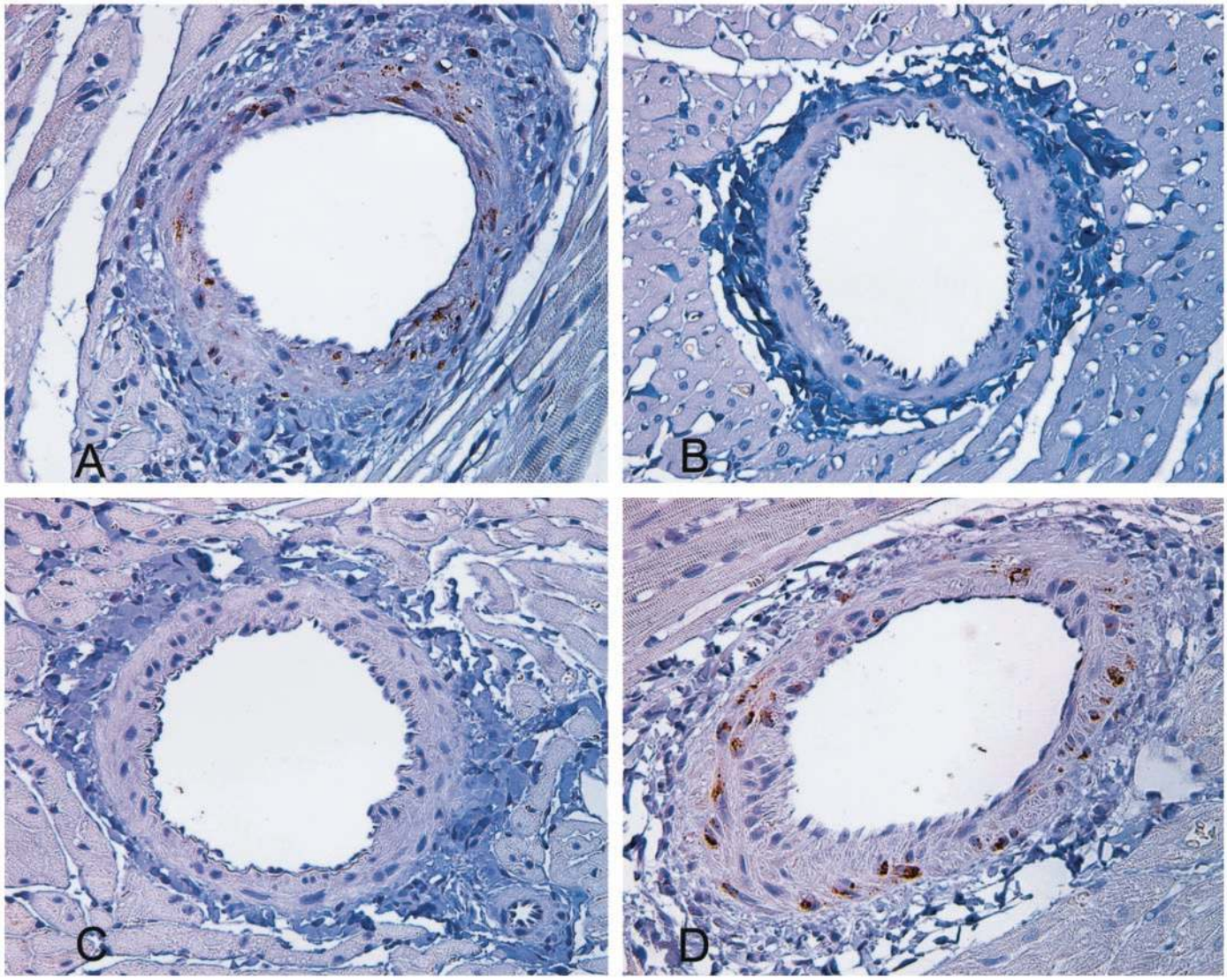

FIG. 3. Immunohistochemistry for osteopontin. Photomicrographs of sections processed with a monoclonal antibody against osteopontin. Vehicle-treated animals did not express osteopontin in the heart (not shown). Ang II/salt treatment induced osteopontin expression in the media of coronary arteries (A). 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).

