Activation of Adenosine 2A Receptors Preserves Structure and Function of Podocytes

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ABSTRACT

Adenosine 2A receptor (A2AR) activation was recently shown to be renoprotective in diabetic nephropathy. A2AR are found in glomeruli and have been shown to associate with the podocyte cytoskeletal protein α-actinin-4, but the effect of their activation on podocyte structure and function is unknown.

Podocyte injury was induced in C57BL/6 mice with puromycin aminonucleoside, and the selective A2AR agonist ATL313 was found to attenuate the resulting albuminuria and foot process fusion. The selective A2AR antagonist ZM241385 reversed the effects of ATL313.

In vitro, A2AR mRNA and protein were expressed in a conditionally immortalized podocyte cell line, and A2AR-like immunoreactivity co-localized with the actin cytoskeleton. Treatment with ATL313 also blocked the increased podocyte permeability to albumin and disruption of the actin cytoskeleton that accompanied puromycin aminonucleoside–induced injury in vitro. ATL313 was ineffective, however, in the presence of the A2AR antagonist and in A2AR-deficient podocytes. It was concluded that A2AR activation reduces glomerular proteinuria, at least in part, by preserving the normal structure of podocyte foot processes, slit diaphragms, and actin cytoskeleton.


Glomerular disorders are the major causes of end-stage renal disease (ESRD) in humans. Podocyte loss, effacement, and dysfunction along with alterations of the podocyte-specific structural proteins and cytoskeleton are key factors that lead to glomerulosclerosis.1–5 Podocytes are highly specialized, terminally differentiated epithelial cells that serve as an important constituent of the glomerular filtration barrier.6 They are anchored to the glomerular basement membrane (GBM) and the capillary tufts and thought to play a pivotal role in pathophysiology of many forms of human and experimental glomerular disease, including minimal-change disease, IgA nephropathy, focal and segmental glomerulosclerosis (FSGS), and diabetic nephropathy (DN).

Both nephrin and podocin are complex proteins that contribute to the assembly and reinforcement of the slit diaphragm by binding to the actin cytoskeleton via CD2-associated protein.7,8 Mutations in nephrin and podocin mRNA and protein expression lead to human glomerular disease.9 Moreover, reduced α3β1 integrin, the major integrin anchoring podocytes to the GBM structures,10,11 collagen IV, fibronectin, and laminin7,12 support the central role of podocytes in glomerular disease.

Adenosine has diverse physiologic and pathophysiologic effects that depend on its interaction with the receptor subtypes A1, A2A, and A2B, and A3.13,14 Recently, Adenosine 2A receptor (A2AR) activation was shown to be renoprotective in diabetic nephropathy. A2AR are found in glomeruli and have been shown to associate with the podocyte cytoskeletal protein α-actinin-4, but the effect of their activation on podocyte structure and function is unknown.

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recently, we reported a renoprotective effect of adenosine 2A receptor (A₂₃R) activation in DN. Streptozotocin (STZ)-induced diabetes in rodents led to marked proteinuria and decreased renal function that was attenuated with continuous subcutaneous administration of A₂₃R agonists. It is interesting that, in these studies, diabetic kidney disease was associated with reduced podocin and nephrin mRNA expression, but their expression was restored in animals treated with A₂₃R agonists. A₂₃R are found in glomeruli and recently A₂₃R were found to associate with α-actinin-4, a cytoskeletal protein, which when mutated is thought to lead to FSGS. These results raise the possibility that A₂₃R agonists may have a direct effect on podocytes; therefore, we sought to determine whether A₂₃R agonists had direct effects on podocytes to preserve glomerular filtration barrier function. We describe here the first characterization of A₂₃R expression in the kidney podocyte as well as the functional analysis of podocyte A₂₃R both in vivo and in vitro. We used puromycin aminonucleoside (PAN) to induce proteinuria and effacement of podocyte foot processes, as demonstrated by electron microscopy (EM), effects that were attenuated with A₂₃R agonists. Podocytes grown in culture expressed A₂₃R and when exposed to PAN showed cytoskeletal disorganization and increased albumin permeability, effects that were attenuated by A₂₃R agonists. These results suggest that A₂₃R expressed on podocytes may serve as an important target to preserve podocyte function in glomerular disease.

