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Mineralocorticoid Receptor Antagonist Reduces Renal Injury in Rodent Models of Types 1 and 2 Diabetes Mellitus

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To determine whether mineralocorticoid receptor (MR) activation plays a role in diabetic renal injury and whether this role differs in types 1 and 2 diabetes mellitus, we examined the effect of a MR antagonist on renal injury in rodent models of type 1 (streptozotocin-treated rat) and type 2 (*db/db* mouse) diabetes. We studied three groups of 8-wk-old, uninephrectomized Wistar rats for 4 wk: diabetic streptozotocin- (55 mg/kg) treated rats (n = 11), diabetic streptozotocin-treated rats receiving the MR antagonist eplerenone (n = 15), and nondiabetic rats (n = 9). In addition, we studied three groups of 8-wk-old mice for 16 wk: diabetic *db/db* mice (n = 10), diabetic *db/db* mice treated with eplerenone (n = 8), and nondiabetic, *db*/+ littermates (n = 11). Diabetic rats and mice developed albuminuria and histopathological evidence of renal injury, including glomerular hypertrophy, mesangial expansion, and tubulointerstitial injury as well as increased renal cortical levels of MR protein, MR mRNA, TGFβ mRNA, and osteopontin mRNA. All of these changes were significantly reduced by treatment with eplerenone except for the elevated MR levels. The beneficial effects of eplerenone were not attributable to changes in blood pressure or glycemia. In summary, MR expression was increased in kidneys of diabetic rodents, and MR antagonists effectively reduced diabetic renal injury irrespective of the species or specific cause of the diabetes. Thus, these data suggest that MR activation is a critical factor in the early pathogenesis of renal disease in both type 1 and type 2 diabetes mellitus. (*Endocrinology* 147: 5363–5373, 2006)

DIABETES MELLITUS IS the predominant cause of chronic renal failure in the United States. Despite the widespread use of renoprotective drugs, angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs), the incidence of end stage renal disease due to diabetes has doubled over the past decade (1–8). Several small studies suggest that addition of a mineralocorticoid receptor (MR) antagonist to ACE inhibitor or ARB therapy has a beneficial effect on proteinuria in patients with type 2 diabetes (9–11). However, the Food and Drug Administration has placed a contraindication on the use of the selective MR antagonist eplerenone in patients with type 2 diabetes and microalbuminuria due to the potential adverse renal effects of MR antagonists. Furthermore, a recent study in patients with type 2 diabetes demonstrates that MR antagonists worsen endothelial function (12). Endothelial dysfunction is a predictor of cardiovascular events (13, 14) and is associated with microalbuminuria in patients with diabetes (15, 16). Thus, it is unclear whether MR antagonists have beneficial or adverse effects on diabetic renal disease. In addition, studies have not examined the renal effects of MR antagonists in patients with type 1 diabetes or in the absence of cotreatment with ACE inhibitors or ARBs.

The overall goal of the present study was to determine in rodent models whether MR blockade has adverse or beneficial effects on diabetic renal disease. Because all of the clinical data are in patients with type 2 diabetes who are generally receiving ACE inhibitor or ARB therapy, we assessed the renal effects of MR antagonists in rodent models of both type 1 and type 2 diabetes and in the absence of these potentially confounding therapies. Furthermore, because the clinical studies were performed in patients with different degrees of renal disease, we studied diabetic rodents with and without a reduction in renal mass. To ensure that our findings are not unique to one species, we studied both rats and mice.

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Abbreviations: ACE, Angiotensin converting enzyme; ARB, angiotensin receptor blocker; ED-1, rat monocyte/macrophage marker for ectodermal dysplasia protein; GV, glomerular volume; MMS, mesangial matrix score; MR, mineralocorticoid receptor; PAS, periodic acid Schiff; PRA, plasma renin activity; RAA, renin-angiotensin-aldosterone.

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Materials and Methods

Experimental animals

Uninephrectomized, 8-wk-old male Wistar rats (Charles River Laboratories, Wilmington, MA) and 8-wk-old male *db/db* mice and *db*/+ heterozygous littermates (background strain C57BLKS/J) (Jackson Laboratory, Bar Harbor, ME) were purchased. Animals were kept in a room lighted 12 h/day at an ambient temperature of 22 ± 1 C. Animals had free access to drinking water and Purina Rodent Chow (5001 for rats [0.4% sodium before administration of streptozotocin and citrate, then 1.6% sodium for the remainder of the study] and for mice 5010 [0.3%

sodium]) (Purina, St. Louis, MO). Institutional Animal Care and Use Committee at Harvard University approved experimental procedures.

Methods

Streptozotocin study. Rats received an ip injection of streptozotocin (55 mg/kg) or sodium citrate 1 wk after nephrectomy. Three experimental groups were studied: 1) nondiabetic (sodium citrate treated) rats; 2) rats with streptozotocin-induced diabetes; and 3) rats with streptozotocin-induced diabetes treated with eplerenone. Afternoon tail vein blood glucose levels were measured daily in streptozotocin-treated rats and every 2 wk in the sodium citrate-treated rats using a one touch ultra-glucose meter (Lifescan, Inc., Milpitas, CA). Streptozotocin-treated rats with diabetes (blood sugars > 300 mg/dl on d 3) were included in the study. From d 3 to 28, diabetic rats received daily sc injections of ultralente insulin (Eli Lilly Co., Indianapolis, IN) in the late afternoon to maintain blood sugar levels between 450 and 550 mg/dl. Eplerenone was administered via the rat chow (1.36 mg eplerenone per gram chow) from d 3 to 28 to achieve an eplerenone dose of approximately 100 mg/kg·d. Rats were housed in individual metabolic cages and daily food intake, water intake, body weight, and urine output were recorded. Systolic blood pressure was measured in conscious animals by tail-cuff plethysmography (blood pressure analyzer, model 179; IITC Life Science, Woodland Hills, CA) on d 0, 14, and 28. On d 28, rats were anesthetized with isoflurane and blood and tissue samples were harvested.

Db/db study. We studied three experimental groups: diabetic *db/db* mice, diabetic *db/db* mice receiving eplerenone, and nondiabetic *db/+* littermates. *Db/db* mice with urine glucose 500 mg/dl or greater at 12 wk of age were deemed to be diabetic. Eplerenone was administered via chow (0.6 mg eplerenone per gram chow) from age 8 to 25 wk to achieve an eplerenone dose of approximately 100 mg/kg·d. Mice were group housed (four per cage) except when placed in individual metabolic cages for collection of 24-h urines. Body weight was obtained at 8 and 25 wk of age. Systolic blood pressure was measured in conscious animals by tail-cuff plethysmography (blood pressure analyzer, model 179, IITC Life Science) at age 25 wk. At 25 wk of age, mice were anesthetized with isoflurane and blood and tissue samples were harvested.

