Adenosine A$_{2A}$ receptor activation attenuates inflammation and injury in diabetic nephropathy

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Dystrophy mellitus is the most common cause of end-stage renal disease (ESRD), responsible for more than 40% of all cases in the United States (1, 52), and this number is likely to continue to increase unabated. Current therapy, including blood pressure and glucose control and treatment with blockers of the renin-angiotensin system, has been modestly successful in delaying the progression of renal failure. More robust outcomes might be achieved through interventions that reverse pathophysiological changes caused by diabetic nephropathy.

Inflammation in the genesis of diabetes as well as its complications has attracted recent interest. Evidence suggests that monocytes/macrophages and their adherence to endothelial cells contribute to the pathogenesis of diabetic nephropathy (19, 54). The subsequent immune response leads to fibrosis and matrix deposition and progressive renal insufficiency. Increased kidney macrophages, mainly in the glomeruli and interstitium, and kidney expression of monocyte chemoattractant protein (MCP-1) were correlated with the duration and severity of renal injury in diabetes (13). Infiltrated macrophages release lysosomal enzymes, nitric oxide, reactive oxygen species (ROS), transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), and interleukin-1 (IL-1) (14, 34, 41) that may play a pivotal role in the development and progression of diabetic nephropathy. These data support the role of macrophages and potentially other bone marrow-derived cells in the genesis and maintenance of inflammation, increased ROS, and endothelial dysfunction in the pathogenesis of diabetic complications.

Adenosine has many diverse functions, all of which are dependent on its interaction with the receptor subtypes: A$_1$, A$_{2A}$, A$_{2B}$, and A$_3$ (28, 39). Activation of adenosine A$_{2A}$ receptors (A$_{2ARs}$) has potent anti-inflammatory effects (10, 37, 49, 50). A$_{2AR}$s are found in the glomeruli (53) as well as in monocyte/macrophages, neutrophils, T cells, and other bone marrow-derived cells (20) that are poised, on activation, to abrogate the immune response. Recently, highly selective A$_{2A}$ agonists have demonstrated a high degree of potency in blocking inflammation primarily by activating bone marrow-derived cells (15). Therefore, we tested the hypothesis that A$_{2A}$ agonists administered chronically, in a dose that does not produce abrogation of the immune response. Recently, highly selective A$_{2A}$ agonists administered chronically, in a dose that does not produce systemic hemodynamic effects, attenuate inflammation and renal injury associated with diabetic nephropathy. Our results demonstrate that early administration of A$_{2A}$ agonists markedly reduces macrophage infiltration, inflammation, functional and histological changes associated with diabetic nephropathy. We believe that A$_{2A}$ agonists represent a new class of compounds in the prevention and treatment of diabetic complications.

METHODS

Induction of diabetes. Experiments were conducted in 14-wk-old conscious Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 245–255 g and approved by the University of Virginia Animal Research Committee. Additional experiments were conducted in 7- to 8-wk-old C57BL/6 mice (wild-type (WT);
Charles River Laboratories] and mice that lack A2AR (A2A-KO; C57BL/6 background) weighing ~20 g (11). After an overnight fast, under brief vaporized halothane anesthesia (Halothan Vapor 19.1), animals were given a single intravenous injection via tail vein of vehicle or streptozotocin (STZ; Sigma, St. Louis, MO; rats 50 mg/kg body wt; mice 100 mg/kg body wt dissolved in lactic Ringer solution). After STZ injection, a 5% dextrose solution was administered subcutaneously (rat 5 ml; mice 2 ml). Establishment of diabetes was confirmed at 48 h after STZ induction and at weekly intervals by measuring fasting (8 h) blood glucose levels >250 mg/dl (Accu-Chek glucometer, Boehringer Mannheim, Indianapolis, IN).