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,

TABLE 2. Effect of treatment on plasma and serum constituents

Treatment groups	Plasma aldosterone (pg/ml)	Serum corticosterone (ng/ml)	Plasma renin activity (ng/ml·h)	Serum Na^+ (mM)	Serum K^+ (mM)
Vehicle/salt	56 ± 10	184 ± 27	3.9 ± 0.6	144 ± 1	4.3 ± 0.2
	(n = 10)	(n = 10)	(n = 10)	(n = 10)	(n = 10)
Angiotensin II/salt	58 ± 14	122 ± 18[a]	0.3 ± 0.1[a]	144 ± 1	3.7 ± 0.1[a]
	(n = 10)	(n = 10)	(n = 3)	(n = 9)	(n = 10)
Angiotensin II/ salt/eplerenone	224 ± 57[a,b]	161 ± 31	1.7 ± 0.8[a,b]	143 ± 0.3	4.4 ± 0.04[a,b]
	(n = 10)	(n = 10)	(n = 6)	(n = 10)	(n = 10)
Angiotensin II/ salt/ADX	nd[a,b]	nd[a,b]	20.6 ± 3.7[a,b]	140 ± 1[a,b]	5.9 ± 0.3[a,b]
	(n = 10)	(n = 10)	(n = 9)	(n = 9)	(n = 9)
Angiotensin II/ salt/ADX/aldosterone	271 ± 33[a,b,c]	nd[a,b]	0.7 ± 0.4[a,c]	146 ± 1[b,c]	3.3 ± 0.1[a,b,c]
	(n = 10)	(n = 10)	(n = 2)	(n = 10)	(n = 10)

Values are expressed as means ± SEM. nd, Not detected, many plasma renin activity values were below limit of detection.
[a] Significantly different from vehicle/salt, $P < 0.05$.
[b] Significantly different from ang II/salt, $P < 0.05$.
[c] Significantly different from ang II/salt/ADX, $P < 0.05$.

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 increases 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. Urine 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