Assays

Urine albumin was assayed using an enzyme immunoassay (ALPCO Diagnostics, Windham, NH) for rats and DCA 2000 microalbumin kit (Bayer, Elkhart, IN) for mice. Serum sodium and potassium were measured by direct potentiometry with the ion-selective electrode (COBAS Integra 400; Roche Diagnostics, Indianapolis, IN). Serum and urine creatinine was measured using the Roche reagent (COBAS Integra 400; Roche Diagnostics). The plasma renin activity (PRA) was measured by radioimmunometric assay (DiaSorin, Stillwater, MN). Serum aldosterone was measured by solid-phase RIA (Diagnostic Products Corp., Los Angeles, CA).

Tissue processing and histological evaluation

For each animal, renal tissue for mRNA and protein analysis was placed in liquid nitrogen immediately after collection. The remaining renal tissue was fixed in buffered 10% formalin and embedded in paraffin blocks. Coronal kidney sections (5 μm) stained with periodic acid Schiff (PAS) and hematoxylin and eosin were examined by light microscopy by a pathologist unaware of the treatment group assignment for estimation of glomerular volume and glomerular mesangial expansion. Glomerular volume ($\bar{V}_G$) was estimated using the formula: $\overline{V_G} = (\beta/k)(\overline{A_G})^{3/2}$ where $\beta = 1.38$, a shape coefficient for spheres, k = 1.1, a size distribution coefficient, and $\bar{A}_G$ = average area of the cross-section of the glomerular tuft (17). This area was determined by standard point counting using a 361-point ocular grid on 50 glomeruli per rodent. Mesangial matrix expansion was estimated from examination of PAS-stained kidney sections. For each glomerulus, mesangial matrix expansion was graded on a semiquantitative scale from 0 to 4+ as described by other investigators (18–21): 0 indicates no expansion; 1+ indicates matrix expansion occupying up to 25% of a glomerulus; and 2+, 3+, and 4+ indicate matrix expansion occupying 25–50, 50–75, and more than 75% of a glomerulus, respectively. The mesangial matrix score for each animal was the average score of 30 glomeruli.

Immunohistochemistry

Briefly, 5-μm paraffin-embedded kidney sections were deparaffinized. The sections with heat-induced antigen retrieval were microwave treated (800 W; General Electric, Louisville, KY) at 199 F for 30 min in the preheated 10 mM citrate buffer (Dako, Carpinteria, CA). Slides were preincubated with blocking solution containing normal horse serum (Vector, Burlingame, CA) for 10 min. Staining was performed by avidin biotin complex method according to the manufacturer's protocol (Vector). Osteopontin was detected by mouse primary antibody (dilution 1:300; University of Iowa, Iowa City, IA) and ED-1 (ectodermal dysplasia protein) by mouse primary antibody (no. MAB1435, dilution 1:800; Chemicon International, Temecula, CA), followed by horse anti-mouse (rat adsorbed) IgG as secondary antibody (dilution 1:200; Vector). Negative control sections were incubated with secondary antibody alone or an irrelevant horse IgG (Vector). Nuclei were counterstained by Mayer's hematoxylin. A pathologist unaware of the treatment group assignment examined sections of rat renal cortex immunostained for ED-1 to estimate the extent of macrophage/monocyte infiltration in the glomerular and tubulointerstitial compartments. For each rat, the average number of ED-1-positive cells in one glomerulus was determined based on examining 20 randomly chosen glomeruli. The extent of the macrophage/monocyte component of the inflammation in the tubules and interstitium was estimated by counting the number of ED-1-positive cells per high power field (×40 magnification) in the renal cortex (excluding glomeruli). The tubulointerstitial inflammatory score for each animal was the average score of 20 randomly chosen high-power fields.

Western blot analysis

Protein was extracted by homogenizing renal cortex with lysis radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Protein extracts (40 μg) were combined with an equal volume of 2× Laemmli loading buffer, boiled for 5 min, and size fractionated by electrophoresis on 10% SDS-polyacrylamide gels. Proteins were transferred from the gel to a nitrocellulose membrane by electroblotting. Membranes were incubated with 5% nonfat dried milk in PBS for 1 h and then incubated with 1:500 MR antibody (rabbit polyclonal antibody H300; no. sc-11412, Santa Cruz) overnight at 4 C. After incubation, samples were washed, incubated with peroxidase-conjugated secondary antibody, and analyzed using enhanced chemiluminescence (PerkinElmer Life Sciences, Boston, MA).

Analysis of mRNA expression by real-time PCR

Total mRNA was extracted from kidney cortex using the RNeasy mini kit (QIAGEN Sciences, Germantown, MD). cDNA was synthesized from 1.5 μg RNA with the first-strand cDNA synthesis kit (Amersham, Buckinghamshire, UK). PCR amplification reactions were performed in duplicate using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) and using the ΔΔCT method to determine mRNA levels. Gene expression was normalized to β-actin mRNA levels or 18S rRNA. PCR amplification performed with the QuantiTect SYBR green PCR kit (QIAGEN) used the primers shown in Table 1 that were designed with Primer Express (version 2.0; Applied Biosystems) and synthesized by Integrated DNA Technologies (Coralville, IA). PCR amplification using TaqMan gene expression assays (proprietary primers and probes designed and synthesized by Applied Biosystems) were used to detect TGFβ, CD68, vascular endothelial growth factor, plasminogen activator inhibitor-1, and 18S rRNA.

Data analysis

The statistical significance of the differences between group means for the data were determined by one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons. Differences in means with $P \leq 0.05$ were considered statistically significant. Values are expressed as mean ± SE.

TABLE 1. Real-time PCR primers used in the studies

	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
Rat		
MR	GCTTTGATGGTAGCTGCG	TGAGCACCAATCCGGTAG
Osteopontin	CCGAGGTGATAGCTTGGCTTA	GCAACTGGGATGACCTTGAGA
β-Actin	AGGCCAACCGTGAAAAGATG	ACCAGAGGCATACAGGGACAAC
Mouse		
MR	GTGGACAGTCCTTTCACTACCG	TGACACCCAGAAGCCTCATCTC
Osteopontin	TGATCAGGACAACAACGGAA	TCTCCTGGCTCTCTTTGGAA
β-Actin	AGCCATGTACGTAGCCATCC	CTCTCAGCTGTGGTGGTGAA

Results

Metabolic parameters in nondiabetic and diabetic streptozotocin-treated rats

Figure 1A shows the daily blood glucose levels in the nondiabetic (sodium citrate treated) rats (n = 9), streptozotocin-treated rats not receiving eplerenone [n = 11 (three rats that died in the third week after streptozotocin were excluded from analysis)], and streptozotocin-treated rats receiving eplerenone (n = 15). The daily insulin dose administered to streptozotocin-treated animals increased over the 28-d study but was not affected by eplerenone treatment (Fig. 1B). Daily food intake and fluid balance were similar in both groups of diabetic rats and food intake was increased, compared with nondiabetic rats (Table 2). Rats appeared healthy and gained weight throughout the study, although weight gain was less in the two diabetic groups as compared with the nondiabetic group (Fig. 1C).