**Drug delivery.** Rats were anesthetized with ketamine (80 mg/kg ip) and xylazine (8 mg/kg ip). Osmotic minipumps (model 2002; ALZA, Palo Alto, CA) containing vehicle or ATL146e (10 ng·kg⁻¹·min⁻¹) 3 days before (ATL146e − 3D) or 7 days after (ATL146e +7D) induction of diabetes (prepared in PBS containing <0.01% DMSO) were inserted subcutaneously. In another experiments, osmotic minipumps containing ATL313 (1 ng·kg⁻¹·min⁻¹) were inserted subcutaneously in mice or rats 3 days before STZ induction of diabetes (15). These doses of A2A agonists do not have systemic hemodynamic effect (38). After recovery from surgery, rats and mice were housed in individual cages under standard controlled conditions.

**Study protocol.** In this study, we used two protocols for rats. In the first protocol, rats (n = 28) were placed in metabolic cages. Twenty-four-hour urinary collection for albumin excretion (UAE) rate was obtained at baseline. Rats were then randomly divided into a control group (n = 8) and diabetes groups (n = 20). The diabetes group was randomly divided into vehicle (n = 8), ATL146e −3D (n = 8), or ATL146e +7D (n = 4) groups. Twenty-four-hour urine collections were obtained at 2, 4, and 6 wk in all groups. At the end of the study, animals were anesthetized and tissue and plasma were collected and animals were euthanized.

In the second protocol, rats (n = 12) were randomly divided into a control group, diabetes group treated with vehicle, and diabetes group treated with ATL313 (n = 4 each group). Twenty-four-hour urine collections were obtained at 6 wk in all groups for measurement of UAE.

To determine the specificity of A2A agonist effect, WT and A2A-KO mice were randomly divided into control, diabetes, or diabetes + ATL313 groups (n = 4 each group) and 24-h urine collections were obtained at 4 wk in all groups for measurement of UAE.

**Blood pressure measurement.** Systolic blood pressure (SBP) was measured via the tail-cuff method as described previously (15) (IITC model 179, IITC/Life Science Instruments, Woodland Hills, CA). Rats or mice were allowed to rest quietly for 10 min at 26°C. All measurements were performed at the same time for all groups to prevent any diurnal variations and were measured twice and then averaged.

**Histochernistry and immunohistochemistry.** Kidneys from rats were fixed in 4% paraformaldehyde and embedded in paraffin, and 5-μm sections were cut. Sections were stained with Gomori’s trichrome reagents. Sections were examined (WKB) in a masked fashion under X400 magnification by light microscopy (Zeiss AxioSkop). The measured parameters were 1) interstitial fibrosis, 2) size of the mesangium, and 3) the tubular basement membrane thickness. Assessment of fibrosis was based on the intensity of trichrome staining as well as change from normal morphology, compared with normal age-matched control rat tissue. A semiquantitative score (0–4+) was assigned based on the masked reading, as previously described (12).

Immunohistochemistry for macrophages was performed in rats using mouse anti-rat ED1+ monoclonal antibody (Serotec, Oxford, UK) on paraffin sections as described previously (23, 35). Sections were incubated with primary antibody (1 μg/ml) followed by a biotinylated goat IgG anti-rat (Vector Laboratories, Burlingame, CA) secondary antibody. A peroxidase reaction was performed according to the manufacturer’s protocol. Sections were viewed using a Zeiss Axioskop microscope, and digital images were taken using a SPOT RT Camera (software version 3.3; Diagnostic Instruments, Sterling Heights, MI). The number of macrophages was counted in eight consecutive nonoverlapping fields (interstitium) or glomerulus (glomeruli) on blinded fashion under ×400 magnification and averaged.

