Direct fibrogenic effects of aldosterone on normotensive kidney: an effect modified by 11β-HSD activity

Andrew S. Brem, David J. Morris, Yan Ge, Lance Dworkin, Evelyn Tolbert, and Rujun Gong

Division of Kidney Diseases and Hypertension, Rhode Island Hospital, Department of Laboratory Medicine, Miriam Hospital, Brown Medical School, Providence, Rhode Island

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Brem AS, Morris DJ, Ge Y, Dworkin L, Tolbert E, Gong R. Direct fibrogenic effects of aldosterone on normotensive kidney: an effect modified by 11β-HSD activity. Am J Physiol Renal Physiol 298: F1178–F1187, 2010. First published March 3, 2010; doi:10.1152/ajprenal.00532.2009.—Aldosterone (Aldo) can be a profibrotic factor in cardiovascular and renal tissues. This study tests the hypothesis that prolonged Aldo exposure is able to directly induce fibrotic changes in the kidney of a normal nonhypertensive animal. Immortalized rat proximal tubule cells (IRPTC) containing 11β-hydroxysteroid dehydrogenase (11β-HSD1) but no mineralocorticoid receptors (MR) and mouse inner medullary collecting duct cells (IMCD) containing 11β-HSD2 and MR were examined. IRPTC exposed to Aldo or corticosterone (10 nM) for 48 h demonstrated no change in collagen production as assessed by Sirius red staining. In contrast, IMCD treated with Aldo exhibited a marked increase in the expression of collagen, fibronectin, and connective tissue growth factor (CTGF), whereas corticosterone alone had no effect. The Aldo-induced overexpression of collagen, fibronectin, and CTGF was substantially attenuated by the MR antagonist RU-318 and by the 11β-HSD end product 11-dehydrocorticosterone, but not by the glucocorticoid receptor antagonist RU-486. In vivo, early fibrotic changes with elevated collagen, fibronectin, and CTGF expression were observed in kidneys isolated from normotensive adrenalectomized mice receiving a continuous infusion of Aldo (8 μg·kg⁻¹·day⁻¹) for 1 wk. These changes were not present in corticosterone-treated mice. Aldo-induced changes were attenuated in adrenally intact mice and in mice treated with RU-318 or 11-dehydrocorticosterone. Thus, extended Aldo exposure produces fibrotic changes in cells containing MR and in normal kidneys. MR antagonists and the end products of 11β-HSD attenuate these fibrogenic effects.

fibrosis; renal epithelia; kidney; mineralocorticoids; 11β-hydroxysteroid dehydrogenase; glucocorticoids

MECHANISMS OF KIDNEY INJURY and the potential for ensuing fibrosis are current and relevant topics in the renal literature. Thus far, the renin-angiotensin system has garnered most of the attention since investigators discovered many of the pathologic pathways (11, 25, 44) and therapeutic agents are available to treat affected patients. However, there is now mounting evidence that steroids, mineralocorticoids, glucocorticoids, and possibly some of their metabolic derivatives may also influence the fibrotic process. Both glucocorticoids and mineralocorticoids have the ability to induce transforming growth factor-β (TGF-β) (26), connective tissue growth factor (CTGF; a downstream TGF-β fibrotic factor) (23, 34), and the expression and action of PAI-1 (4, 12). What is becoming apparent is that the fibrotic process following injury is multifaceted and offers opportunities for intervention beyond interruption of angiotensin activation.

The physiologic role for mineralocorticoids and specifically aldosterone (Aldo) lies in the regulation of sodium and potassium homeostasis. Classical studies conducted in the toad bladder and later in the kidney collecting duct demonstrated that mineralocorticoids bind to specific intracellular receptors, which are then translocated to the cell nucleus, and induce synthesis of selected proteins before electrolyte transport occurs (14, 15, 35). Thus, Aldo-stimulated transepithelial sodium transport begins after 30 to 90 min of exposure and lasts several hours before gradually subsiding. In contrast to the isolated renal epithelial laboratory models like toad bladder and collecting duct cells, acute administration of Aldo does not predictably produce changes in renal electrolyte transport in adrenally intact animals; it requires adrenalectomy to clearly show evidence of Aldo activity (5, 31, 39). This observation is consistent with the idea that other adrenally produced (and possibly modified peripherally) compounds may physiologically attenuate or regulate the electrolyte-transporting functions of Aldo.