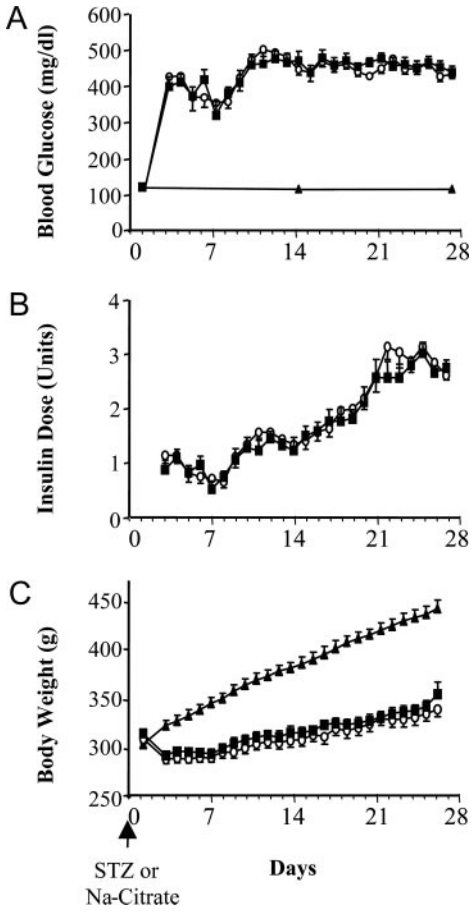

FIG. 1. Daily blood glucose levels (A), insulin doses (B), and body weights (C) in nondiabetic rats (*triangle*, n = 9), streptozotocin-treated rats (*square*, n = 11), and streptozotocin-treated rats receiving eplerenone (*circle*, n = 15) over the 28 d after streptozotocin or Na citrate treatment. STZ, Streptozotocin.

Systolic blood pressure measured at baseline was similar in all three groups (Table 2). In contrast, systolic blood pressure measured on d 14 and 28 was elevated in the streptozotocin and streptozotocin/eplerenone groups as compared with the nondiabetic control group. Systolic blood pressure tended to be lower in the streptozotocin/eplerenone group, compared with the streptozotocin group, although this did not reach statistical significance. PRA, serum aldosterone, and serum potassium did not differ significantly between the streptozotocin and nondiabetic groups. Treatment with streptozotocin and eplerenone significantly increased PRA, aldosterone, and potassium (Table 2). Serum creatinine did not differ between groups. Renal cortical levels of MR mRNA and protein were increased in streptozotocin-treated rats, compared with the nondiabetic rats. Treatment with eplerenone did not have a significant effect on MR expression (Fig. 2).

Renal injury in streptozotocin-treated rats

Diabetic rats not receiving eplerenone had increased kidney weights and increased kidney to body weight ratios, compared with nondiabetic rats (Table 2). Treatment of diabetic rats with eplerenone for 1 month significantly reduced renal hypertrophy. On d 0, albuminuria was similar in the nondiabetic, streptozotocin, and streptozotocin/eplerenone groups, 0.2 ± 0.05, 0.4 ± 0.15, and 0.3 ± 0.04 mg per 24 h, respectively. At the end of the study, albuminuria was elevated in the streptozotocin group, compared with both the nondiabetic animals and streptozotocin-treated animals receiving eplerenone (see Fig. 4A). Twenty-four-hour urinary creatinine levels were similar across groups showing similar efficacy of urine collection in the three groups.

Histological sections of kidneys from the streptozotocin-treated rats revealed glomeruli with signs of hypertrophy of the tuft, mild expansion of the mesangial matrix, and a slight increase of mononuclear cell elements in the mesangial areas (Fig. 3B). These changes were not present in the nondiabetic rats (Fig. 3A) and were recognized only focally in diabetic rats receiving eplerenone (Fig. 3C). Estimated glomerular volume (GV) and mesangial matrix scores (MMS) were increased in diabetic rats not receiving eplerenone (GV: 0.98 ± 0.06 × 10^{-6} μm^3; MMS: 3.09 ± 0.29), compared with non-

TABLE 2. Metabolic measurements in the streptozotocin study

	Nondiabetic (n = 9)	STZ (n = 11)	STZ + eplerenone (n = 15)
Systolic blood pressure (mm Hg)			
d 0	112 ± 4	111 ± 3	111 ± 3
d 14	108 ± 2	122 ± 3[a]	120 ± 4[a]
d 28	110 ± 3	124 ± 2[b]	117 ± 3[a]
Daily food intake (g)	29 ± 0.4	37 ± 0.8[b]	38 ± 0.5[b]
Daily fluid balance (ml)[c]	22 ± 3	37 ± 6	38 ± 5
Left kidney weight (g)	2.8 ± 0.2	4.3 ± 0.2[b]	3.6 ± 0.1[b,d]
Kidney to body weight ratio (g/kg)	6 ± 0.3	13 ± 0.7[b]	11 ± 0.2[b,d]
Blood measurements			
PRA (ng/ml·h)	0.2 ± 0.1	1.4 ± 0.6	3.3 ± 0.5[b,e]
Serum aldosterone (ng/dl)	8.5 ± 0.8	22.5 ± 5.4	44.5 ± 7[b,e]
Serum Na^{+}[f]	143 ± 0.8	144 ± 0.8	146 ± 0.8[a]
Serum K^{+}	4.8 ± 0.1	5.0 ± 0.2	5.5 ± 0.1[b,e]
Serum creatinine (mg/dl)[g]	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Creatinine clearance (ml/h)[g]	0.42 ± 0.15	0.34 ± 0.11	0.31 ± 0.17

Data given as mean ± SE. STZ, Streptozotocin.
[a] $P < 0.05$ *vs.* non-DM.
[b] $P < 0.01$ *vs.* non-DM.
[c] Daily fluid balance equals 24-h fluid intake minus 24-h urine output.
[d] $P < 0.01$ *vs.* STZ.
[e] $P < 0.05$ *vs.* STZ.
[f] Corrected serum Na^{+} = measured serum Na^{+} + [(glucose − 100) × 0.024].
[g] n = 4–12/group.

diabetic rats (GV: 0.8 ± 0.02 × 10^{-6} μm^3; MMS: 0.99 ± 0.1) and diabetic rats receiving eplerenone (GV: 0.76 ± 0.03 × 10^{-6} μm^3; MMS: 1.46 ± 0.14) (Fig. 4, B and C). Furthermore, the number of ED-1-positive cells in glomeruli of diabetic rats not receiving eplerenone (8.75 ± 0.67 cells/glomerulus) was elevated, compared with nondiabetic rats (5.17 ± 0.44 cells/glomerulus) and diabetic rats receiving eplerenone (5.99 ± 0.88 cells/glomerulus) (Fig. 4E).

Other changes in the streptozotocin-treated rats not receiving eplerenone were mild distension of the proximal tubules with irregular and low cuboidal epithelial cells; focal reabsorption protein granules in the glomerular visceral epithelial cells, proximal tubules, and distal tubules; and focal mononuclear cell infiltrates in the interstitium, mostly surrounding tubules with reactive changes. Such changes were not observed in the nondiabetic rats and were recognized only rarely in kidney sections from diabetic rats receiving eplerenone. There was a significant increase in the number of ED-1-positive cells in the tubulointerstitial compartment of diabetic rats, compared with nondiabetic rats, and eplerenone treatment tended to reduce this increase in inflammatory cells (nondiabetic *vs.* streptozotocin, $P = 0.005$, streptozotocin *vs.* streptozotocin + eplerenone, $P = 0.076$ by ANOVA with *post hoc* Tukey's) (Figs. 4F and 3, G–I).