RESULTS

Effect of A₂₃R Agonists on Urinary Albumin Excretion Rate in PAN-Induced Proteinuria

In a preliminary study, PAN (50, 75, and 100 mg/kg) was injected into the tail vein of C57BL/6 mice, and urine was collected for 24 h at 0, 3, 6, and 10 d after PAN injection (n = 2 per group). We found that proteinuria was maximal on day 6 after a dosage of 100 mg/kg (367% of control; Figure 1A).

Next, we determined the effect of continuous administration of ATL313 (1 ng/kg per min) in vivo on PAN-induced proteinuria. As shown in Figure 1B, PAN injection (100 mg/kg) significantly increased urinary albumin excretion (UAE; 259% of control; P < 0.01) after 6 d, an effect completely blocked by ATL313 treatment (107% of control; P < 0.01 to PAN treatment). The effect of ATL313 was reversed with the selective A₂₃R antagonist ZM241385 (5 ng/kg per min; 212% of control; P < 0.05 to ATL313 group). PAN injection was associated with a significant reduction in podocin and zonula occludens-1 (ZO-1) mRNA expression compared with control mice (35 and 73% of control, respectively). ATL313 administration reversed this reduction (185 and 131% of control), respectively; however, there was no significant difference in nephrin mRNA expression among all groups (control 100 ± 10%; PAN 120 ± 25% [P < 0.7 of control]; PAN + ATL313 64 ± 9% [P < 0.3 of control and P < 0.08 of PAN]).

Figure 1. Effect of ATL313 on UAE in PAN-induced nephrosis. Urine was collected during a 24-h period for measurement of UAE by ELISA after PAN injection. (A) Dosage and time (days after PAN injection) response curve of PAN injection (n = 2 per symbol). (B) Effect of continuous 6-d infusion of vehicle (PAN), ATL313 (PAN + ATL313), or ATL313 + ZM241385 (PAN + ATL313 + ZM241385) on UAE in PAN-induced glomerular injury (100 mg/kg, intravenously, day 0) on day 6 after PAN injection. Data are means ± SEM. *P < 0.05, **P < 0.01 versus untreated control; + P < 0.05, ++ P < 0.01 versus PAN + ATL313 group.

ATL313 Reverses Morphologic Changes Associated with PAN-Induced Injury

PAN-induced proteinuria is associated with podocyte foot process effacement; therefore, we sought to determine whether the reduction of PAN-induced proteinuria was reversed with A₂₃R agonists. In comparison with the control group (Figure 2, A and B), the foot processes in the PAN group (Figure 2, C and D) were effaced, retracted, widened, and shortened. The effect of PAN on podocyte foot process ultrastructure was attenuated with ATL313 (Figure 2, E and F); ZM241385 (Figure 2, G and H) reversed the protective effect of ATL313 on podocyte foot process. The number of open slit diaphragm per micrometer of GBM length was reduced in PAN (0.26 ± 0.1) in comparison with control (1.47 ± 0.3; P < 0.05) or with ATL313 (2.03 ± 0.2; P <
ZM241385 significantly reversed the effect of ATL313 (0.66 ± 0.3; P < 0.01 compared with ATL313; Figure 3). These results suggest that the reduction of PAN-induced proteinuria by ATL313 is associated with preservation of podocyte foot process ultrastructure and raises the possibility of a direct effect of A2AR activation on podocytes.

In contrast to EM results, light microscopic analysis of hematoxylin and eosin staining of the renal cortex and medulla revealed no histologic changes among all groups (data not shown).

A2AR Are Present in Murine Podocytes
We used cultured podocytes to determine whether they express A2AR and to determine the functional effects of podocyte A2AR activation in vitro. We detected A2AR mRNA in immortalized podocytes by reverse transcription–PCR (RT-PCR; n = 6; Figure 4A). We also found that the band identified by PCR is indeed an amplified product of A2AR mRNA by using a direct sequencing. We also used a well-characterized mAb to A2AR and showed labeling of A2AR-like immunoreactivity in podocytes with particular concentration in “spindle-like” extensions that resemble foot processes (Figure 4B, arrows). All batches of podocytes in these studies expressed both the Wilms’ tumor gene (WT-1) and synaptopodin (Figure 4, C and D, respectively).