**Quantitative real-time PCR.** Total RNA was extracted from rat kidneys using RNeasy Mini Kit (Qiagen, Gmbh, Hilden, Germany). The quality of RNA was confirmed by size separating total RNA using 2% agarose gel with subsequent staining with ethidium bromide. Single-strand cDNA was synthesized using iScript cDNA Synthesis Kits (Bio-Rad, Hercules, CA) for two-step real-time RT-PCR. Gene-specific primers for neprhin, podocin, and fibronectin were designed using Beacon Designer Probe/Primer Design Software (Premier Biosoft International, Palo Alto, CA). The sense primers were GCAGTGGGCTAAAGTGGG, GCCGGAGACTCGAGGACC, and GGAGTGGAAGTGTGAGCGAC, and the antisense primers were GAGGTCACAGGGCTTAATAAG, GGAATCACCAGCCTT- TTG, and GTGGGTCGGGTTGGTGAATAG (Integrated DNA Technologies, Coralville, IA), respectively. The corresponding cDNA product sizes were 124, 140, and 97 bp, respectively. Amplification products were verified by melting curves and agarose gel electrophoresis. Quantitative real-time PCR was performed using MyIQ Single Color Real-Time PCR Detection System iCycler (Bio-Rad). Reactions were performed in duplicate, and threshold cycle numbers were averaged. Samples were calculated with normalization to GAPDH. Fold overexpression was calculated according to the formula 2^(-ΔΔCt), where Rt is the threshold cycle number for the reference gene observed in the test sample, Er is the threshold cycle number for the experimental gene observed in the test sample, Rr is the threshold cycle number for the reference gene observed in the control sample, and En is the threshold cycle number for the experimental gene observed in the control sample.

**Analytic methods.** UAE was measured by ELISA using Nephrat kit for rats or Albuwell M for mice (Exocell, Philadelphia, PA) as described previously (25). Plasma and urine MCP-1, TNF-α, and IFN-γ were measured by ELISA (Pharmingen, San Diego, CA) (25). Plasma creatinine concentration was determined at the end of the study using a colorimetric assay according to the manufacturer’s protocol (Sigma).

**Statistical analysis.** Comparisons between groups were examined by one-way ANOVA by using SPSS version 13.0 software for Windows (SPSS, Chicago, IL) program. Multiple comparisons of individual pairs of effect means were conducted by using the least squares methods of pooled variance. Data are expressed as means ± SE. Statistical significance was identified at P < 0.05.

**RESULTS**

Effects of A2A agonists on blood glucose, body weight, and SBP in rats. Tables 1 and 2 summarize the data for blood glucose, body weight, and SBP in rats treated with ATL146e or ATL313. Blood glucose levels increased significantly in the diabetes groups treated with vehicle or A2A agonists 48 h after STZ injection and continued to be elevated throughout the study period. Control rats gained weight as expected. The increase in body weight in vehicle and treatment groups was modest. In rats treated with ATL146e, there were no significant changes in SBP 3 wk after STZ induction of diabetes between all groups. SBP was significantly higher after 6 wk in vehicle and ATL146e +7D groups compared with control and ATL146e −3D groups. In rats treated with ATL313, there was no significant change in SBP between groups throughout the study.
Effects of A2A agonists on renal function in diabetes.

In rats treated with ATL146e, UAE rate was $135 \pm 100/23 \pm 9262$ g/24 h at baseline and increased to $2,629 \pm 272 g/24 h$ ($P < 0.0001$) at week 6 in the diabetic group treated with vehicle (Fig. 1A). ATL146e administered to diabetic rats reduced albuminuria at week 6.

**Table 1. Changes in BG, BW, and SBP in control, untreated diabetic, and diabetic rats treated with ATL146e −3D or ATL146e +7D**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetes</th>
<th>Diabetes + ATL146e −3D</th>
<th>Diabetes + ATL146e +7D</th>
</tr>
</thead>
<tbody>
<tr>
<td>BG, mg/dl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>78 ± 4</td>
<td>88 ± 4</td>
<td>90 ± 5</td>
<td>78 ± 3</td>
</tr>
<tr>
<td>Week 1</td>
<td>72 ± 3</td>
<td>385 ±18†</td>
<td>361 ±15†</td>
<td>402 ±12†</td>
</tr>
<tr>
<td>Week 6</td>
<td>81 ± 3</td>
<td>386 ±14†</td>
<td>399 ±16†</td>
<td>480 ±18†</td>
</tr>
<tr>
<td>BW, g</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>282 ± 9</td>
<td>291 ± 9</td>
<td>284 ±12†</td>
<td>269 ± 4</td>
</tr>
<tr>
<td>Week 6</td>
<td>510 ±12†</td>
<td>354 ±16*</td>
<td>370 ±14†</td>
<td>313 ±22*</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>115 ± 3</td>
<td>115 ± 2</td>
<td>113 ± 1</td>
<td>118 ± 3</td>
</tr>
<tr>
<td>Week 3</td>
<td>118 ± 3</td>
<td>117 ± 1</td>
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<tr>
<td>Week 6</td>
<td>116 ± 2</td>
<td>141 ± 2‡</td>
<td>120 ± 4</td>
<td>150 ±2‡</td>
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</table>

Data are means ± SE. *P < 0.05, †P < 0.001, ‡P < 0.0001 to baseline.