That Aldo may have a pathological role in promoting fibrosis separate from its physiological function is a relatively new concept (10, 22, 36). As alluded to earlier, Aldo, through activation of the enzyme serum glucocorticoid kinase (SGK), promotes the expression of CTGF, TGF-β, and fibronectin in cultured mesangial cells (41). Mice genetically lacking SGK and treated with the mineralocorticoid DOCA and a high-salt diet demonstrate significantly less renal fibrosis and proteinuria, suggesting that this pathway is important in the disease pathogenesis (3). In the heart, there is evidence of increased collagen type III protein after animals are treated 48 h with the mineralocorticoid DOCA, an observation that occurs before the onset of hypertension (45). Lastly, in several models of kidney injury, animals treated with Aldo antagonists show a decrease in renal fibrosis independent of blood pressure (2, 28, 38). Taken together, all these findings suggest that mineralocorticoids may have a significant role in deleterious fibrotic processes and their activity needs to be tightly controlled.

Since the majority of all the previous studies of the fibrotic actions of Aldo in the kidney were undertaken using various injury models including partial nephrectomy, high-salt diet, and/or pharmacologic administration of mineralocorticoids, the present experiments were conducted to test the hypothesis that a lower dose Aldo could cause fibrotic changes in the kidney independent of prior injury or hypertension. Two renal epithelial cell lines were chosen for study; a cell line derived from proximal tubules, which does not contain mineralocorticoid receptors (MR) but does express the glucocorticoid-metabolizing enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD)
type 1 (7), and a cell line derived from collecting ducts, which contains MR and the isoform 11β-HSD type 2 (see RESULTS). We also examined the kidneys isolated from otherwise normal adrenalectomized (ADX) and adrenally intact Aldo-treated mice for fibrotic changes. Our novel experimental findings include 1) that Aldo in the absence of hypertension, prior renal injury, or dietary sodium loading can, over time, induce fibrotic changes in renal tissue, 2) that these fibrotic changes appear to occur only in cells, which contain MR, and perhaps most significantly, 3) that the Aldo-induced changes are directly attenuated by MR antagonists as well as metabolic products of both renal isoforms of 11β-HSD.

METHODS

Cell Studies

Dr. J. Ingelfinger originally provided the immortalized rat proximal tubular cells (IRPTC). In previously published studies, our laboratory established that IRPTC only contain 11β-HSD1 (7) and these proximal tubule-derived cells do not to contain classical MR. Cells were grown in 100-mm dishes containing DMEM-F12 with 5% FBS until confluent and then transferred to serum-free media during the studies with Aldo or corticosterone (Cort). The mouse inner medullary collecting duct cells (IMCD) were purchased from American Type Culture Collection (Manassas, VA) and also were grown in 100-mm dishes or slides containing DMEM-F12 with 5% FBS until confluent and then transferred to serum-free media during the studies with Aldo or Cort. As indicated in RESULTS, IMCD express only 11β-HSD2 and contain MR.

Sirius Red Assay and Staining

Sirius Red specifically stains collagens I and III (27, 42), early markers of fibrosis. To assess the quantitative production of fibrotic substances in cultured renal epithelial cells, the cells were grown to confluence and treated as indicated. Cells were harvested and sonicated for Sirius Red assays. In brief, ammonium sulfate was added to the sonicated cells and the contents were incubated under a slow constant rocking motion at 4°C for 24 h and then centrifuged. The pellet was resuspended in acetic acid. An aliquot of the resuspended sample was mixed with Sirius Red (50 μM) solution (Sigma, St. Louis, MO) made up in 0.5 M acetic acid. After 30 min of constant mixing, the pellet was prepared by centrifugation and resuspended in 1 ml of potassium hydroxide. The optical density of the samples was individually read with a spectrophotometer set to a wavelength of 540 nm.

In separate but related studies, epithelial cells were grown on coverslips but under the same culture conditions. At the completion of the experiment, the cells were directly stained with Sirius Red and directly examined under the light microscope.

Collagen Synthesis Assay

Collagenase-sensitive [3H]proline incorporation was assessed to measure collagen synthesis (21). Briefly, IMCD cultured on 12-well plates were serum-starved overnight and then assayed by adding 1 μCi/ml [3H]proline (Amersham Pharmacia Biotech, 1 Ci = 37 GBq) along with steroids of interest for 48 h. Cells were removed from tissue culture dishes with trypsin and protein was precipitated overnight with 20% TCA. After centrifugation, pellets were washed three times with 1.0 ml 5% TCA plus 0.01% proline and then dissolved with 0.2 M NaOH, and the solutions were titrated to neutral pH with 0.2 M HCl. Collagenase II (100 μl, 2 mg/ml) in Tris-CaCl2-N-ethylmaleimide buffer was added to each tube, and samples were incubated for 1 h at 37°C. Samples were then placed on ice, proteins were precipitated with 10% TCA for 1 h and centrifuged at 18 g for 10 min, and radioactivity of the supernatant fraction was determined by liquid scintillation counting.

