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Local renal aldosterone production induces inflammation and matrix formation in kidneys of diabetic rats

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Abstract

Recently, we reported the presence of a local renal aldosterone production. In the present study, we tested the hypothesis that local aldosterone production in the kidney contributes to renal inflammation, matrix formation and albuminuria associated with diabetes. We evaluated changes in renal aldosterone content (RAC), aldosterone synthase expression, nuclear factor κB (NFκB), tumour necrosis factor α (TNFα), interleukin-6 (IL-6), transforming growth factor β (TGFβ), glomerular fibronectin, collagen type IV and urinary albumin extraction (UAE) in response to the aldosterone synthase inhibitor FAD286. Studies were conducted in adrenalectomized, normoglycaemic (control) or diabetic rats for 14 weeks. The FAD286 was administered during the last 10 weeks of the study. Plasma aldosterone levels were not detectable in any of the study groups. Compared with control rats, diabetic rats had higher levels of RAC by 488% ($P < 0.01$), NFκ B by 293% ($P < 0.01$), TNFα by 356% ($P < 0.01$), IL-6 by 378% ($P < 0.01$), TGFβ by 337% ($P < 0.01$) and UAE by 1122% ($P < 0.01$), and increased glomerular fibronectin and collagen type IV immunostaining. In diabetic rats, FAD286 reduced RAC ($P < 0.01$), UAE ($P < 0.05$), NFκ B mRNA, TNFα mRNA, IL-6 mRNA and TGFβ mRNA by 51, 41, 41 and 52% and also their proteins and decreased glomerular fibronectin and collagen type IV immunostaining. In conclusion, diabetes increases local aldosterone production in the kidney, which contributes to development of renal inflammation, matrix formation and albuminuria. Inhibition of aldosterone production in the kidney could be helpful in management of diabetic nephropathy.

Diabetes is a major contributing factor to development of end-stage renal disease and to increased cardiovascular morbidity and mortality (Schiffrin *et al.* 2007). Diabetic nephropathy is associated with increased urinary albumin excretion (UAE) and progressive accumulation of extracellular matrix (Mauer *et al.* 1984; Wolf & Ziyadeh, 2007). Despite the use of angiotensin-converting enzyme inhibitors and angiotensin receptor blockers, the progression of diabetic nephropathy continues, leading to development of end-stage renal disease (Nakao *et al.* 2003).

Growing evidence suggests that aldosterone contributes to development of diabetic complications in the kidney (Miric *et al.* 2001; Sato *et al.* 2003; Fujisawa *et al.* 2004). The mechanisms by which aldosterone contributes to the development of diabetic nephropathy are not well elucidated. Candidates which may mediate the effects of aldosterone in diabetic nephropathy include several inflammatory and growth factors. Nuclear factor κ B (NFκ B) mediates inflammatory responses by enhancing the release of tumour necrosis factor α (TNFα) and interleukin-6 (IL-6) in the kidney (Sharma & Ziyadeh, 1994; Barnes & Karin 1997; Siragy *et al.* 2003; Kalantarinia *et al.* 2003; Bacher & Schmitz, 2004; Schott-Ohly *et al.* 2004). Similarly, production of transforming growth factor β (TGFβ) is upregulated in

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diabetes, leading to an increase in synthesis of extracellular matrix (Nakamura *et al.* 1993; Young *et al.* 1995; Pfeiffer *et al.* 1996; Park *et al.* 1997; Schott-Ohly *et al.* 2004). Recently, we presented evidence for local aldosterone production in the kidney (Xue & Siragy, 2005). In the present study, we hypothesized that aldosterone production in the kidney contributes to development of diabetic nephropathy via enhancement of renal production of inflammatory and growth factors. Our data suggest that in presence of diabetes, reduction of the local renal aldosterone production with the aldosterone synthase inhibitor FAD286 (Fiebeler *et al.* 2005) ameliorates renal inflammation, matrix formation and albuminuria.