Renal cortical levels of osteopontin mRNA were increased in streptozotocin-treated rats not receiving eplerenone, compared with both nondiabetic rats and with streptozotocin-treated rats receiving the MR antagonist (Fig. 4D). Immunohistochemical staining for osteopontin revealed strong and extensive expression of osteopontin in renal cortical tubules of diabetic rats not receiving eplerenone (Fig. 3E). The reaction was negative in nondiabetic rats (Fig. 3D) and only focal in the histological sections from diabetic rats receiving eplerenone (Fig. 3F).

Metabolic parameters in db/db mice

At 8 wk of age, *db/db* mice were significantly heavier than *db/+* mice (Table 3). Over the ensuing 16 wk, weight increased in all groups. One mouse died over the 16-wk study and was not included in the analysis; this *db/db* mouse died secondary to a skin infection. At 25 wk of age, plasma glucose levels were markedly elevated in the two *db/db* groups as compared with *db/+* mice (Table 3). Systolic blood pressure measured at 25 wk did not differ significantly among the three groups of mice (Table 3). Plasma aldosterone and PRA were similar in the *db/+* and *db/db* mice not receiving eplerenone. *Db/db* mice receiving eplerenone had elevated PRA and aldosterone levels, compared with untreated *db/db* and *db/+* mice. Renal cortical levels of MR mRNA were increased in diabetic *db/db* mice, compared with *db/+* mice. Treatment with eplerenone did not significantly change MR expression in *db/db* mice (Table 3).

Renal injury in db/db mice

At 25 wk of age, albuminuria was increased in *db/db* mice, compared with nondiabetic *db/+* mice, and *db/db* mice receiving eplerenone (Fig. 5A). Kidney to body weight ratios were reduced in both groups of *db/db* mice, compared with lean, *db/+* littermates due to the increased weight of the obese *db/db* mice (Table 3). Kidney weights were similar in all three groups (Table 3).

Histological sections of kidneys from the *db/db* mice not receiving eplerenone showed signs of hypertrophy of the glomerular tuft, with mild mesangial matrix expansion and slightly increased mononuclear cells in the mesangial areas (Fig. 6B). These findings were not present in the *db/+* mice (Figs. 6A) and showed less intensity and frequency in the kidney sections of *db/db* mice receiving eplerenone (Figs. 6C). Estimated glomerular volume and mesangial matrix scores

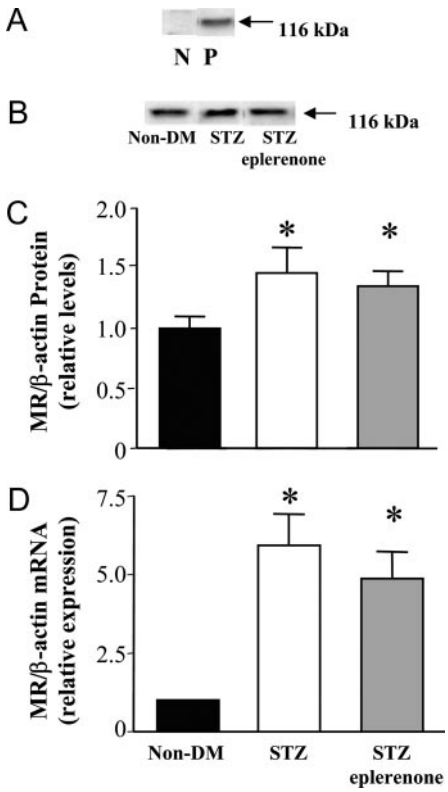

FIG. 2. MR protein and mRNA in STZ study. Representative Western blot for MR from lysates of human embryonic kidney (HEK) 293 cells, which do not express MR (N, negative control) and HEK 293 cells transfected with MR expression vector (kindly provided by Marc Lombès (Faculté de Médecine Xavier BICHAT, Paris, France). P, Positive control (A). Representative Western blot for MR from renal cortical lysates (40 μg) in nondiabetic rats (non-DM), streptozotocin-treated rats (STZ), and streptozotocin-treated rats receiving eplerenone (STZ + eplerenone) (B). Levels of MR protein (relative to β-actin protein) (C) and MR mRNA (relative to β-actin mRNA) (D) on d 28 in three treatment groups. *, $P < 0.05$ *vs.* non-DM group. C, n = 3 (non-DM group) and 8–9 (STZ groups). D, n = 9–15 animals/group. Data are mean ± SE.

were increased in *db/db* mice, compared with the nondiabetic littermates and the *db/db* mice receiving eplerenone (Fig. 5, B and C). Additional findings not specific to early diabetic changes were observed in other renal structures in isolated cases of *db/db* mice and included focal protein reabsorption droplets in the tubules and focal mononuclear inflammatory infiltrates in the renal papillae.

Diabetic *db/db* mice had increased renal cortical mRNA levels of osteopontin (Fig. 7A), TGFβ (Fig. 7B), and CD68 (Fig. 7C), compared with the nondiabetic littermates and diabetic *db/db* mice receiving eplerenone. Plasminogen activator inhibitor-1 and vascular endothelial growth factor mRNA levels did not differ between the three treatment groups (data not shown).

Discussion

In answer to the goals of this study, MR antagonist reduced renal injury in animal models of both type 1 and type 2 diabetes and reduced albuminuria and histopathological evidence of renal damage. MR antagonists were beneficial in both rats and mice, and in rats with reduced renal mass due to uninephrectomy. Thus, the beneficial effect of a MR antagonist on the kidney is independent of the cause of the diabetes and universally effective in reducing several markers of renal injury.

In humans with diabetes, hyperglycemia leads to glomerular hypertrophy and eventually diffuse glomerulosclerosis characterized by an increase in mesangial matrix and cellular elements, thickening of the glomerular basement membrane and functional and structural adaptations in the visceral epithelial cell including variable effacement of the foot processes. This progressive glomerular injury causes leakage of albumin into Bowman's space and the tubular system, with development of albuminuria (22, 23). Development of renal injury is accelerated by reductions in nephron number as occurs with uninephrectomy. As demonstrated in the current studies, hyperglycemic *db/db* mice and streptozotocin-treated rats develop albuminuria and some of the early histopathological changes observed in diabetes—glomerular hypertrophy and mesangial expansion (24–29; for reviews see Refs. 30 and 31). Interruption of the renin-angiotensin-aldosterone (RAA) system with ACE inhibitors or ARBs reduces glomerulosclerosis and albuminuria in uninephrectomized and nonnephrectomized rats treated with streptozotocin (24–27). In addition, blockade of the RAA system with the MR antagonist spironolactone has been reported to reduce some features of diabetic renal injury in streptozotocin-treated rats, specifically renal fibrosis, renal macrophage infiltration, and renal elevations in TGFβ mRNA levels (32, 33).