Measurement of ECM Production and Degradation

Production of radioactive matrices. Matrices of IMCD cells were obtained as described previously (21). Briefly, cells were seeded into 6-well plates at 2 × 10^5 cells/well. 1-[2,3,4,5-3H]proline was added to the culture on the second day and daily thereafter. The medium was changed every 2 days. 1-[2,3,4,5-3H]proline was added to the culture medium at 1 μCi/ml on days 1, 3, and 5 after seeding. One week after the cells were seeded, cultures were washed with PBS, and the cells were removed by addition of 1 ml 2.5 mM NH4OH, 0.1% Triton X-100 per well for 1 min. The matrices left in each well were then washed extensively with PBS and distilled water and kept covered with sterile water at 4°C until further use.

Degradation of the matrices by IMCD cells. Labeled matrices were washed twice with 2 ml of serum-free DMEM/F12 medium before addition of IMCD at 2 × 10^5 cells/well in DMEM/F12 medium supplemented with 5% FBS. Medium was removed after 24-h incubation, allowing cells to attach to and recover from plating. Cells were washed three times with PBS to remove proteolytic enzyme inhibitors potentially present in the serum and then incubated for 24 h in 2 ml of serum-free DMEM/F12 containing 0.2% lactalbumin hydrolyzate and 4 μg/ml plasminogen. Exogenously added agents were dissolved in medium at the concentrations as indicated. At the end of the experiment, supernatants containing digested matrix were collected. Matrix remaining on the plates was also collected after being digested with 2 ml of 2 N NaOH at 37°C for 18 h. All samples were measured in a scintillation counter. Background values obtained with medium in the absence of cells were subtracted from these values. Counts for the supernatant and residual undigested matrix were considered to be 100%. The percentage of ECM degradation was expressed as the value of supernatant counts divided by that of the sum of counts.

HPLC Identification of Steroids

Renal cell homogenates were prepared in the presence of aprotinin and leupeptin as above and then are incubated with appropriate co-factors for amounts of time indicated for the kinetics experiments. At the completion of the incubation, the reaction is stopped with methanol and the sample is analyzed by HPLC. The steroids present in the supernatant are separated by HPLC using a Dupont Zorbax C8 column eluted at 44°C at a flow rate of 1 ml/min using 60% methanol for 10 min. The individual steroids are identified through comparison to known standards by monitoring radioactivity on-line with a Packard Radiomatic Flo-One/Beta series A-500 counter connected to a Dell Optiplex 425 S/L computer running Flo-One for Windows (version 2.0A). Each point in the kinetics experiments represents an average of three observations.

Animal Studies

Male C57BL/6 mice that weighed between 20 and 25 g were housed in the Central Research Facilities of Rhode Island Hospital, which is an American Association for Accreditation of Laboratory Animal Care-accredited facility, and studies were performed under an approved Institutional Animal Care and Use Committee protocol. Mice were anesthetized and received either a sham operation or bilateral adrenalectomy. Mice were offered 0.9% saline as drinking water after adrenalectomy for the duration of the study period to ensure survival. In following experiments, mice also received an Alzet micro pump implanted subcutaneously. The pump contained either vehicle or indicated steroids. Mice were randomly assigned to one of the following groups (n = 4): 1) sham control: sham-operated mice were given vehicle (DMSO) as a continuous subcutaneous infusion of vehicle by micro pump for 7 days, 2) sham + Aldo: sham-operated mice received a continuous subcutaneous infusion of Aldo by micro...
pump at a dose of 8 μg·kg⁻¹·day⁻¹ for 7 days, or 3) ADX control: ADX mice were given vehicle as a continuous subcutaneous infusion of vehicle by micro pump for 7 days, 4) ADX + Aldo: ADX mice received a continuous subcutaneous infusion of Aldo by micro pump at a dose of 8 μg·kg⁻¹·day⁻¹ for 7 days, 5) ADX + Cort: ADX mice received a continuous subcutaneous infusion of Cort by micro pump at a dose of 8 μg·kg⁻¹·day⁻¹ for 7 days, 6) ADX + Aldo + A: ADX mice received a continuous subcutaneous infusion of Aldo and 11-dehydro-corticosterone (A) by micro pump at a dose of 800 μg·kg⁻¹·day⁻¹ for 7 days, 7) ADX + Aldo + Cort: ADX mice received a continuous subcutaneous infusion of Aldo and Cort by micro pump at a dose of 800 μg·kg⁻¹·day⁻¹ for 7 days, and 8) ADX + Aldo + RU-318: ADX mice received a continuous subcutaneous infusion of Aldo and RU-318 by micro pump at a dose of 800 μg·kg⁻¹·day⁻¹ for 7 days. Seven days after surgery, animals were killed and bladder urine was collected and pooled for electrolyte analysis. The kidneys were perfused with iced saline and segments were fixed in fixation solutions, frozen for cryostat sectioning, or snap-frozen in liquid nitrogen, and stored at −80°C.