Methods

Animal preparation

Study protocols were approved by the University of Virginia Animal Care and Use Committee. Sprague–Dawley rats (Harlan Teklad, Madison, WI, USA) weighing 245–255 g were housed in a well ventilated room (21 ± 1° C, 12 h–12 h light–dark cycle). The animals were randomly divided into a normoglycaemic control group (n = 8) and a diabetic group (n = 16). Under general anaesthesia (ketamine 60 mg kg^{-1}, i.p.), all animals underwent bilateral adrenalectomy (ADX) according to a previously published method (Kalantarinia *et al.* 2003). After adrenalectomy, rats received dexamethasone (12 μ g kg^{-1} day^{-1}, s.c.; Sigma, St Louis, MO, USA) in sesame oil. Following adrenalectomy diabetes was induced by streptozotocin (65 mg kg^{-1}, i.p.; Xue & Siragy, 2005); in the control group, the same volume of saline was injected intraperitoneally. Studies were conducted for 14 weeks. At the beginning of week 5 after development of diabetes, the aldosterone synthase inhibitor FAD286 (4 mg kg^{-1} day^{-1}, gavage; Novartis Pharmaceuticals, East Hanover, NJ, USA) or normal saline (n = 8 per group) was given to the diabetic rats for 10 weeks. Blood glucose and urinary albumin were monitored weekly throughout the study. At the end of the experiments, animals were killed by overdose of anaesthetic (Ketamine, 60 mg kg^{-1}, i.p.), and plasma and kidneys were harvested and stored at −80° C for aldosterone measurements and for renal molecular and immunostaining studies.

Blood pressure measurement

At the beginning and at the end of the experiments, the systolic blood pressure (BP) was measured three times at 10 min intervals using SC1000 BP Analysis System (Hatteras, Cary, NC, USA). The mean values of the recorded BP were calculated.

Urine albumin excretion and aldosterone assays

Urine albumin excretion was determined by enzyme-linked immunosorbent assay (Nephrat (tm) kit, Exocell, Inc., Philadelphia, PA, USA) according to manufacturer's instructions (Kalantarinia *et al.* 2003). Aldosterone assay was performed as previously described (Xue & Siragy, 2005). Briefly, each sample of plasma (50 μ l) and homogenized kidney tissue (79–94 mg) was extracted by methylene chloride (1:2 v/v). After evaporation of the methylene chloride using a vacuum centrifuge, the extract was dissolved into enzyme immunoassay (EIA; Cayman, Ann Arbour, MI, USA) buffer (1:1 v/v) and added to the assay wells (50 μ l per well). Each well was coated with aldosterone AChE Tracer (50 μ l; Cayman) and antiserum (50 μ l; Cayman) and incubated at 4°C overnight. Finally, the plate was developed by Ellman's Reagent (Cayman) and read at a wavelength 405 nm (Xue & Siragy, 2005).

Quantitative real time reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot

The procedures for mRNA measurement were performed as previously described (Xue & Siragy, 2005). The frozen kidneys were thawed and homogenized on ice. Gene-specific primers for NFκB, TGFβ1 and β-actin were designed using the Genbank. The exon–intron boundaries

were determined using the University of California Santa Cruz (UCSC, CA, USA) Genome Bioinformatics Site (http://genome.ucsc.edu). The corresponding cDNA primers were selected from *AF079314*, *X66539*, *M26744*, *AY550025*, *NM012538* and *BC063166*, the gene codes for rat NFκ B, TNFα , IL-6, TGFβ 1, aldosterone synthase (CYP11B2) and β -actin sequences, respectively. The specificity of the primers was verified by melting curves (iCycler, Bio-Rad, Hercules, CA, USA) and amplified product size using agarose gel electrophoresis. Quantitative real-time RT-PCR was performed using iCycler, and threshold cycle number was determined using iCycler software version 3.1 (Bio-Rad).