The current studies extend these findings to show that MR blockade significantly reduces albuminuria, glomerular hypertrophy, early mesangial matrix expansion, and osteopontin expression in both streptozotocin-treated uninephrectomized rats and *db/db* mice. Furthermore, we demonstrate that MR blockade reduces glomerular inflammatory infiltrates in streptozotocin-treated rats and renal cortical expression of TGFβ and CD68, a macrophage antigen, in *db/db* mice. These results in the *db/db* animal model are consistent with a preliminary report demonstrating a reduction in renal inflammation, collagen deposition, glomerulosclerosis, and albuminuria with MR blockade in another model of type 2 diabetes, the Otsuka Long-Evans Tokushima fatty rat (34). Thus, blockade of the RAA system at the level of MR reduces renal injury in diabetes. This beneficial effect of MR blockade occurred in diabetic animals treated with eplerenone despite an increase in serum aldosterone and presumably up-regulation of the RAA system and higher angiotensin II levels.

The effects of MR blockade in reducing inflammation, fibrosis, and albuminuria in diabetic rodents are consistent with studies in nondiabetic hypertensive rodents in which MR antagonists are shown to reduce proteinuria and inflammation and injury of renal vessels (35–37). In these rodent models, MR blockade has widespread cardiovascular benefits, reducing vascular injury and inflammation in heart, brain, and kidney as well as decreasing end organ damage (*e.g.* myocardial necrosis, cardiac fibrosis, stroke, and renal injury) (38). Similarly, in diabetic rats, MR blockade reduced both renal and cardiac fibrosis (33). The renoprotective effects of MR blockade in diabetic rodents are consistent with clinical studies showing a decrease in albuminuria with MR

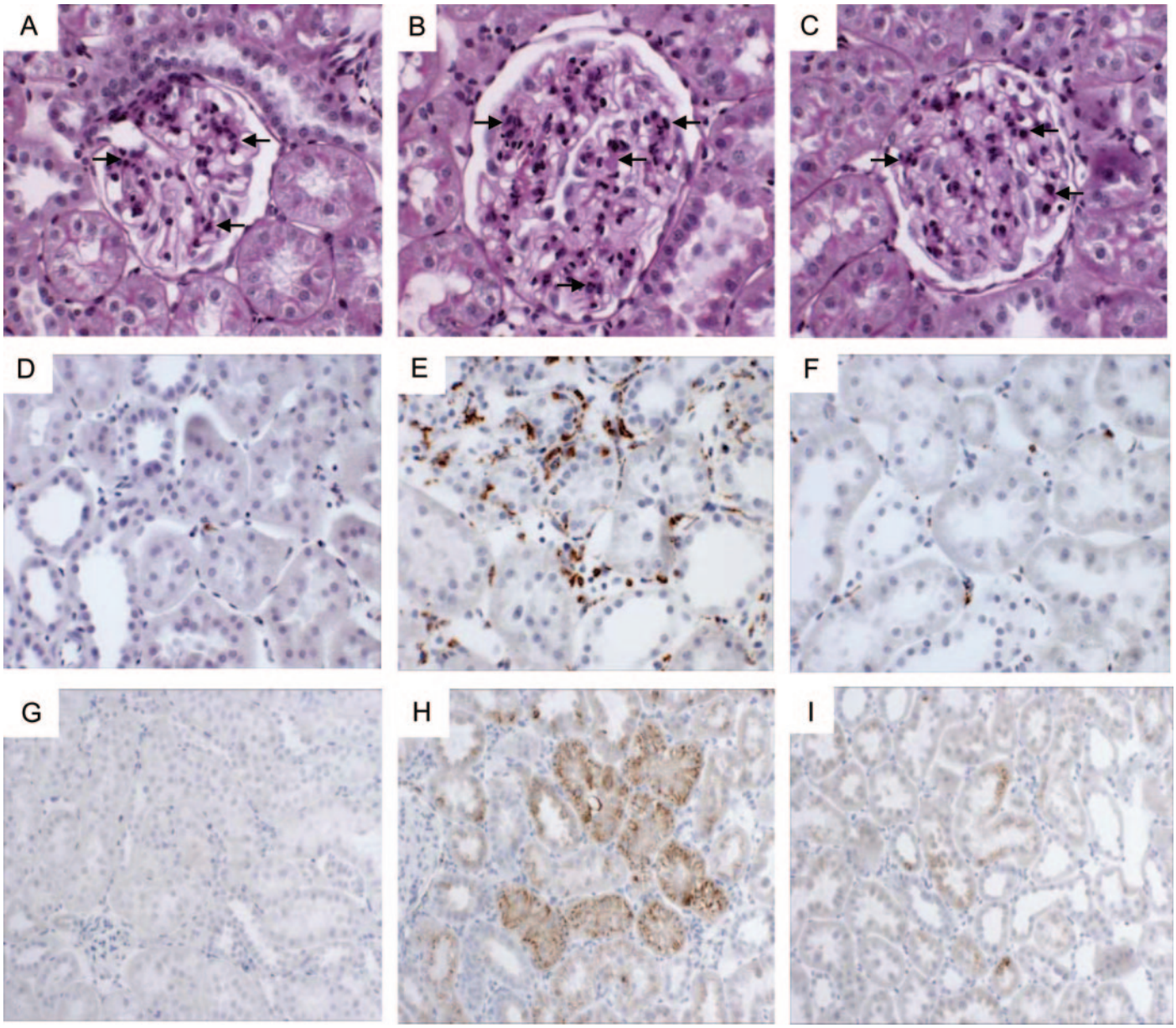

FIG. 3. Renal histopathology and immunohistochemical staining for osteopontin and ED-1 in representative kidney sections from nondiabetic (A, D, and G), streptozotocin-treated (B, E, and H), and streptozotocin/eplerenone-treated (C, F, and I) rats. A–C, PAS staining (×40). Streptozotocin-treated rats (B) have renal damage characterized by glomerular hypertrophy, mild expansion of the mesangial matrix, and presence of mononuclear cell elements in several mesangial areas, compared with diabetic rats receiving eplerenone (C) and nondiabetic rats (A). *Arrows* indicate regions of mesangium with matrix elements and mononuclear cells for the specific group. D–F, Immunostaining for osteopontin with hematoxylin counterstaining of nuclei (×20). There is prominent staining (*brown*) for osteopontin in tubular epithelial cells from streptozotocin-treated rats as compared with nondiabetic and eplerenone/streptozotocin-treated rats. G–I, Immunostaining for ED-1 with hematoxylin counterstaining of nuclei (×40). The tubulointerstitial area of streptozotocin-treated rats presents many mononuclear cells with positive staining (*brown*) for ED-1 as compared with nondiabetic and eplerenone/streptozotocin-treated rats.

antagonists in individuals with hypertension (39–41) and studies showing a beneficial effect of spironolactone on albuminuria in patients with type 2 diabetes, most of whom were receiving ACE inhibitor or ARB therapy (9–11).