**Blood Pressure**

Just before death, mice were anesthetized and prepared for a measurement of mean arterial pressure. A polyethylene catheter was inserted into the femoral artery and a computer running WINDAS software (DATAQ Instruments, Akron, OH) continuously monitored the blood pressure using a pressure transducer.

**Renal Histology**

Formalin-fixed kidneys were embedded in paraffin and prepared in 3-μm-thick sections. For general histology, sections were processed for hematoxylin/eosin, periodic acid Schiff, and Masson-Trichrome staining. A semiquantitative morphometric score index was used to evaluate the degree of renal interstitial ECM accumulation (22, 25). Presence of ECM was revealed by trichrome staining of collagen. The magnitude of renal interstitial collagen accumulation was graded from 0 to 3 (21): 0, absent; 1, mild; 2, moderate; and 3, severe. A mean score was calculated using the values obtained in 20 random high-power (×400) fields in cortex and medulla per mouse in four mice per group. All sections were examined without knowledge of the treatment protocol.

**Glomerular Morphometry**

Glomerular volume was assessed on sections of methacrylate-embedded kidney tissue using a computerized image analysis system consisting of a high-resolution digital camera (MicroPublisher 3.3 RTV, QMAGING, Burnaby, BC, Canada) attached to a microscope (Olympus BX41) and to a computer (16). Images were displayed at a pixel resolution of 1,024 × 768 pixels (spatial resolution = 0.11 μm per pixel). Fifty glomeruli from each mouse were recorded. Glomerular surface area was obtained using the Image Pro Plus version 5.1 software (Media Cybernetics). Manual corrections were performed as needed. Glomerular volume was derived from the harmonic mean of the glomerular surface area. The mesangial area was measured by capturing the nucleus-free, PAS-positive area within each glomerulus. The nuclei of 50 glomeruli were counted to determine the relative glomerular cell number.

**Immunoperoxidase Staining**

Kidneys were removed and postfixed in 3% PFA/PBS before being embedded in paraffin wax. Paraffin wax sections (3 μm) were cut using a Leica RM2135 rotary microtome (Leica Microsystems) and mounted onto Superfrost microscope slides. Deparaffinized and dehydrated kidney sections were incubated in 1% H₂O₂ in methanol for 30 min to block endogenous peroxidase activity. Masked antigens were retrieved by microwaving the sections in TEG buffer (10 mM Tris, 0.5 mM EGTA, pH 9.0) for 7 min at 800 W and 5 min at 400 W. Sections were left to cool down before treatment with 50 mM NH₄Cl in PBS for 30 min. Sections were blocked with nonimmune serum from goat and then incubated overnight at 4°C in a humidified chamber with the 11B-HSD1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or 11B-HSD2 antibody (Chemicon-Millipore, Temecula, CA) diluted in 0.1% nonimmune serum with 0.3% Triton X-100 in PBS. Sections were rinsed with PBS and then incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin, diluted 1:200, for 1 h at room temperature. After being rinsed in PBS, the reaction was visualized using 3,3′-diaminobenzidine-tetrahydrochloride. Sections were counterstained with Mayer’s hematoxylin, dehydrated, and coverslips were mounted with Permount (Fischer Scientific, Fair Lawn, NJ). Immunostaining was examined using a Zeiss Axioshot light microscope (Carl Zeiss), and photographs were taken using a Spot RT Color digital camera (Diagnostic Instruments) (20). For a negative control, preimmune IgG from the same species replaced the primary antibody and no staining occurred.

**Total Kidney Collagen Assay**

Total kidney collagen was quantified as described (21) previously by determining the amount of hydroxyproline on the assumption that collagen contains 12.7% hydroxyproline by weight. The final results were expressed as microgram of collagen per milligram of kidney.