**Table 2. Changes in BG, BW, and SBP in control, untreated diabetic, and diabetic rats treated with ATL313**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetes</th>
<th>Diabetes + ATL313</th>
</tr>
</thead>
<tbody>
<tr>
<td>BG, mg/dl</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>85 ± 3</td>
<td>79 ± 4</td>
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<tr>
<td>Week 6</td>
<td>108 ± 9</td>
<td>333 ±17†</td>
<td>308 ±27†</td>
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<tr>
<td>BW, g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>271 ± 3</td>
<td>280 ± 3</td>
<td>270 ± 5</td>
</tr>
<tr>
<td>Week 6</td>
<td>442 ±16†</td>
<td>295 ±5*</td>
<td>333 ±8‡</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>138 ± 4</td>
<td>129 ± 2</td>
<td>133 ± 4</td>
</tr>
<tr>
<td>Week 6</td>
<td>130 ± 2</td>
<td>128 ± 2</td>
<td>137 ± 2</td>
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</table>

Data are means ± SE. *P < 0.05, †P < 0.0001 to baseline.

**Fig. 1. Effects of ATL146e on urinary albumin excretion (UAE) rate (UAER) and plasma creatinine in diabetic rats.** A: urine collections were obtained for measurement of UAE in rats by ELISA at baseline, 2, 4, and 6 wk of the study. B: plasma creatinine concentration was determined in rats at the end of the study (6 wk). Open bars, control rats; filled bars, diabetic rats; light gray bars, diabetic rats treated with ATL146e −3D; dark gray bars, diabetic rats treated with ATL146e +7D. Data are means ± SE. *P < 0.05, **P < 0.001, ***P < 0.0001 to control; +P < 0.05, ++P < 0.01, +++P < 0.001 to diabetes.

**Effects of A2A agonists on renal function in diabetes.** In rats treated with ATL146e, UAE rate was $135 \pm 23 \mu g/24 h$ at baseline and increased to $2,629 \pm 272 \mu g/24 h$ ($P < 0.0001$) at week 6 in the diabetic group treated with vehicle (Fig. 1A). ATL146e administered to diabetic rats reduced albuminuria at
week 6 in ATL146e −3D or ATL146e +7D groups to 614 ± 79 μg/24 h (P < 0.001 from vehicle) and 568 ± 109 μg/24 h (P < 0.001 from vehicle), respectively. At week 6, plasma creatinine was significantly higher in diabetic animals treated with vehicle (1.6 ± 0.3 mg/dl; P < 0.01) compared with the control (0.25 ± 0.02 mg/dl), ATL146e −3D (0.29 ± 0.05 mg/dl), and ATL146e +7D (0.6 ± 0.3 mg/dl) groups (Fig. 1B).

We next examined a new A2A agonist, ATL313, a compound that has similar selectivity as ATL146e at the A2AR but has a longer T1/2 life (16). As shown in Fig. 2A, UAE rate was 295 ± 61 μg/24 h in the control group and increased to 1,726 ± 334 μg/24 h (P < 0.01) at week 6 in the diabetes group treated with vehicle, effects significantly reduced by ATL313 administration to 521 ± 55 μg/24 h (P < 0.005 from vehicle).