The primers were as follows. For NFκ B: forward sequence, 5′-TCTGGGCCATATGTGGAGAT-3′; reverse sequence, TGCTTCTCTCCCCAGGAATA; length 106 bp. For TNFα : forward sequence, TGCCTCAGCCTCTT-CTCATT; reverse sequence, TTGGGAACTTCTCCTCC-TTG; length 103 bp. For IL-6: forward sequence, GCCCTTCAGGAACAGCTATG; reverse sequence, TGA-AGTAGGGAAGGCAGTGG; length 101 bp. For TGFβ 1: forward sequence, TGAGTGGCTGTCTTTTGACG; reverse sequence, TGGGACTGATCCCATTGATT; length 146 bp. For CYP11B2: forward sequence, TGAGACGTG-GTGTGTTCTTGC; reverse sequence, GGCCTCCAA-GAAGTCCCTTGC; Length 126 bp. For β -actin: forward sequence, AGCCATGTACGTAGCCATCC; reverse sequence, ACCCTCATAGATGGGCACAG; length 115 bp.

The Western blot analysis was performed as previously described (Xue & Siragy, 2005). Antibodies for NFκ B (1:1000 dilution), TGFβ (1:2000 dilution), fibronectin (1:500 dilution), collagen type IV (1:500 dilution; Santa Cruz, CA, USA), TNFα (1 μ g ml^{-1}; R&D Systems, Minneapolis, MN, USA), IL-6 (1 μ g ml^{-1}; R&D Systems) or CYP11B2 (CHEMICON International, Inc., Temecula, CA, USA) were used for measurement of respective proteins. Each band density was normalized to the corresponding density of β -actin.

Immunohistochemistry

This method was performed as previously described (Xue & Siragy, 2005). The kidney sections were incubated overnight with NFκ B (1:100 dilution), TGFβ (1:500 dilution), fibronectin (1:50 dilution) or collagen IV (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies at −4° C, followed by 1 h of incubation with secondary antibody (IgG) conjugated with biotin (Sigma) at room temperature. Immunoreactive signals were detected with an avidin–biotin immunoperoxidase reaction (Sigma) and visualized by exposure to diaminobenzidine (Sigma). The non-specific binding was controlled by using the same species immunoglobulin G isotypes (Sigma) or omitting the primary antibody for negative comparison. The immunostaining was evaluated by light microscopy.

Periodic acid-Schiff (PAS) staining

The kidney sections were deparaffinized and sequentially treated with 1% periodic acid for 10 min (Sigma), Schiff's reagent for 10 min (Sigma), followed by Carazzi Haematoxylin for 2 min (Sigma) and differentiated in acid alcohol. Sections were well washed between treatments with tap water. Finally, the sections were mounted and examined by light microscopy.

Statistical analysis

Comparisons among different treatment groups were made by two-way ANOVA. Data are expressed as means ± S.E.M. Statistical significance was identified at $P < 0.05$.

Results

Plasma and renal aldosterone content, renal aldosterone synthase expression, blood glucose and urine albumin excretion in diabetic rats and response to aldosterone synthase inhibition

At the start of the study, there were no differences in body weight, BP, 24 h urine volume, blood glucose or UAE between adrenalectomized normoglycaemic (control) rats, diabetic rats and diabetic rats treated with FAD286. Throughout the study, there were no significant differences in BP between different animal groups (102 ± 2.8 mmHg for adrenalectomized control group, 102 ± 4.3 mmHg for adrenalectomized diabetic rats and 101 ± 6.1 mmHg for adrenalectomized diabetic rats treated with FAD286). In adrenalectomized normoglycaemic rats, there were no significant changes in 24 h urine volume, blood glucose or UAE rate (Fig. 1), while their body weight increased from 249 ± 6 g at baseline to 450 ± 19 g at end of week 14 of the study ($P < 0.01$). Similarly, diabetic rats and diabetic rats treated with FAD286 increased in their body weight from 247 ± 5 and 247 ± 4 g, respectively, at baseline to 436 ± 22 and 437 ± 25 g, respectively, at the end of week 14 of the study ($P < 0.01$). Plasma aldosterone levels were not detectable in any of the studied groups (Fig. 1*A*). Compared with the adrenalectomized normoglycaemic control group, adrenalectomized diabetic rats had higher 24 h urine volume (105 ± 11 *versus* 20 ± 4 ml, $P < 0.01$), renal aldosterone content (Fig. 1*B*), aldosterone synthase (CYP11B2) mRNA and protein expression (Fig. 1*C*), blood glucose (Fig. 1*D*) and UAE (Fig. 1*E*). In adrenalectomized diabetic rats, FAD286 treatment did not influence blood glucose (Fig. 1*D*) but increased urine volume to 123 ± 12.1 ml ($P < 0.01$) and caused significant reduction in renal aldosterone content (Fig. 1*B*) and UAE (Fig. 1*E*). The FAD286 caused a slight but not significant increase in renal aldosterone synthase mRNA and protein expression compared to adrenalectomized diabetic rats (Fig. 1*C*).