Tubulointerstitial disease is a key histological correlate of impaired renal function and a predictor of long-term renal function in diabetes (42, 43). Osteopontin, along with other chemokines, is postulated to play a role in tubulointerstitial disease by stimulating macrophage/monocyte infiltration (44, 45). Osteopontin mRNA and protein levels are increased in tubules of streptozotocin-treated rats (29, 46–48), and most, but not all, studies show a reduction in tubulointerstitial damage and osteopontin expression with ACE inhibition (46–48). In the current studies, there was increased immunostaining for osteopontin in tubules and infiltration of macrophages/monocytes in tubulointerstitium of diabetic rats, compared with nondiabetic rats. Furthermore, osteopontin and CD68 mRNA levels were increased in the

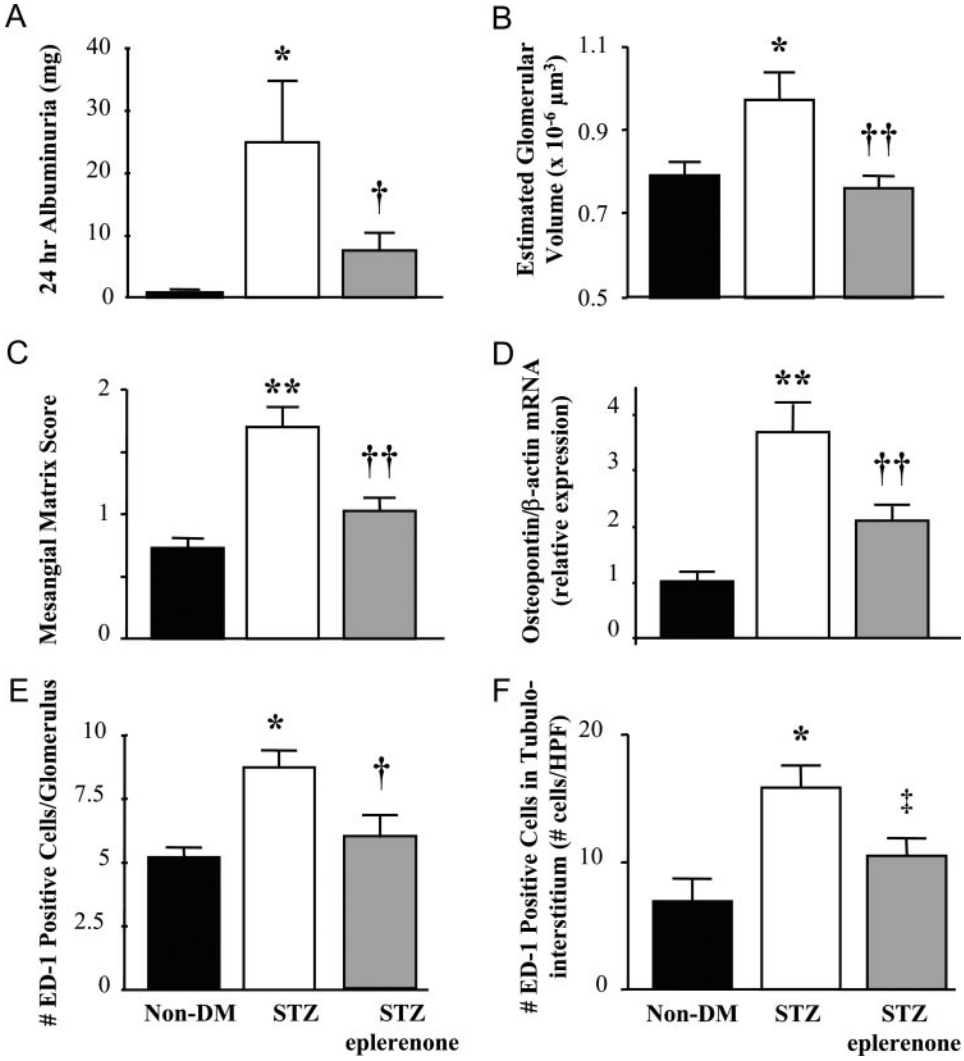

FIG. 4. Twenty-four-hour albuminuria (A), estimated GV (B), MMS (C), osteopontin mRNA levels (D), the number of ED-1-positive cells per glomerulus (E), and number of ED-1-positive cells per high-power field (HPF) in the tubulointerstitium (F) measured on d 28 in nondiabetic (non-DM) rats (*black bars*), streptozotocin-treated (STZ) rats (*open bars*), and streptozotocin-treated rats receiving eplerenone (*gray bars*). *, $P < 0.05$; and **, $P < 0.01$ *vs.* non-DM group; †, $P < 0.05$; ††, $P < 0.01$; and ‡, $P = 0.076$ *vs.* streptozotocin group (n = 9–15 animals/group). Data are mean ± SE.

renal cortex of diabetic *db/db* mice, compared with nondiabetic mice. MR blockade reduced osteopontin expression and these measures of renal inflammation, consistent with close association between osteopontin and macrophage infiltration (46–48). MR blockade may reduce osteopontin expression through indirect mechanisms. However, a recent report in cultured rat aortic endothelial cells suggests that aldosterone directly stimulates osteopontin transcription (49). Thus, diabetes leads to an increase in chemokine expression in the renal cortex of diabetic rodents that can be prevented by blockade of the RAA system at the level of the MR.

MR expression was increased in kidneys of diabetic streptozotocin-treated rats and diabetic *db/db* mice. Thus, it is possible that increased intrarenal expression of MR may be one mechanism contributing to increased MR activity in diabetic kidneys. Of interest, a recent study also showed increased MR mRNA levels in kidney biopsies from patients with chronic renal failure and heavy albuminuria, most of whom did not have diabetes (50).

In these studies, the streptozotocin-treated rats received daily, individualized doses of sc insulin to maintain blood sugar levels at target levels and gained weight over the study. Daily blood glucose monitoring demonstrated similar glycemic control in streptozotocin-treated animals irrespective of eplerenone treatment, suggesting that the decrease in renal injury with eplerenone treatment was not mediated by changes in glycemia. We do not have an overall assessment of glycemic control in the *db/db* study, but plasma glucose levels measured at the end of the study were not altered by eplerenone treatment. Eplerenone treatment caused a slight insignificant decrease in systolic blood pressure in the streptozotocin-treated rats and no change in the *db/db* mice. These results are consistent with previous hypertensive, nondiabetic studies showing a beneficial effect of MR blockade on cardiac and renal injury without a significant change in systolic blood pressure (35–37). However, it is possible that, as described for ACE inhibitors (24), MR antagonists reduce glomerular capillary hypertension. Aldosterone acting via the MR inhibits depolarization-induced vasoconstriction in microdissected, perfused rabbit renal afferent arterioles (51), and in conscious dogs, an aldosterone infusion increases renal blood flow and glomerular filtration rate by 15 and 20%, respectively (52). Furthermore, studies of cultured rat mesangial cells show that MR activation stimulates ERK 1/2 activity and cellular proliferation (53), suggesting that aldo-