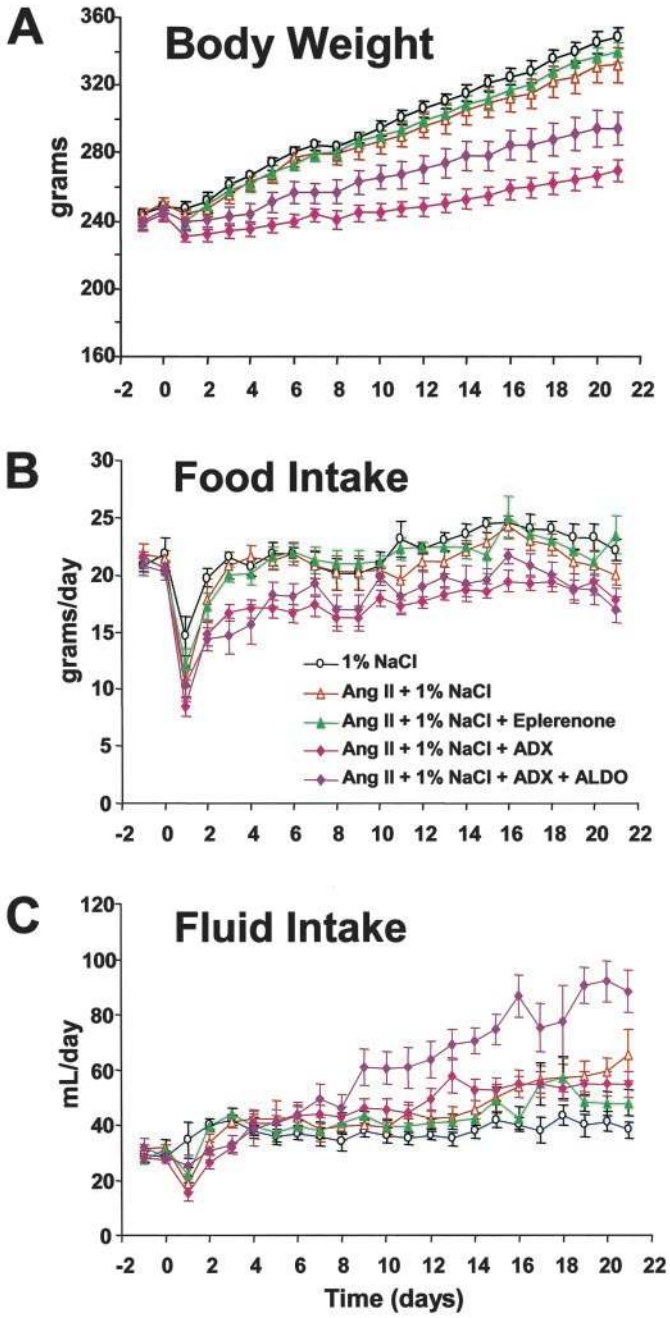

FIG. 4. Effect of treatments on body weight, and food and fluid intake. Rats were housed in individual metabolic cages, 2 d before initiation of the treatments. Surgical implantation of minipumps and adrenalectomy was performed on d 0. A 1%-NaCl drinking solution was started in all animals immediately after surgery. Animals were killed on d 21. Values represent mean ± SEM.

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 reparation or regeneration (11, 13). It can activate macrophages and T-cells to migrate and produce other cytokines via $\alpha_v\beta_3$ or CD44 receptors (14, 15). In addition, osteopontin has the ability to induce vascular smooth muscle cell proliferation and migration via $\alpha_v\beta_3$ 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 aldo 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 aldo 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, aldo/salt-treated 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-L-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 aldo 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 aldo-deficient and in aldo-replaced rats. Consequently, we cannot exclude that these changes may have contributed to the myocardial changes observed in these two groups.