**Western Immunoblot Analysis**

The kidney tissue or cultured cells were lysed in RIPA buffer supplemented with protease inhibitors (1% Nonidet P-40, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 5 μg EDTA in PBS). Protein concentrations were determined using a bicinchoninic acid protein assay kit (Sigma). Samples with equal amounts of total protein (80 μg/ml) were fractionated on 7.5 to 10% SDS-polyacrylamide gels under reducing condition and analyzed by Western immunoblot as described previously (19). The antibodies against collagen I and actin were purchased from Santa Cruz Biotechnology. In the Western blot experiments, antibodies against 11β-HSD1 and the MR were obtained from Santa Cruz Biotechnology. The fibronectin and CTGF antibodies were also purchased from Santa Cruz Biotechnology, whereas the 11B-HSD2 antibody was purchased from Chemicon-Millipore.

**Measurement of serum Aldo and cort levels.** The serum Aldo levels were measured from blood samples collected from the mice with Aldo ELA Kits (Cayman Chemical, Ann Arbor, MI) following the instructions provided by the manufacturer.

**Statistical Analyses**

One investigator in a blinded manner performed computerized morphometric analysis as well as histological scoring. For immunoblot analysis, bands were scanned and the integrated pixel density was determined using a densitometer and the NIH image analysis program. All data are expressed as means ± SD. Statistical analysis of the data from multiple groups was performed by ANOVA followed by Student-Newman-Keuls tests. Data from two groups were compared by t-test. P < 0.05 was considered significant.

**RESULTS**

**Cell Culture Studies**

**Characterization of the epithelial cells.** The rat-derived IRPTC used in the current experiments have been previously reported to have characteristics of proximal tubule epithelial cells. These cells express 11B-HSD type 1, the isoform of the glucocorticoid-metabolizing enzyme known to exist in renal proximal tubules with a Kᵣ of 1.6 μM and a Vᵣ of 3.1

pmol-min$^{-1}$-mg protein$^{-1}$ (7, 8, 13, 18), and we documented that they do not contain classical MR by Western blot (data not shown). Our laboratory characterized the mouse-derived IMCD and these cells express NAD-dependent 11β-HSD type 2 by Western blot with an apparent $K_m$ of 61 nM and a $V_{\max}$ of 0.786 pmol-min$^{-1}$-mg protein$^{-1}$. Furthermore, IMCD also contain MR by Western blot. The respective 11β-HSD isoforms contained in both IRPTC and IMCD only show dehydrogenase activity; there is no evidence of reverse reductase in intact cells or in prepared homogenates of these cells.

**Effect of Aldo and Cort on Collagen Formation**

After 48 h of exposure to 10 nM Aldo, IRPTC demonstrated no increase in collagen production as evidenced by Sirius Red. Similarly, IRPTC incubated with 10 nM Cort alone or with Cort plus carbonoxolone (CBX; 1 μM), an inhibitor of 11β-HSD, showed no increased generation of Sirius Red staining by optical density. When experiments were repeated using IMCD, cells exposed to 10 nM Aldo exhibited a 93 ± 11% (means ± SE) increase in Sirius Red staining (collagen) compared with Cort alone. While 10 nM Cort alone had no effect, cells incubated with Cort plus CBX had a 46 ± 13% (means ± SE) increase in collagen production assessed by Sirius Red staining (Fig. 1). This observation is consistent with the view that when 11β-HSD2 is inhibited, Cort can bind to and activate MR. The Aldo-induced changes in Sirius Red staining seen in IMCD required 48 h to occur; no clear effect could be demonstrated after either 3 or 24 h of hormone exposure (Fig. 2A). To confirm the augmented collagen production measured by the optical density studies, IMCD were grown on eight-well coverslips and either left as controls or exposed to 10 nM Aldo for 48 h. Compared with controls (Fig. 2B), Sirius Red staining is markedly amplified in cells exposed to the Aldo (Fig. 2C).

The Aldo-induced increase in Sirius Red staining seen in IMCD could be blocked by the MR antagonist 1 μM RU-318 but not by the glucocorticoid receptor antagonist 1 μM RU-486. 11-Dehydrocorticosterone (1 μM), the metabolic product

![Graph showing effect of steroids on collagen formation in inner medullary collecting duct cells (IMCD).](image)

![Image of collagen formation in IMCD under different conditions.](image)
of 11β-HSD2, also suppressed the Aldo-induced increase in Sirius Red staining (Fig. 3A).