Renal expression of NF*κ* B, TNFα , IL-6, TGF*β* , fibronectin and collagen type IV in diabetes and in response to aldosterone synthase inhibition

Compared with the adrenalectomized normoglycaemic control group, the diabetic rats had increased renal mRNA expression of NF*κ* B by 293% (Fig. 2*A*), TNF*α* by 356% (Fig. 2*B*), IL-6 by 378% (Fig. 2*C*) and TGF*β* by 337% (Fig. 2*D*) and also their respective proteins. Immunostaining for TGF*β* , fibronectin and collagen type IV (Fig. 3) was absent in kidney sections when immunoglobulin G pre-immune serum was used as a control (Fig. 3*A*, *E*, *I* and *M* respectively). In the adrenalectomized normoglycaemic control group, renal immunostaining of TGF*β* , fibronectin and collagen type IV is minimal in glomeruli and tubules, respectively (Fig. 3*B*, *F*, *J* and *N*). Compared with the control group, kidney immunostaining of TGF*β* in glomeruli and tubules (Fig. 3*D* and *G*), and fibronectin (Fig. 3*K*) and collagen type IV (Fig. 3*O*), and PAS staining (Fig. 3*R*) in glomeruli are enhanced in adrenalectomized diabetic rats. Treatment with FAD286 reduced renal mRNA expression of NF*κ* B, TNF*α* , IL-6 and TGF*β* by 51, 41, 41 and 52%, respectively, and also their corresponding proteins (Fig. 2). The FAD286 also reduced renal TGF*β* (Fig. 3*D* and *H*), fibronectin (Fig. 3*K*), collagen type IV (Fig. 3*P*) and PAS staining (Fig. 3*S*).

Discussion

Aldosterone is known to play a role in the development of cardiovascular and renal diseases. However, the mechanisms by which aldosterone contributes to the pathophysiology of these diseases are still unclear. In this study, we hypothesized that diabetes increases local aldosterone production in the kidney, which contributes to the development of diabetic nephropathy via stimulation of renal inflammation and matrix formation. Our study demonstrates that diabetes increases local renal aldosterone synthase expression and aldosterone production. These results confirm and extend our recent report (Xue & Siragy,

2005) demonstrating the presence of a local renal aldosterone production that is upregulated by hyperglycaemia. Aldosterone receptors are present in the kidney (Nishiyama *et al.* 2005), and the presence of local aldosterone production is confirmed in our study by the presence of aldosterone synthase and detectable aldosterone levels in the kidneys 14 weeks post-adrenalectomy, despite the complete absence of detectable levels of aldosterone in the circulation. The production of this hormone in close proximity to its receptors at target sites of action supports the concept of the presence of a local renal aldosterone paracrine system.