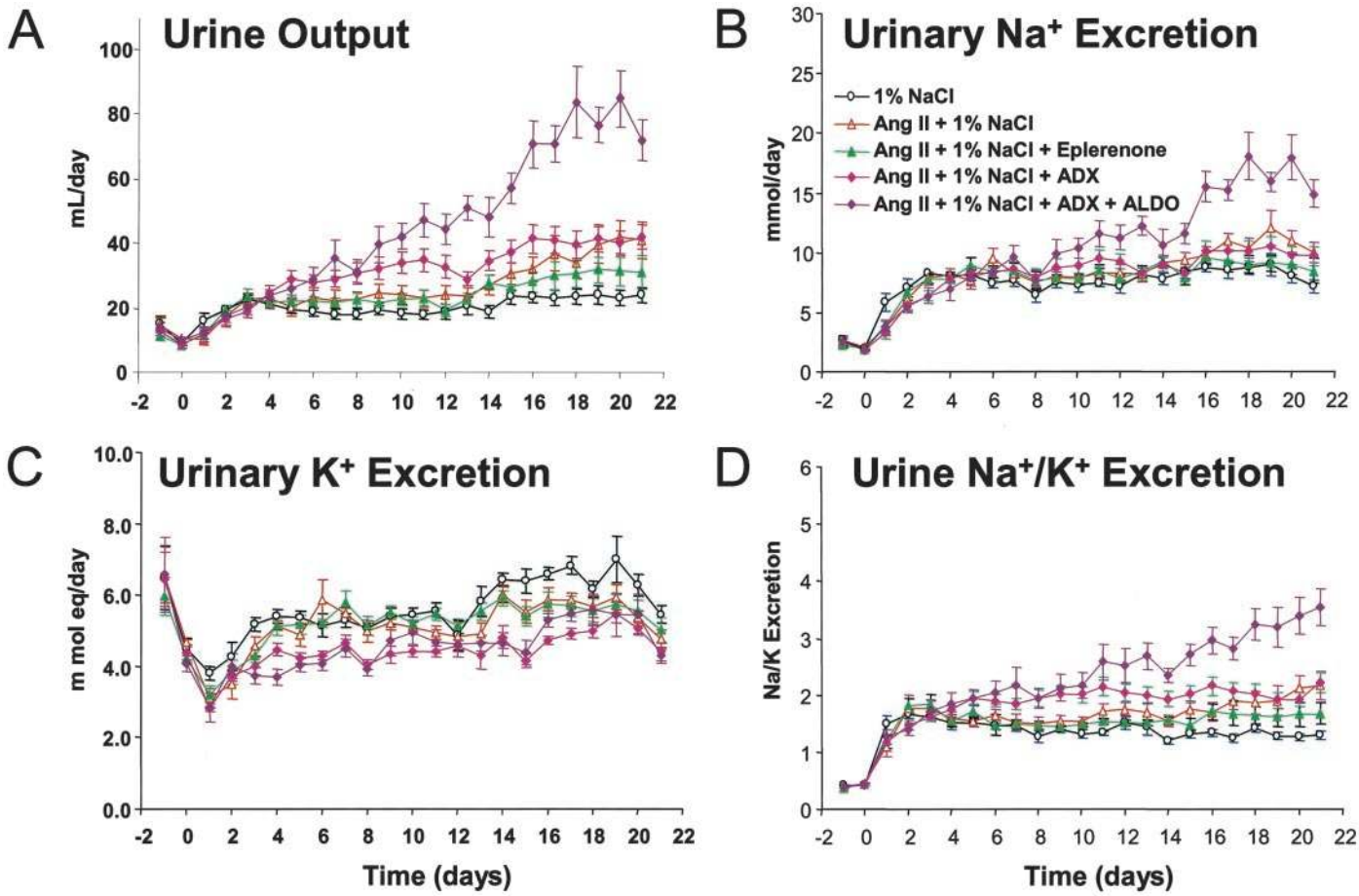

FIG. 5. Effect of treatments on urine output and urinary electrolyte excretion. Urine samples were collected every day for determination of urinary Na+ and K+ concentration. After surgery on d 0, a 1%-NaCl drinking solution was started in all groups. Treatments were maintained for 21 d.

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 epl 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 epl was similar to that of adrenalectomy. Treatment with aldo in ADX animals reversed the beneficial effect of adrenalectomy. These data indicate that aldo mediates, at least in part, the deleterious consequences of Ang II/salt treatment in the rat heart and support a beneficial effect of aldo blockade in the treatment of hypertensive myocardial disease.

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Address all correspondence and requests for reprints to: Ricardo Rocha, M.D., Pharmacia, 4901 Searle Parkway, Skokie, Illinois 60077. E-mail: ricardo.rocha@pharmacia.com.

References

1. **Gavras H, Lever AF, Brown JJ, Macadam RF, Robertson JI, Giacomelli F** 1971 Acute renal failure, tubular necrosis, and myocardial infarction induced in the rabbit by intravenous angiotensin II. Lancet 2:19–22
2. **Giacomelli F, Anversa P, Wiener J** 1976 Effect of angiotensin-induced hypertension on rat coronary arteries and myocardium. Am J Pathol 84:111–138
3. **Mervaala EM, Muller DN, Park JK, Schmidt F, Lohn M, Breu V, Dragun D, Ganten D, Haller H, Luft FC** 1999 Monocyte infiltration and adhesion molecules in a rat model of high human renin hypertension. Hypertension 33: 389–395
4. **Rocha R, Stier Jr CT, Kifor I, Ochoa-Maya MR, Rennke HG, Williams GH, Adler GK** 2000 Aldosterone: a mediator of myocardial necrosis and renal arteriopathy. Endocrinology 141:3871–3878
5. **Fiebeler A, Schmidt F, Muller DN, Park JK, Dechend R, Bieringer M, Shagdarsuren E, Breu V, Haller H, Luft FC** 2001 Mineralocorticoid receptor affects AP-1 and nuclear factor-κB activation in angiotensin II-induced cardiac injury. Hypertension 37:787–793
6. **Morris DJ, Souness GW, Brem AS, Oblin ME** 2000 Interactions of mineralocorticoids and glucocorticoids in epithelial target tissues. Kidney Int 57: 1370–1373
7. **Rocha R, Rudolph AE, Frierdich GE, Nachowiak DA, Kekec BK, Blomme EAG, McMahon EG, Delyani JA,** Aldosterone induces a vascular inflammatory phenotype in the rat heart. Am J Physiol Circ Physiol, in press
8. **Robert V, Silvestre JS, Charlemagne D, Sabri A, Trouve P, Wassef M, Swynghedauw B, Delcayre C** 1995 Biological determinants of aldosterone-induced cardiac fibrosis in rats. Hypertension 26:971–978
9. **Fujisawa G, Dilley R, Fullerton MJ, Funder JW** 2001 Experimental cardiac fibrosis: differential time course of responses to mineralocorticoid-salt administration. Endocrinology 142:3625–3631
10. **Denhardt DT, Noda M, O'Regan AW, Pavlin D, Berman JS** 2001 Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. J Clin Invest 107:1055–1061