Validation of A2AR Expression in Murine Podocytes
To confirm the specificity of the A2AR mAb, we used both a specific (antigenic A2AR peptide; Figure 5A) and an irrelevant (Figure 5B) peptide in competition experiments. Only the antigenic peptide blocked labeling, indicating that the A2AR mAb specifically recognizes an epitope of the A2AR used in generating the antibody.

We also used the small interfering RNA (siRNA) technique to knock down A2AR in podocytes as another ap-
With three of four different A2AR siRNA oligonucleotides (siRNA 1, 2, and 4), we were able to demonstrate knockdown of A2AR mRNA and protein in differentiated podocytes by using RT-PCR (Figure 5C) and immunohistochemistry (Figure 5D), respectively. One construct (siRNA 3) failed to decrease mRNA or protein expression (data not shown). There was no effect of A2AR siRNA oligonucleotides on the control gene (cyclophilin) expression of podocytes (Figure 5C).

ATL313 increases Podocyte cAMP Levels In Vitro

A2AR are known to be coupled through stimulatory G proteins to increase cAMP upon activation.20 We tested whether A2AR activation in podocytes by ATL313 regulated cAMP accumulation. ATL313 (30 nM) increased intracellular cAMP production from a baseline of 55 ± 4 to 91 ± 14 pmol/ml (P < 0.05), an effect completely blocked by ZM241385 (150 nM; 34 ± 3 pmol/ml; P < 0.01 to ATL313). These results provide a functional measure of A2AR activation in podocytes.
ATL313 Attenuates PAN-Induced Increase in Podocyte Permeability In Vitro

We determined the functional effects of ATL313 on podocyte permeability by measuring the transepithelial passage of BSA across differentiated podocytes grown on Transwell chambers as described previously.\(^2\) As shown in Figure 6, differentiated podocytes treated with PAN significantly increased albumin passage through podocytes (\(P < 0.05\) to control). ATL313 treatment completely blocked the effect of PAN on podocyte permeability to albumin, an effect reversed with ZM241385 treatment. These data further demonstrate that functional A\(_{2A}\)R are expressed in podocytes and that activation of podocyte A\(_{2A}\)R directly reduces their permeability to albumin.

ATL313 Attenuates PAN-Induced Disruption of the Podocyte Actin Cytoskeleton In Vitro

Reversal by ATL313 of the observed alterations in podocyte albumin permeability in response to PAN may be due to the ability of A\(_{2A}\)R agonists to preserve the podocyte structural integrity. To examine this possibility, differentiated podocytes were injured using PAN 100 \(\mu\)g/ml alone, and the protective effect of ATL313 (50 nM) with and without ZM241385 (150 nM) was determined. As shown in Figure 7A, the specific activation of A\(_{2A}\)R by ATL313 (which was blocked by ZM241385) prevented the marked disruption of the organization of the actin cytoskeleton produced by treatment of podocytes with PAN. To confirm that the protective effect of ATL313 was indeed mediated by podocyte A\(_{2A}\)R, we demonstrated that the effect of ATL313 was lost in PAN-treated cells in which the A\(_{2A}\)R had been deleted by siRNA (Figure 7B). These results confirmed that the effect of AT313 to preserve the actin cytoskeletal organization after PAN exposure was due to activation of podocyte A\(_{2A}\)R.

Figure 6. Effect of ATL313 on podocyte permeability in vitro. Transepithelial permeability of differentiated podocytes to BSA was measured as described in the Concise Methods section. After 14 d of culture on Transwell filter chambers (3-\(\mu\)m pore) at 37\(^\circ\)C, podocytes were pretreated with vehicle or ATL313 (50 nM) with or without ZM241385 (150 nM) for 1 h and then exposed to PAN (100 \(\mu\)g/ml) for 24 h. The lower chambers were then filled with BSA-containing medium, and the upper chambers were sampled at 1, 3, and 6 h (\(n = 3\) different experiments). Data are means ± SEM. *\(P < 0.0001\) versus untreated podocytes (normal); +\(P < 0.0001\) versus PAN + ATL313 group.