ATL313 reduces proteinuria in diabetic nephropathy through A2ARs. To confirm the selectivity of ATL313, we used WT and A2A-KO mice treated with ATL313. Table 3 summarizes the effect of ATL313 on blood glucose, body weight, and

<table>
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<tr>
<th>BG, mg/dl</th>
<th>Control</th>
<th>Diabetes</th>
<th>Diabetes + ATL313</th>
<th>Control</th>
<th>Diabetes</th>
<th>Diabetes + ATL313</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 4</td>
<td>78 ± 3</td>
<td>340 ± 17‡</td>
<td>375 ± 12‡</td>
<td>79 ± 3</td>
<td>347 ± 41‡</td>
<td>355 ± 13‡</td>
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</tbody>
</table>

Data are means ± SE. *P < 0.01, †P < 0.001, ‡P < 0.0001 to baseline. WT, wild type; A2A-KO, A2A knockout.
SBP. ATL313 had no effect on blood glucose, body weight, or SBP compared with vehicle-treated wild-type or diabetic mice. As shown in Fig. 2B, both WT and A2A-KO mice show increased UAE in the diabetes group (34.3 ± 3 μg/24 h, P < 0.0001 and 80.4 ± 11 μg/24 h, P < 0.01, respectively) from control group (11.7 ± 1 and 25 ± 2 μg/24 h, respectively) after 4 wk. ATL313 treatment significantly decreased albuminuria in the WT mice (20.7 ± 0.8 μg/24 h, P < 0.001 to vehicle) but not the A2A-KO mice (73.5 ± 14 μg/24 h, P = not significant to vehicle). The absence of effect of ATL313 on proteinuria in A2A-KO mice is similar to that observed with ATL146e on ischemia-reperfusion injury in A2A-KO mice (15).

**Effects of ATL146e on kidney matrix deposition in diabetes.**

Diabetic nephropathy has been shown to be associated with an increase in kidney matrix deposition and fibrosis. Therefore, we investigated whether ATL146e could decrease fibronectin expression in diabetic kidney. We assessed kidney fibronectin mRNA by real-time PCR. As shown in Fig. 3, diabetes led to a 3.5-fold increase in fibronectin mRNA (P < 0.01 to control) 6 wk after diabetes, an effect attenuated by ATL146e treatment (ATL146e − 3D, 0.6-fold increase from control; P < 0.05 to vehicle and ATL146e + 7D, 0.2-fold increase from control; P < 0.05 to vehicle). Figure 4 shows the effect of ATL146e treatment on kidney histology after 6 wk of diabetes. Trechome stain of the kidneys from control (A), diabetic rats treated with vehicle (B), ATL146e − 3D (C), or ATL146e + 7D (D) at 6 wk of the study. Semiquantitative histological score is shown in Table 4. The diabetes group showed a significant increase in the interstitial fibrosis (206%), size of the mesangium (235%), and thickness of the tubular basement membrane (277%) from the control group, effects significantly attenuated by ATL146e treatment to control levels.

**Effects of ATL146e on macrophage recruitment in the kidney tissue in diabetes.** We next examined macrophage (ED1+ cells) infiltration in rat kidneys at 6 wk after induction of diabetes (Fig. 5). At 6 wk, diabetes (B) led to an increase in ED1+ cells in the glomeruli (7.6-fold; P < 0.05) and in the interstitium (3.4-fold; P < 0.01) from control (A). ATL146e treatment reduced macrophage density in the glomeruli (ATL146e − 3D, 1.9-fold from control; ATL146e + 7D, 4.4-fold from control) and the interstitium (ATL146e − 3D, 1.6-fold from control; P < 0.05 to vehicle and ATL146e + 7D, 1.5-fold from control; P < 0.01 to vehicle; C and D, respectively).

**Effects of ATL146e on inflammatory cytokines.** Increased inflammatory cytokines is a major feature of and an important predictor of diabetic nephropathy. Therefore, we further assessed the anti-inflammatory effect of adenosine 2A receptor agonist with ATL146e treatment in rats. Plasma MCP-1 and IFN-γ concentrations were not different between all groups (data not shown). In contrast, urinary MCP-1 and IFN-γ were 36 ± 4 ng/24 h and 16 ± 0.5 pg/24

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**Table 4. Histological score with trichrome stain**