11. **deBlois D, Lombardi DM, Su EJ, Clowes AW, Schwartz SM, Giachelli CM** 1996 Angiotensin II induction of osteopontin expression and DNA replication in rat arteries. Hypertension 28:1055–1063
12. **Kupfahl C, Pink D, Friedrich K, Zurbrugg HR, Neuss M, Warnecke C, Fielitz J, Graf K, Fleck E, Regitz-Zagrosek V** 2000 Angiotensin II directly increases transforming growth factor β1 and osteopontin and indirectly affects collagen mRNA expression in the human heart. Cardiovasc Res 46:463–475
13. **Senger DR, Ledbetter SR, Claffey KP, Papadopoulos-Sergiou A, Peruzzi CA, Detmar M** 1996 Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the αvβ3 integrin, osteopontin, and thrombin. Am J Pathol 149:293–305
14. **Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Jansson M, Zawaideh S, Rittling SR, Denhardt DT, Glimcher MJ, Cantor H** 2000 Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. Science 287:860–864
15. **O'Regan AW, Hayden JM, Berman JS** 2000 Osteopontin augments CD3-mediated interferon-gamma and CD40 ligand expression by T cells, which results in IL-12 production from peripheral blood mononuclear cells. J Leukoc Biol 68:495–502
16. **Gadeau AP, Campan M, Millet D, Candresse T, Desgranges C** 1993 Osteopontin overexpression is associated with arterial smooth muscle cell proliferation *in vitro*. Arterioscler Thromb 13:120–125
17. **Cowan KN, Jones PL, Rabinovitch M** 2000 Elastase and matrix metalloproteinase inhibitors induce regression, and tenascin-C antisense prevents progression, of vascular disease. J Clin Invest 105:21–34
18. **Belton O, Byrne D, Kearney D, Leahy A, Fitzgerald DJ** 2000 Cyclooxygenase-1 and -2-dependent prostacyclin formation in patients with atherosclerosis. Circulation 102:840–845
19. **Young W, Mahboubi K, Haider A, Li I, Ferreri NR** 2000 Cyclooxygenase-2 is required for tumor necrosis factor-alpha- and angiotensin II-mediated proliferation of vascular smooth muscle cells. Circ Res 86: 906–914
20. **Stier Jr CT, Adler LA, Levine S, Chander PN** 1993 Stroke prevention by losartan in stroke-prone spontaneously hypertensive rats. J Hypertens Suppl 11:S37–S42
21. **Stier Jr CT, Benter IF, Ahmad S, Zuo HL, Selig N, Roethel S, Levine S, Itskovitz HD** 1989 Enalapril prevents stroke and kidney dysfunction in salt-loaded stroke-prone spontaneously hypertensive rats. Hypertension 13: 115–121
22. **Rocha R, Chander PN, Khanna K, Zuckerman A, Stier Jr CT** 1998 Mineralocorticoid blockade reduces vascular injury in stroke-prone hypertensive rats. Hypertension 31:451–458
23. **Young MJ, Funder JW** 1996 Mineralocorticoids, salt, hypertension: effects on the heart. Steroids 61:233–235
24. **Tomita H, Egashira K, Ohara Y, Takemoto M, Koyanagi M, Katoh M, Yamamoto H, Tamaki K, Shimokawa H, Takeshita A** 1998 Early induction of transforming growth factor-beta via angiotensin II type 1 receptors contributes to cardiac fibrosis induced by long-term blockade of nitric oxide synthesis in rats. Hypertension 32:273–279