**Effect of Aldo ± Inhibitors on Fibrogenesis in IMCD**

For these experiments, IMCD were grown to confluence in six-well plates and exposed to Aldo (10 nM), Aldo plus RU-318 (1 μM), or Aldo plus 11-dehydrocorticosterone (1 μM). The expression of fibronectin, collagen I, and CTGF was all decreased relative to actin when cells were exposed to Aldo plus RU-318 or Aldo plus 11-dehydrocorticosterone (Fig. 3B).

**Effect of Aldo on ECM Synthesis and Degradation**

As seen in Fig. 4A, IMCD cells incubated in the presence of 10 nM Aldo generated a nearly 3.5-fold increase in ECM synthesis compared with controls. When the 11β-HSD end product 11-dehydrocorticosterone (Fig. 4A; concentrations from 1 μM to 10 nM) was also included in the incubation medium, the effect of Aldo was blunted in a dose-dependent fashion; 1 μM almost completely negated the effect of Aldo. The influence of Aldo on matrix degradation was also significant, although less dramatic; it decreased degradation by ~40% (Fig. 4B). Again, 11-dehydrocorticosterone attenuated the effects of Aldo on matrix degradation in a dose-dependent manner.

**Whole Animal Studies**

**Effectiveness of the adrenalectomy.** To confirm the effectiveness of the adrenalectomy procedure, we measured sodium and potassium in urine obtained by bladder tap just before the animals were killed. All the ADX mice demonstrated sodium wasting (pooled urine from controls: 21 mM Na, 104.3 mM K, Na/K ratio 0.20 vs. pooled urine from ADX: 416 mM Na, 211.7 mM K, Na/K ratio 1.97) with a marked increase in the urine sodium/potassium ratio as expected. Mean arterial blood pressure measurements were also obtained just before the mice were killed. There were no significant differences in mean blood pressure among the groups (mean values ranging from 88 to 93 mmHg). In experimental animals, the 1 wk of Aldo exposure was not long enough to produce hypertension. Plasma Aldo levels were 671 ± 134 pg/ml in controls, 3,410 ± 357 pg/ml in adrenally intact mice infused with Aldo, 57 ± 27 pg/ml in ADX mice, 2,868 ± 742 pg/ml in ADX mice infused

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**Fig. 3.** Effect of steroid receptor antagonists on fibrotic changes in IMCD. **A:** MR antagonist RU-318 (1 μM) and 11-dehydrocorticosterone (A; 1 μM) completely suppressed Aldo-induced collagen formation at 48 h. The glucocorticoid receptor antagonist RU-486 had no effect (*P < 0.05 vs. RU-318 or A-treated group). **B:** representative immunoblots show that MR antagonist RU-318 or A could markedly abrogate Aldo-induced production of fibronectin and connective tissue growth factor (CTGF).

**Fig. 4.** A attenuates the effect of Aldo on ECM synthesis (A) and degradation (B) in IMCD. **A:** IMCD cells were cultured in media supplemented with [3H]proline for 48 h in the presence of 10 nM Aldo ± A (1 μM, 0.1 μM, 10 nM, respectively). Results of labeled matrix synthesis were calculated as described in METHODS. Values are expressed relative to vehicle-treated control. *P < 0.05 vs. other groups. **B:** IMCD cells reseeded on the 3H-labeled matrix and were then incubated for 24 h in the presence of 10 nM Aldo ± A (1 μM, 0.1 μM, 10 nM, respectively). Results of labeled matrix degradation were calculated as described in METHODS. Values are expressed relative to vehicle-treated control. *P < 0.05 vs. other groups. Each bar represents means ± SD for 6 observations. Aldo appears to exert a greater effect on matrix synthesis than matrix degradation.
with Aldo, and 46 ± 34 pg/ml in ADX mice infused with Cort.

**Histological Changes Induced by Aldo**

Continuous infusion of Aldo for 1 wk induced early renal interstitial ECM accumulation independent of prior injury or systemic hypertension (Fig. 5, A–D). This effect was clearly more prominent in mice that underwent adrenalectomy before exposure to the Aldo. Cort, the naturally occurring glucocorticoid in mice, had no effect and the kidneys from mice treated with Cort resembled those from control adrenalectomized mice. The histological fibrotic changes seen in Aldo-treated mice correlated directly with increases in the expression of type I collagen. Thus, prolonged and continuous exposure to Aldo can induce fibrotic interstitial changes in an otherwise normal mammalian kidney.

In a separate confirmatory set of experiments, ADX mice were treated with Aldo alone or together with 11-dehydrocorticosterone, Cort, or RU-318 for 1 wk as outlined above (Fig. 6, A–C). Mice treated with both Aldo and either Cort or 11-dehydrocorticosterone (Fig. 6A) demonstrated little in the way of fibrotic change and there was no increase in fibronectin or collagen expression. Since both renal isoforms of 11β-HSD effectively locally metabolize Cort to 11-dehydrocorticosterone, the results for these two steroids should be the same. RU-318 also blunted the effect of Aldo as expected.