TABLE 3. Metabolic measures in the *db/db* study

	db/+ (n = 11)	*db/db* (n = 10)	*db/db* + eplerenone (n = 8)
Systolic blood pressure (mm Hg)			
25 wk	111 ± 4	121 ± 5	126 ± 2
Body weight (g)			
8 wk	24 ± 0.4	37 ± 0.6[a]	36 ± 0.8[a]
25 wk	32 ± 0.5	56 ± 2.6[a]	55 ± 2.0[a]
Kidney weight (KW)			
Average KW (mg)	223 ± 6	239 ± 9	221 ± 5
KW to body weight (mg/g)	7.0 ± 0.3	4.2 ± 0.2[a]	4.1 ± 0.1[a]
Plasma measurements[b]			
Glucose (mg/dl)	159 ± 21	769 ± 41[a]	680 ± 70[a]
Aldosterone (ng/dl)	36 ± 6.3	43 ± 5.7	133 ± 12[a,c]
PRA (ng/ml·h)	13 ± 3.7	11 ± 2.2	21 ± 1.9[d,e]
Creatinine (mg/dl)	0.20 ± 0.01	0.23 ± 0.02	0.25 ± 0.02
MR mRNA levels in renal cortex[f]	1.0 ± 0.4	2.7 ± 0.5[a]	2.1 ± 0.4

Data given as mean ± SE.
[a] $P < 0.01$ *vs. db/+*.
[b] n = 6–10/group.
[c] $P < 0.01$ *vs. db/db*.
[d] $P < 0.05$ *vs. db/+*.
[e] $P < 0.05$ *vs. db/db*.
[f] n = 8/group.

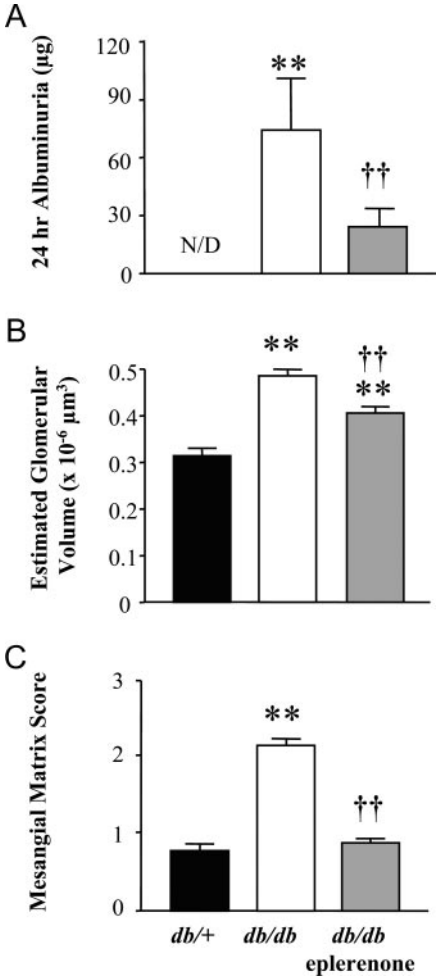

FIG. 5. Twenty-four-hour albuminuria (A), GV (B), and MMS (C) at 25 wk of age in nondiabetic *db/+* mice (*black bars*, n = 11), *db/db* mice (*open bars*, n = 10), and eplerenone-treated *db/db* mice (*gray bars*, n = 8). N/D, Urine albumin concentration assay limit of 5 µg/ml or less in all samples. **, $P < 0.01$ *vs. db/+* control mice; ††, $P < 0.01$ *vs. db/db* diabetic mice. Data are mean ± SE.

sterone may have direct effects on mesangial cells that contribute to glomerular injury.

There are several factors that should be considered when interpreting these studies. The current studies used a preventive model, *i.e.* eplerenone was initiated either at the start or early in the course of the development of the disease. Human studies have all involved a reversal model, *i.e.* damage is present before initiating MR antagonist therapy. Thus, whereas our results are consistent with what is likely occurring in humans, direct extrapolation should be performed cautiously. Second, in the streptozotocin study, all of the animals had a reduced renal mass due to uninephrectomy. Uninephrectomy accelerates renal injury in this diabetic model, and it is possible that it plays a role in the beneficial renal effects of MR blockade. However, the ability of eplerenone to reduce renal injury in the diabetic *db/db* mice with intact kidneys suggests that there is a general ability of MR blockade to reduce diabetic nephropathy. Third, whereas the streptozotocin-treated animal had daily blood glucoses controlled with insulin, glucose levels were not monitored or controlled in the *db/db* mice. At the end of the study, the *db/db* animals had higher blood glucose levels than the rats. It is known that over time *db/db* mice develop insulin deficiency and therefore in our study may have become a combined type 1/type 2 model of diabetes. However, this same scenario applies to many patients with type 2 diabetes. Fourth, even though glucose levels were controlled in streptozotocin-treated rats, they were still very elevated. Thus, in neither animal model can we determine the relative importance on renal damage of the increased glucose (glucose toxicity) *vs.* metabolic derangements. However, the MR antagonist reversed the damage regardless of the underlying causes. Fifth, even though we did not find a consistent change in blood pressure with eplerenone treatment, we cannot exclude a contribution of a blood pressure reduction on eplerenone's beneficial effects, given the limits of the tail-cuff blood pressure measurements. However, none of the animals developed what one would categorize as hypertension. Sixth, because each method to assess renal injury could have been insensitive to detect an effect of diabetes or MR blockade, we examined multiple indices of renal injury that measure different aspects of injury: albuminuria, renal histopathology, and gene expression of TGFβ and osteopontin. Detailed analyses of glomerular structure by electron microscopy were not performed because tissues were not preserved in a manner that would allow for these studies. Observers unaware of treatment group performed the assessments of renal injury. Across all indices of damage, there was an increase in injury in the diabetic animals, compared with the nondiabetic animals, and this increase in injury is comparable with that observed by other investigators. For example, the increase in albuminuria to 75 µg per 24 h, the 55% increase in glomerular volume and the roughly 2-fold increase in mesangial expansion that we observed in the db/db mice are of similar magnitude to the changes