Figure 7. Effect of ATL313 on the podocyte actin cytoskeleton after injury with puromycin treatment in vitro. Differentiated murine podocytes were transfected with a scrambled oligonucleotide (A) or specific siRNA oligonucleotide to knockdown A\(_{2A}\)R (B). The actin cytoskeleton (green) and nuclei (DAPI, blue) were labeled in untreated cells (control) or in cells after incubation with PAN (100 \(\mu\)g/ml), PAN + ATL313 (50 nM), or PAN + ATL313 + ZM243185 (150 nM). Bar = 40 \(\mu\)m.
DISCUSSION

Podocytes contribute to the maintenance of the glomerular filtration barrier, and abnormalities of podocyte structure and function lead to a number of glomerular diseases; therefore, treatments that preserve podocyte structure may lead to a reduction of proteinuria and preservation of glomerular and kidney functions. Here we demonstrate that PAN-induced nephrosis, a model of human minimal-change disease, was associated with proteinuria and characteristic foot process effacement. An agonist of A2A R, ATL313, reversed this effect. By RT-PCR, immunohistochemistry, and functional assessment, we established that podocytes grown in culture expressed A2A R. When exposed to PAN, the actin cytoskeleton becomes disorganized, leading to an increase in permeability to albumin. ATL313 restores normal actin cytoskeletal arrangement and normal albumin permeability; therefore, activation of A2A R expressed on podocytes may serve as a therapeutic strategy in diseases that lead to podocyte dysfunction.

Previously, in an STZ-induced model of DN, we demonstrated that an A2A R agonist, ATL146e, led to marked reversal of injury. The induction of proteinuria with STZ was markedly reduced by ATL146e, an effect that was associated with preservation of estimated GFR and glomerular structure. Both nephrin and podocin mRNA levels were reduced after STZ-induced diabetes, an effect completely restored with A2A R agonist treatment. Because the expression of nephrin and podocin is limited to podocytes, we questioned whether the protective effect of A2A R agonists in reducing proteinuria may in part be due to direct effects on podocytes. In this study, we subjected mice to PAN to examine the in vivo and in vitro effects of A2A R agonists on the maintenance of podocyte integrity.

In this study, we demonstrated by RT-PCR and immunohistochemical localization that differentiated murine podocytes express A2A R mRNA and protein, respectively. Confocal microscopy revealed A2A R-like immunoreactivity both intracellularly and along the periphery of the podocyte. The specificity of immunohistochemical labeling with the A2A R mAb is well characterized in brain but has not previously been examined in podocytes. The specificity of the labeling using the A2A R mAb was confirmed by preadsorption experiments with the competing immunizing peptide and in A2A R siRNA “knockdown” experiments; labeling was absent or markedly attenuated in both cases. Furthermore, our data provide the first demonstration of the expression of functional A2A R in podocytes and coupling to cAMP production. Given the marked tissue-protective effect of A2A R in other tissues, it was of interest to understand the biologic significance of A2A R expression in podocytes and whether their activation could reverse podocyte injury.

Advances in podocyte biology have led to insights into the pathogenesis of glomerular disease. Podocytes play a key role in the maintenance of the glomerular filtration barrier, and normal podocyte function is intimately linked to its complex cytoskeletal architecture. The podocyte backbone is mainly formed by abundantly rich actin cytoskeleton and α-actinin-4, which enable podocytes to alter continually their shape and surround the glomerular capillaries to form a filtration slit diaphragm. In vivo, podocyte differentiation is required for normal filtration barrier function. Podocyte injury leads to alterations of the foot process/slit diaphragm complex and a reduction in podocyte number with loss of glomerular permeability. Given the importance of podocin and normal albumin permeability; therefore, activation of A2A R expressed on podocytes may serve as a therapeutic strategy in diseases that lead to podocyte dysfunction.

In our study, we investigated the effect of A2A R activation in vivo after PAN-induced nephrosis. Although mice are more resistant to PAN than rats, we used a different approach and demonstrated a dosage and time course of proteinuria in male mice that were administered an intravenous injection of puromycin to ensure an optimal effect. We used male mice because female mice are relatively resistant. Previous studies successfully induced PAN nephrosis in mice after a high dosage (500 mg/kg subcutaneously), after a high-cholesterol diet, or with multiple doses (20 mg/kg subcutaneously or 90 mg/kg intraperitoneally). We found that PAN led to an increase in UAE associated with foot process effacement and a reduction in podocin and ZO-1 mRNA expression. A2A R agonist treatment significantly reduced UAE in PAN-induced proteinuria and reversed the podocyte effacement observed by EM. Foot process effacement depends on disruption of the actin cytoskeletal network. Previous reports showed that mRNA expression and immunostaining for nephrin declined immediately after PAN injection in rats and before the incidence of proteinuria. Although we found a significant reduction in podocin and ZO-1 mRNA expression after PAN injection compared with control mice, there was no significant difference in nephrin mRNA expression among all groups. We believe that mice may be different from rats regarding nephrin expression with PAN nephrosis.