<table>
<thead>
<tr>
<th></th>
<th>Tubular Basement Membrane Thickness</th>
<th>Size of the Mesangium</th>
<th>Interstitial Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.4 ± 0.4</td>
<td>1.5 ± 0.3</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>Diabetes</td>
<td>3.2 ± 0.2*</td>
<td>3.3 ± 0.1*</td>
<td>1.5 ± 0.2*</td>
</tr>
<tr>
<td>Diabetes + ATL146e − 3D</td>
<td>1.5 ± 0.2‡</td>
<td>1.5 ± 0.2‡</td>
<td>3.2 ± 0.2‡</td>
</tr>
<tr>
<td>Diabetes + ATL146e + 7D</td>
<td>2.1 ± 0.3†</td>
<td>2.1 ± 0.3†</td>
<td>3.2 ± 0.3†</td>
</tr>
</tbody>
</table>

Values represent means ± SE *P < 0.01 to control; †P < 0.05; ‡P < 0.01 to diabetes. Sections were examined (WKB) in a masked fashion under 400× magnification by light microscopy (Zeiss Axioskop). A semiquantitative score (0–4+) was assigned based on the masked reading.

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**Fig. 4. Effect of ATL146e on renal tissue fibrosis.** Representative rat kidney sections were subjected to histological staining with trichrome stain for normal control rat (A), diabetic treated with vehicle (B), diabetic rats treated with ATL146e − 3D (C), and diabetic rats treated with ATL146e + 7D (D) after 6 wk. A–D: photograph from separate animals. Magnification ×400. Semiquantitative analysis is shown in Table 4.
h at baseline and increased in diabetes group at 4 and 6 wk after STZ induction. At 6 wk, urinary MCP-1 and IFN-γ increased following STZ induction (705% of baseline; \( P < 0.001 \) and 298% of baseline; \( P < 0.001 \)), respectively. ATL146e treatment before or after induction of diabetes significantly decreased urine MCP-1 to 38% of control (\( P < 0.001 \) to diabetes) and 50% of control (\( P < 0.001 \) to diabetes), respectively, and IFN-γ to 118% of control (\( P < 0.01 \) to diabetes) and 130% of control (\( P < 0.05 \) to diabetes), respectively, 6 wk after diabetes. There was a significant correlation between urinary MCP-1 and IFN-γ with UAE (\( r = 0.82 \)) and (\( r = 0.6 \)), respectively, between all groups at 6 wk of the study (Fig. 6, A and B). Similarly, urinary TNF-α excretion was 54.6 ± 8.5 pg/24 h at baseline and increased to 1,586% of baseline (\( P < 0.01 \) in the diabetes group at 6 wk after diabetes. ATL146e administration before or after diabetes significantly decreased the increase in TNF-α excretion to 312% of control (\( P < 0.05 \) to diabetes) and 352% of control (\( P < 0.05 \) to diabetes), respectively (Fig. 6C). There was a significant correlation between urinary TNF-α excretion and UAE (\( r = 0.76 \)) at 6 wk of the study. Plasma TNF-α was not detectable in all groups at any point of the study periods (data not shown).

Effect of ATL146e on the expression of podocyte slit diaphragm-associated molecules podocin and nephrin. As shown in Fig. 7, diabetes was associated with a significant reduction in podocin and nephrin mRNA expression compared with control rats (podocin: 56% decrease from control, \( P < 0.005 \); nephrin: 36% decrease from control, \( P < 0.05 \)). ATL146e administration before or after induction of diabetes restored podocin and nephrin mRNA expression to control levels.

DISCUSSION

The present study demonstrates that 6 wk after induction of diabetes in Sprague-Dawley rats, marked albuminuria associated with reduction of podocyte specific gene mRNAs, reduction of renal function, and early histological changes of diabetic nephropathy are established. Furthermore, these changes were associated with evidence of inflammation. Macrophage infiltration and urinary excretion of TNF-α, IFN-γ, and MCP-1 were increased in diabetic animals as well as kidney fibronectin mRNA. Administration of A₂A agonists led to a marked reversal of albuminuria along with restoration of podocin and nephrin transcripts, histological changes, plasma creatinine, and urinary proinflammatory cytokines. Given the known effect of A₂A agonists to block inflammation through immune regulation of hematopoietic cells (15, 36, 37, 50) along with our finding of their effects on podocyte gene expression, these results suggest the possibility that A₂A agonists protect kidneys from diabetic nephropathy through actions on hematopoietic cells and/or kidney-derived cells. Given the increasingly recognized role of inflammation in diabetic nephropathy (13, 33, 54), we conducted studies to examine whether A₂A agonists reduce injury associated with a chronic model of STZ-induced diabetic nephropathy. ATL146e, an A₂AR agonist, has a higher affinity and selectivity for human and rat A₂ARs than the widely used CGS-21680 (44) and blocks inflammation and renal ischemia-reperfusion injury primarily by activating bone marrow-derived cells (15). ATL313 is similarly effective with longer duration than ATL146e (16).