Continuous infusion of Aldo for 1 wk also induced glomerular enlargement, a finding that was unexpected in the absence of overt injury (Fig. 7, A–D). As before, the changes were aggravated by prior adrenalectomy. Both total glomerular volume and mesangial area were increased after continuous Aldo exposure. Mesangial cell number also appeared to be higher in these glomeruli. Glomeruli from mouse kidneys continuously exposed to Cort were no different from controls in their morphology. The experiment was repeated in ADX mice treated with Aldo (8 μg·kg⁻¹·day⁻¹) ± Cort, 11-dehydrocorticosterone, or RU-318 (each at 800 μg·kg⁻¹·day⁻¹). Glomeruli from mice coinfused with Cort, 11-dehydrocorticosterone, or RU-318 were smaller than those from mice treated with Aldo alone, very similar to the patterns observed in Fig. 7.

**Confirmation of the Presence of Both 11β-HSD Isoforms**

One hypothesis emanating from the present experiments is that the metabolic product of renal 11β-HSD, in this case...
11-dehydrocorticosterone, is one adrenally derived factor, which can directly inhibit or attenuate the actions of Aldo in the kidney. Since both renal isoforms of 11β-HSD function in the dehydrogenase mode (7, 8, 18), it was important to demonstrate their presence and locale for these studies. In Fig. 8, we were able to show that 11β-HSD1 was expressed by segments of proximal tubules, whereas 11β-HSD2 is mainly expressed by distal tubules in mouse kidneys. This pattern is consistent with prior observations in both the rodent, canine, and human kidney (8, 17, 18). [The online version of this article contains supplemental data.]

DISCUSSION

Many studies of Aldo and renal disease have shown a role for Aldo in the inflammatory and fibrotic response phase following the injury. Since functioning tissue is lost with extensive fibrosis, research efforts have generally focused on limiting inflammation and suppression of the fibrotic processes so that healing will occur with normal tissue rather than scar. Given the ability for Aldo to activate inflammatory and fibrotic pathways, the use of mineralocorticoid antagonists seems to be a promising therapeutic maneuver. However, with the current available evidence, one cannot be confident that mineralocorticoids actually cause fibrotic changes in the absence of pre-existing renal injury, hypertension, or potassium deficiency. The present experiments are unique and are among the first to show that MR-responsive renal epithelial cells undergo fibrotic changes after prolonged direct exposure to Aldo in the absence of induced injury or hypertension. In contrast to Aldo-induced electrolyte transport which is normally a relatively short-term defined event, responsive renal epithelial cells require a prolonged exposure to the Aldo before any fibrotic changes develop and the changes appear to be limited to renal epithelial cells that contain classical MR.

Since excess Aldo produces hypertension, electrolyte abnormalities, and now fibrotic changes in responsive epithelia, it would be reasonable to assume that there should be a naturally occurring counterbalance to the effects of this hormone. Ap-
proximately 50 years ago, researchers described a curious paradox regarding Aldo and its actions on the kidney (5, 39). Aldo reproducibly induced transepithelial sodium transport in preparations like the toad bladder and isolated perfused renal collecting ducts (35, 40) yet Aldo could not reliably induce renal sodium reabsorption in adrenally intact animals (5, 31). If animals were ADX before treatment with physiological doses of Aldo, renal sodium reabsorption was readily seen. This was the first evidence that Aldo itself was physiologically tightly regulated by naturally occurring endogenously generated factors probably emanating from the adrenal gland.

Uete and Venning (43) published some of the first experiments on endogenous Aldo antagonists. Cortisol or its 11-dehydro metabolite cortisone essentially blocked the antinatriuretic response to both DOCA and Aldo in the ADX rat model. This blunting effect of cortisol and cortisone was dose dependent. Morris and colleagues (31) confirmed this observation. Alberti and Sharp (1) expanded these observations and suggested a mechanism by demonstrating that cortisone functioned as an Aldo antagonist. Cortisone had no direct effect on sodium transport by itself but it was able to either displace Aldo from its receptor or limit the translocation of the Aldo-MR complex from the cytosol to the nucleus. The end result was to prevent Aldo-induced sodium reabsorption. Our laboratories and others reported similar findings with 11-dehydrocorticosterone and cortisone in concentrations from 1 μM to as low as 20 nM blocking the translocation of physiological concentrations of Aldo to the cell nucleus (9, 32, 33). Now, in the present studies, 11-dehydrocorticosterone, the 11-keto derivative of Cort-like spironolactone and RU-318, also prevents the fibrotic changes directly induced by Aldo in MR-responsive epithelial cells.