The observed increase in renal aldosterone production in diabetes is most likely to be related to increased intrarenal angiotensin II production (Siragy *et al.* (2003), which is supported by the fact that blockade of the angiotensin subtype 1 (AT_1) receptor decreases aldosterone synthase expression and aldosterone levels in the kidney (Xue & Siragy, 2005). In the present study, concomitant with the increase in aldosterone production, there was an increase in expression of NFκ B, TNFα , IL-1, TGFβ and fibronectin, and fibrosis in the kidneys of the diabetic animals. These results suggest that renal inflammation and matrix formation contribute to the development of diabetic kidney disease as evidenced by the presence of albuminuria. Previously, we demonstrated increased renal inflammation in diabetes (Kalantarinia *et al.* 2003; Siragy *et al.* 2003), a process that precedes development of albuminuria (Kalantarinia *et al.* 2003). Inhibition of renal aldosterone synthase with FAD286 in diabetic animals reduced renal aldosterone tissue content, NFB, TNFα , IL-6, TGFβ , fibronectin, collagen type IV and albuminuria despite a lack of influence on blood glucose. These results suggest a direct contribution of aldosterone synthase to renal inflammation and albuminuria independent of changes in blood glucose. Similarly, an absence of reduction in BP during FAD286 treatment confirms the direct renoprotective effects of aldosterone synthase inhibition. In addition, our study shows that inhibition of aldosterone synthase minimizes the development of albuminuria in diabetic rats. These results are consistent with previous reports showing that the development of proteinuria (Abbate *et al.* 1998) and increased matrix formation (Wolf *et al.* 1992) in diabetes is related to enhanced stimulation of TGFβ (Kagami *et al.* 1994).

Previous studies demonstrated that blockade of aldosterone receptors leads to improvement of renal function in diabetic patients (Sato *et al.* 2003). However, conflicting reports exist concerning circulating aldosterone levels in diabetes (Beretta-Piccoli *et al.* 1983; Cronin *et al.* 1995; Perez *et al.* 1997; Luik *et al.* 2003), ranging from normal to low levels. In the present study, depletion of circulating aldosterone by adrenalectomy in diabetic animals did not abolish renal aldosterone or totally prevent the development of renal inflammation, matrix formation or albuminuria, indicating the importance of the locally produced aldosterone in the kidney in development of diabetic nephropathy.

Besides the adrenal glands, biosynthesis of aldosterone has been reported in blood vessels and brain, although the pathophysiological relevance of these findings is still not well elucidated (Young *et al.* 1995; Takeda *et al.* 1997; Silvestre *et al.* 1998; Ahmad *et al.* 2004; Gomez-Sanchez *et al.* 1997, 2005). Our findings are consistent with a recent report that FAD286 (Fiebeler *et al.* 2005) decreases albuminuria in a human renin and angiotensinogen double transgenic rat model. The major difference between this report (Fiebeler *et al.* 2005) and our present study is that in the former study animals developed albuminuria secondary to severe hypertension, while in our study there were no significant differences in BP levels between adrenalectomized normoglycaemic control, adrenalectomized diabetic and FAD286-treated adrenalectomized diabetic animals. Absence of elevated BP did not prevent development of albuminuria in the diabetic rats. It is possible that the mechanisms contributing to development of albuminuria in diabetes are different from those in hypertension. Furthermore, absence of circulating aldosterone in the present study did not prevent development of proteinuria and provides evidence for the importance of local renal aldosterone production in the development of diabetic nephropathy.

In conclusion, there is a local aldosterone system in the kidney and its activity is increased in diabetes. Increased renal production of aldosterone contributes to development of renal inflammation, matrix formation and albuminuria. Inhibition of aldosterone synthase is a potential therapeutic tool in management of diabetic kidney disease via reducing renal inflammation and matrix formation.

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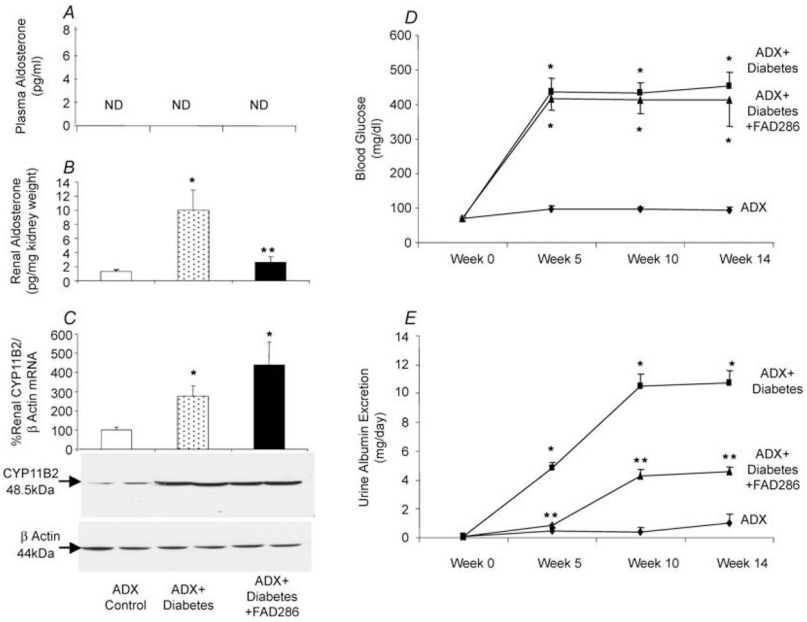