In the present study, we demonstrated a significant increase in UAE after 2 wk of diabetes in conscious animals that continued to be elevated throughout the study period. Administration of A₂A agonists significantly reduced albuminuria.
Fig. 6. Effect of ATL146e on inflammatory cytokines. Twenty-four-hour urine collections were obtained for measurement of MCP-1 (A), IFN-γ (B), and TNF-α (C) in rats with a correlation analysis with UAE using linear regression analysis in control, diabetes, and diabetes + ATL146e treatment groups at 6 wk of the study. Open bars, control rats; filled bars, diabetic rats; light gray, diabetic rats treated with ATL146e −3D; dark gray bars, diabetic rats treated with ATL146e +7D. Values are means ± SE. *P < 0.01, **P < 0.001, ***P < 0.0001 to control; +P < 0.05, ++P < 0.01, +++P < 0.0001 to diabetes.

Fig. 7. Effect of ATL146e on slit diaphragm-associated molecules (podocin and nephrin) mRNA expression in rats. RT-PCR was performed on whole rat kidney total RNA in control, diabetes, and diabetes rats treated with ATL146e (ATL146e −3D and ATL146e +7D) after 6 wk. Podocin and nephrin mRNA expressions were normalized with GAPDH. Open bars, control rats; filled bars, diabetic rats; light gray bars, diabetic rats treated with ATL146e −3D; dark gray bars, diabetic rats treated with ATL146e +7D. Values represent means ± SE. *P < 0.05, **P < 0.01 to control; +P < 0.05, ++P < 0.01, +++P < 0.0001 to diabetes.
Similarly, ATL146e administered either before or after STZ-induced diabetes reduced the increase in plasma creatinine in diabetes. Loss of A<sub>2A</sub> agonist effect on A<sub>2A</sub>-KO mice as shown in our results (current study and Ref. 15) indicates ATL146e and ATL313 are selective at A<sub>2A</sub>Rs. It is interesting to note that proteinuria is greater in A<sub>2A</sub>-KO mice than WT mice, which suggests that endogenous A<sub>2A</sub>Rs might contribute to kidney protection from diabetes in a similar manner than they do in kidney ischemia-reperfusion injury (15). In these mice, blood glucose and blood pressures were the same for both groups; thus these factors are unlikely to explain the difference in proteinuria observed.

Other findings from the present study indicate that the increase in kidney fibronectin mRNA in diabetes is reduced by ATL146e. Increased expression of renal fibronectin is an early event in the pathogenesis of diabetic renal disease and its accumulation in the kidney is thought to lead to the development of glomerulosclerosis (30). In diabetes, both TGF-β and fibronectin stimulate matrix production and blocking matrix degradation (5, 7, 21, 55).

The mechanism by which A<sub>2A</sub> agonists mediate protection in STZ-induced diabetic nephropathy is not known; however, we speculate that A<sub>2A</sub> agonists may attenuate kidney injury in diabetes either indirectly through effects on hematopoietic cells, or directly through effects on podocytes, or vascular endothelium. Both nephrin and podocin are crucial complex proteins in the assembly and reinforcement of the slit diaphragm by binding to the actin cytoskeleton via CD2-associated protein (17, 47). Previous studies showed a reduction in nephrin mRNA and protein expression in human (4) and STZ diabetic rat models (6, 26). Data on podocin are controversial ranging from no change (4), decreased protein expression (27), to increased mRNA expression (27). α3β1-integrin is the major integrin that anchors podocytes (43, 48) to collagen, fibronectin, and laminin present on glomerular basement membranes (GBM) (17, 18). Deregulation of these proteins and podocytes has been described to be associated with glomerular disease including DN (31, 40). Whether the effect of ATL146e on restoring podocin and nephrin is due to reducing inflammation or has a direct effect on podocyte is not clear. Additional studies need to explore the relationship between diabetes, podocytes, and A<sub>2A</sub>Rs.