Synthetic spironolactones, acting as competitive inhibitors against MR, effectively block the translocation of the Aldo-activated receptors from the cytoplasm to the cell nucleus, the

Fig. 7. Continuous infusion of Aldo for 1 wk induced glomerular enlargement; this effect was enhanced by adrenalectomy. A: representative micrographs of glomeruli with PAS staining (magnification ×800). B: glomerular morphometry reveals glomerular volume for each group. *P < 0.05, #P < 0.05 vs. other groups. C: glomerular morphometry was used to assess mesangial area for each group. *P < 0.05, #P < 0.05 vs. other groups. D: glomerular cell numbers for each group are shown. *P < 0.05, #P < 0.05 vs. other groups.

Fig. 8. 11β-Hydroxysteroid dehydrogenase (11β-HSD1) was predominantly expressed in segments of proximal tubules while 11β-HSD2 was mainly expressed by distal tubules in mouse kidneys. Representative micrographs of immunoperoxidase staining of 11β-HSD1 or 11β-HSD2 in kidneys using preimmune Ig (left), specific anti-11β-HSD1 antibody (middle), or anti-11β-HSD2 antibody (right). Magnification ×400.
first step in the Aldo activation cascade. There is increasing evidence that there are naturally occurring substances, which may function somewhat like endogenous spironolactones. These compounds also appear to prevent or limit activated MR from translocating to the nucleus (32, 33). As previously mentioned, two prime candidates are the 11-keto metabolites of cortisol and Cort. The kidney contains two isoforms of the enzyme 11β-HSD, which generate these metabolites, cortisone and 11-dehydrocorticosterone (6, 8, 18). 11β-HSD1 is present in proximal tubular segments and although bidirectional in most other tissues, it appears to function only as a dehydrogenase in the kidney (8, 13, 18). 11β-HSD2 is located in mineralocorticoid-sensitive collecting duct epithelial cells and has the role of limiting glucocorticoid access to MR. In generating these 11-keto glucocorticoid metabolites, both these renal enzymes now appear to have an expanded physiologic purpose; first, to prevent inappropriate binding of glucocorticoids to MR and second, to locally produce products that limit the actions of Aldo.

The cell culture experiments were important providing clues to the fibrotic actions of Aldo. However, for the findings to be generalizable, studies had to be conducted and the observations confirmed in the whole animal model. To this end, adrenally intact and ADX mice were continuously exposed to Aldo or Cort infused via a mini pump delivery system. After a 7-day exposure, ADX mice treated with Aldo exhibited early signs of renal interstitial collagen I deposition. These changes occurred without prior injury and in the absence of hypertension. Mice with the micro pumps exhibited serum concentrations of Aldo some four- to sixfold higher than observed in adrenally intact control mice. These Aldo concentrations, while not entirely physiologic, have been observed and even exceeded in adrenally intact but stimulated animals (24). In other animal models, even more modest interventions like daily diuretic administration have been shown to increase Aldo levels between two and three times those observed in controls (30, 37). Reungjui and co-workers (37) showed that administration of the diuretic hydrochlorothiazide over a period of 20 wk was associated with aldosterone, a glucocorticoid metabolite, inhibiting aldosterone action in toad bladder.

While the observations induced by Aldo were interesting and provocative, the changes in the glomeruli were more unexpected. Aldo treatment was associated with a significant increase in glomerular size mostly in the form of mesangial expansion. This change could be accounted for by vascular volume expansion but it happened in the absence of overt hypertension making other explanations possible. MR have been described in mesangial cells and recently Terada and colleagues (41) presented evidence that Aldo directly stimulated serum and glucocorticoid-inducible protein kinase in mesangial cells mediating fibrotic signaling. Thus, our observed glomerular changes are very compatible with Terada’s findings as well.

Our experiments allowed us to look at Aldo in a different light. In the short term, Aldo is one of the guardians over the composition of the body fluids, regulating sodium reabsorption and promoting potassium and hydrogen ion secretion. More chronic and unregulated exposure to the hormone results in hypertension, electrolyte abnormalities, and perhaps most importantly, fibrotic changes in responsive tissues. Thus, tightly regulating the effects of this vital hormone endogenously is essential for long-term survival. Disease may follow from the loss of this internal regulation.

DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES