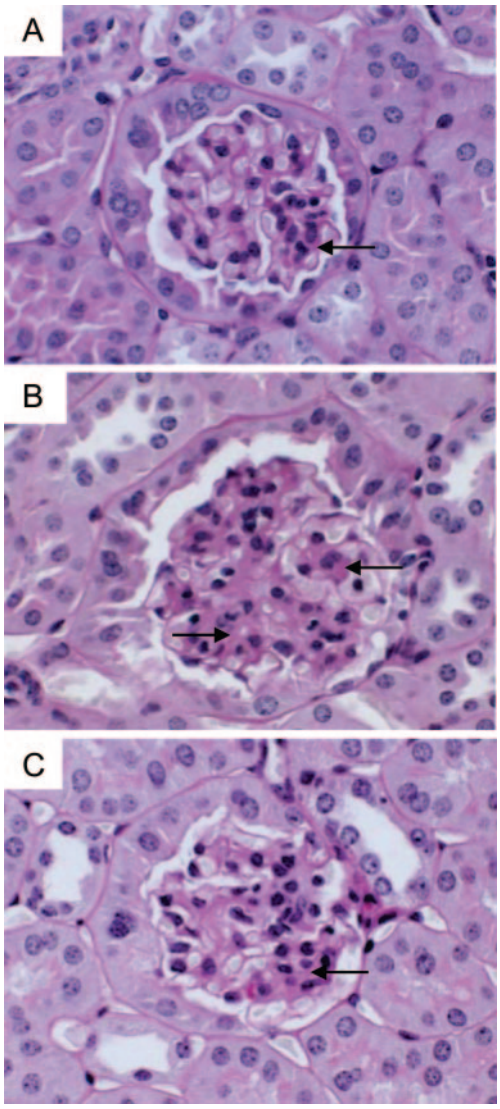

FIG. 6. Renal histopathology of *db/db* mice study. A–C, Kidney sections of *db/+*, *db/db*, and *db/db* mice treated with eplerenone (PAS; ×40). A, Photomicrograph of glomerulus from *db/+* mice showing preservation of the architecture with delicate mesangium and normal mesangial cellularity. B, Photomicrograph from *db/db* mice showing glomerular hypertrophy and a mild increase in mesangial matrix and mononuclear cells, compared with the nondiabetic *db/+* group. C, Photomicrograph of glomerulus from *db/db* mice receiving eplerenone showing a decreased glomerular size and a minor component of matrix elements and mononuclear cells in the mesangium, compared with *db/db* mice not receiving eplerenone. *Arrows* indicate regions of mesangium with matrix elements and mononuclear cells typical for the specific group.

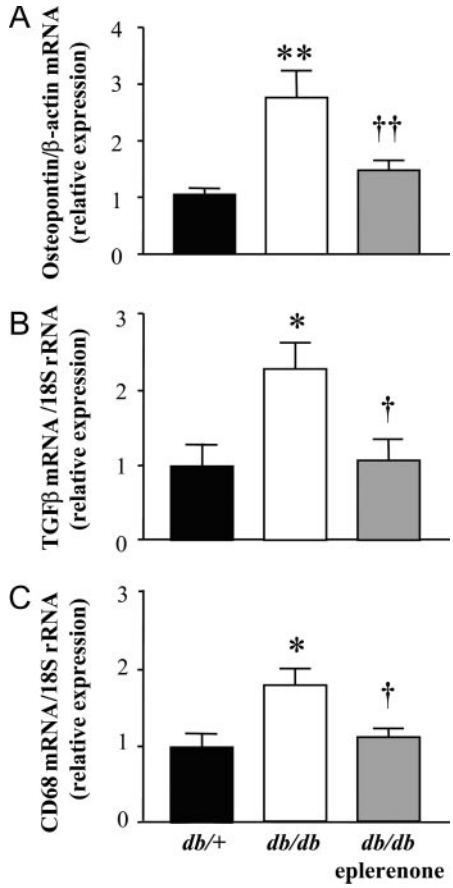

FIG. 7. mRNA expression of osteopontin (A), TGFβ (B), and CD68 (C) at 25 wk of age in nondiabetic *db/+* mice (*black bars*, n = 11), *db/db* mice (*open bars*, n = 10), and eplerenone-treated *db/db* mice (*gray bars*, n = 8). β-Actin mRNA relative to 18S rRNA was similar in all groups (nondiabetic *db/+*: 1.0 ± 0.08; *db/db*: 1.02 ± 0.04 and *db/db* with eplerenone: 1.14 ± 0.08). *, $P < 0.05$; **, $P < 0.01$ *vs.* *db/+* control mice; †, $P < 0.05$; ††, $P < 0.01$ *vs.* *db/db* diabetic mice. Data are mean ± SE.

observed by other investigators (19–21; for reviews see Refs. 30 and 31). Most importantly, MR blockade reduced all our measurements of renal injury. Thus, it is unlikely that the observed changes in a specific measurement were unrelated to the influence of diabetes and MR blockade.

In summary, MR expression was increased in the renal cortex of diabetic, streptozotocin-treated, uninephrectomized rats and diabetic *db/db* mice. In both animal models, blockade of the MR reduced albuminuria, glomerular mesangial expansion, and glomerular hypertrophy, key clinical and morphological characteristics of diabetic nephropathy. MR blockade also reduced renal inflammation and TGFβ and osteopontin expression in renal cortex of diabetic animals. The ability of the MR antagonist to reduce renal injury in a similar fashion in models of both type 1 and type 2 diabetes, and in both rats and mice, makes it unlikely that the MR antagonist is ameliorating injury that is unique to a specific model. These studies support the hypothesis that MR activation promotes renal injury in diabetes.

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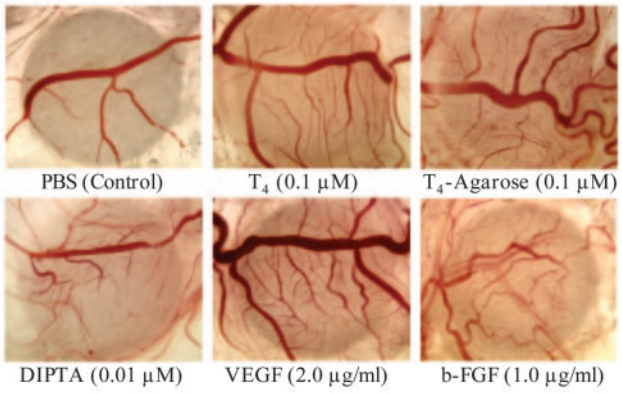
Erratum

The article titled "Proangiogenesis Action of the Thyroid Hormone Analog 3,5-Diiodothyropropionic Acid (DITPA) is Initiated at the Cell Surface and Is Integrin Mediated" by Shaker A. Mousa, Laura O'Connor, Faith B. Davis, and Paul J. Davis (*Endocrinology* **147:**1602–1607, 2006) described the proangiogenic action of the thyroid hormone analog, DITPA, in a chorioallantoic membrane (CAM) model of angiogenesis. In Fig. 2A, representative images from a study were shown, but the images were inadvertently selected by the authors from results of studies with a different thyroid hormone analog. *Below* is a corrected Fig. 2, which in part A includes representative images of the effects of PBS, T_4, T_4-agarose, DITPA, VEGF, and b-FGF. The original table in part B of the figure and the legend are correct and unchanged. *The authors sincerely regret this error.*

A



B

Treatment	Mean Vessel Branch Points ± SD
PBS (Control)	87 ± 9
T_4 (0.1 μM)	148 ± 7*
T_4-agarose (0.1 μM)	167 ± 8*
DITPA (0.01 μM)	134 ± 11*
DITPA (0.1 μM)	170 ± 9*
VEGF (2.0 μg/ml)	168 ± 10*
b-FGF (1.0 μg/ml)	174 ± 8*

Data represent mean ± SD, n=8; *p<0.01, indicating significant stimulation of angiogenesis. PBS, phosphate-buffered saline.