Figure 1. Plasma aldosterone (*A*), renal aldosterone (*B*), representative renal aldosterone synthase (CYP11B2) mRNA (*C*, upper panel) and protein (*C*, lower panel), blood glucose (*D*) and urinary albumin excretion (*E*) in adrenalectomized (ADX) normoglycaemic (ADX control), adrenalectomized diabetic (ADX + Diabetes) and adrenalectomized diabetic rats treated with the aldosterone synthase inhibitor FAD286 (ADX + Diabetes + FAD286)

$^{*}P < 0.01$ from control; $^{**}P < 0.01$ from control or ADX + Diabetes. $n = 8$ each group.

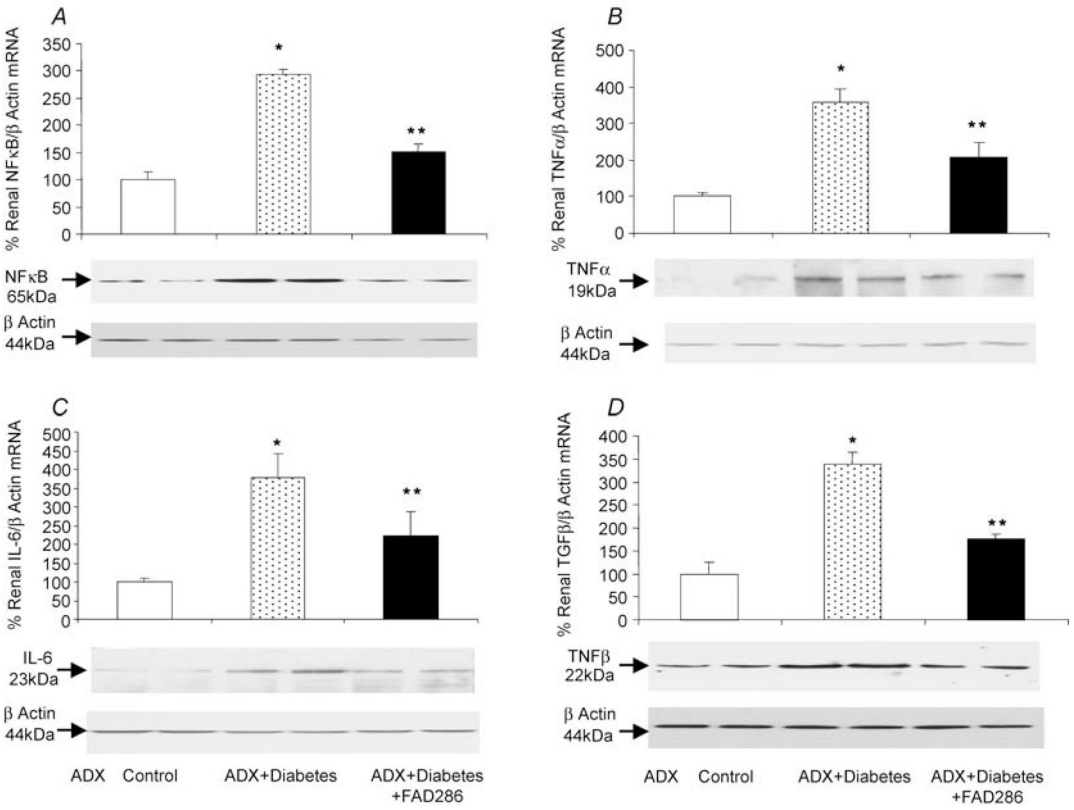

Figure 2. Representative renal NFκB (*A*), TNFα (*B*), IL-6 (*C*) and TGFβ (*D*) mRNA (upper panels) and their protein expression (lower panels) in adrenalectomized normoglycaemic (ADX control), adrenalectomized diabetic (ADX + Diabetes) and adrenalectomized diabetic rats treated with FAD286 (ADX + Diabetes + FAD286)

$^{*}P < 0.001$ from control; $^{**}P < 0.05$ from control or ADX + Diabetes. $n = 8$ each group.