The increase in kidney macrophages was correlated with the duration and severity of renal injury in diabetes (13). In the kidney, MCP-1 is produced by mesangial and tubular epithelial cells (42, 45) and mediates renal interstitial inflammation, tubular atrophy, and interstitial fibrosis (29). In the current study, we found significant elevation of urinary MCP-1 excretion level with the progression of diabetes that correlated with the rate of UAE in the diabetes group. ATL146e significantly reduced urinary MCP-1 to normal levels. There was no change in plasma MCP-1 in all groups indicating the urinary excretion of MCP-1 in diabetes is due to MCP-1 production by the kidney. These data suggest the possibility that renal MCP-1 may contribute to the glomerular and tubulointerstitial lesions in diabetic nephropathy.

Macrophages were found in our study mainly in the interstitium and the glomeruli. In our study, urinary TNF-α significantly correlated with UAE in diabetes. TNF-α is produced mainly by monocytes, macrophages, T and B lymphocytes, and glomerular mesangial cells (3, 22). Both TNF-α and IL-1β have been associated with increasing vascular endothelial permeability (46) and have been detected in isolated GBM in diabetes (33). The relationship between proteinuria, MCP-1, macrophage infiltration, inflammatory cytokines, and renal tissue fibrosis adds further support to the potential role of macrophages in the pathogenesis of DN. The ability of A<sub>2A</sub> agonists to block macrophage entry or secretion of key products is likely a key element in tissue protection.

CD4<sup>+</sup> cells are the primary hematopoietic cells that secrete IFN-γ. Increased IFN-γ with diabetes and the reduction observed with A<sub>2A</sub> agonists treatment implicate the potential role of T cells. IFN-γ is known to induce macrophage priming or activation causing renal injury (24). Further studies are needed to better understand the role of individual hematopoietic elements in the pathogenesis of diabetic complications.

Our studies do not exclude the possibility that A<sub>2A</sub> agonists induce a favorable intraglomerular hemodynamic effect to reduce proteinuria. It is possible that A<sub>2A</sub> agonists mitigate proteinuria through direct effects on the glomerular vascular bed or indirectly by blocking vasoactive inflammatory mediators. Additional studies are necessary to address this issue.

There is unexplained variability in the blood pressure response ranging from an increase (9, 32) to no change in diabetes (8, 51). In our study, we observed similar variabilities. Thus conclusions drawn from blood pressure measurements should be made with caution. SBP increased significantly at 6 wk in the diabetes group, an effect that may be a consequence of ongoing inflammation and endothelial dysfunction. The effect of ATL146e to block inflammation may have resulted in the observed late decrease in SBP possibly secondary to reduced inflammation. It is unlikely that ATL146e has a direct blood pressure-reducing effect at the doses used, as previous studies in rats and mice show that ATL146e infused by osmotic minipumps did not produce an effect on SBP (38) and had not early effects in this study.

In summary, our studies demonstrate that chronic administration of selective A<sub>2A</sub> agonists attenuates renal lesions and functional abnormalities characteristic of diabetic nephropathy. We believe that the renal tissue protective effect of A<sub>2A</sub> agonist is mediated primarily by abrogating the inflammatory response associated with diabetes. Whether A<sub>2A</sub> agonists have direct effects on bone marrow-derived cells or nonbone marrow-derived cells such as podocytes in attenuating the diabetic kidney phenotype similar to effects observed in acute renal ischemia-reperfusion injury is the focus of future studies. We conclude that A<sub>2A</sub> agonists represent a novel therapeutic option for the treatment of diabetic kidney disease and potentially other diabetic complications.

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