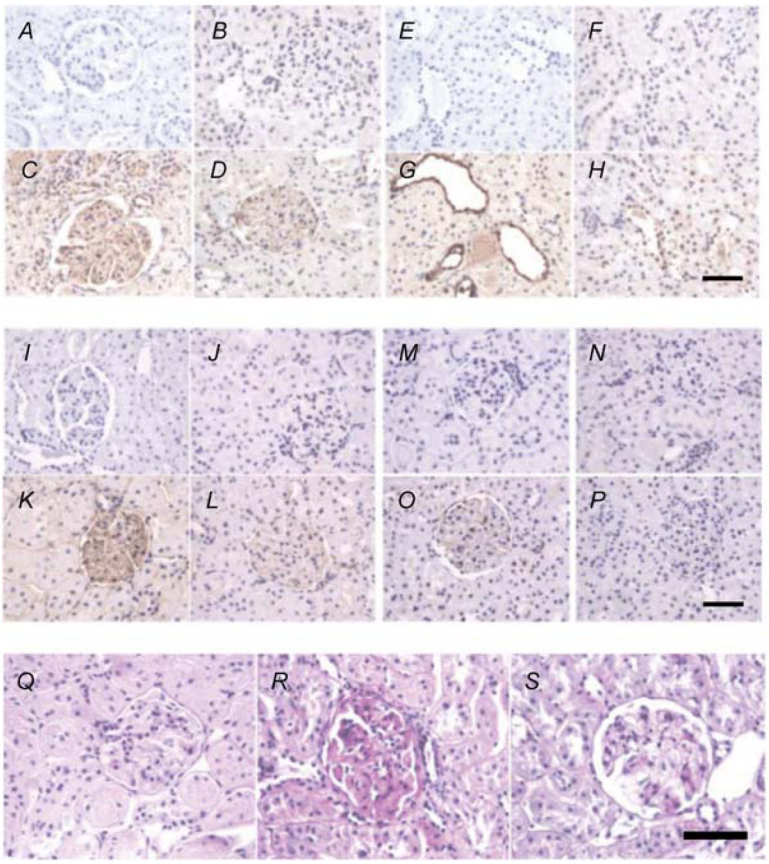

Figure 3. Rat kidney immunostaining of glomeruli and tubules in adrenalectomized normoglycaemic, adrenalectomized diabetic adrenalectomized diabetic rats treated with FAD286

Immunoglobulin G immunostaining was used as negative control for TGFβ (*A* and *E*), fibronectin (*I*) and collagen type IV (*M*) in normoglycaemic adrenalectomized rats. Renal immunostaining (brown) in normoglycaemic adrenalectomized rats is presented for TGFβ (*B* and *F*), fibronectin (*J*), collagen type IV (*N*) and PAS (*Q*). Renal immunostaining (brown) is increased in diabetic adrenalectomized rats for TGFβ (*C* and *G*), fibronectin (*K*), collagen type IV (*O*) and PAS (red staining, *R*). Treatment of adrenalectomized diabetic rats with the aldosterone synthase inhibitor FAD286 reduced renal immunostaining (brown) for TGFβ (*D* and *H*), fibronectin (*L*), collagen type IV (*P*) and PAS (red staining, *S*). Scale bars represent 50 μm.