It is also unlikely that the reduction of proteinuria in this model of PAN-induced nephrosis is due to effects of A2A R agonists on systemic BP. We previously showed that continuous administration of ATL131 (1 ng/kg per min) via osmotic minipumps had no effect on systolic BP in diabetic rats or mice treated up to 6 wk. In addition, a nearly identical prototypical compound, ATL146e (4 mg/kg per min), with a shorter half-life had no effect on systolic BP when infused continuously via osmotic minipumps for 24 to 28 h in rats subjected to ischemia-reperfusion injury. Thus, when used in the dosage range of 1 to 10 ng/kg per min via continuous infusion, it is unlikely that effect of A2A R agonists to reduce proteinuria in PAN-induced nephrosis is mediated through alterations in systemic hemodynamics.

In vivo, we showed that PAN disrupts the highly organized podocyte cytoskeleton, leading to podocyte injury and proteinuria. We also demonstrated that A2A R agonists can reverse podocyte injury and reduce proteinuria in vivo after PAN-induced nephrosis.
actin cytoskeleton. Treatment of podocytes in vitro with ATL313 restored the architecture of the podocyte cytoskeleton, suggesting that the observed effect of ATL313 in vivo to restore normal podocyte structure may be due to direct effects on podocytes. The mechanisms by which this effect occurs are not known; however, there is evidence that the C-terminal domain of the A2AR interact with α-actinin, a major F-actin-cross-linking protein through co-localization, co-immunoprecipitation, and pull-down experiments. Previous observation demonstrated a transient dysregulation of α-actinin in podocytes just before the development of foot process effacement and a concomitant redistribution of α-actinin and actin associated with PAN-induced nephrosis. In addition, α-actinin has been shown to bind β1 integrin subunit and strengthen the podocyte–GBM interaction, thereby stabilizing glomerular architecture. Taken together, these observations lead us to speculate that α-actinin may mediate A2AR agonist’s effect on podocyte actin cytoskeleton. Additional experiments will be needed to explore this mechanism.

In our study, A2AR activation led to an increase in cAMP release from differentiated podocytes. This finding is consistent with the known coupling of A2AR to stimulatory G proteins whereupon activation leads to an increase in cAMP levels. cAMP may be another mechanism by which A2AR agonists protect podocytes from injury. Previous reports showed that treatment of crescentic glomerulonephritis in rats with a PDE4 inhibitor to prevent the degradation of cAMP effectively reduced albuminuria and crescent formation. It is possible that cAMP accumulation resulting from activation of protein kinase A regulates actin filament assembly by phosphorylation of myosin light-chain kinase. In addition, increased cAMP levels in isolated glomeruli via inhibition of PDE4 may suppress the phorbol ester–induced formation of reactive oxygen metabolites.

Furthermore, podocytes are vulnerable to many forms of both immune- and non–immune-mediated injury. PAN nephrosis has been shown to be associated with interstitial infiltration of monocytes and macrophages. In addition, exposure to PAN increased both mRNA expression and protein production of fibronectin and monocyte chemoattractant protein 1. Moreover, podocytes were demonstrated to express a variety of vasoactive and immune substances that may regulate podocyte function. Given the known effect of A2A agonists to block inflammation through immune regulation of hematopoietic cells along with our finding of their anti-inflammatory effect on podocytes, we believe that the reduction of proteinuria after PAN is due to a direct effect of ATL313 on podocytes. Podocytes express A2AR, and activation of A2AR expressed in podocytes grown in culture after exposure to PAN attenuated actin cytoskeletal disruption and albumin permeability. On the basis of these data, we suggest that after injury with PAN, direct activation of A2AR on podocytes restores normal function. These findings raise the possibility that podocyte A2AR might serve as a novel target for diseases that affect podocyte function.

**CONCISE METHODS**

**Puromycin-Induced Proteinuria**

Experiments were conducted in C57BL/6 mice (6 to 8 wk of age; Charles River Laboratories, Wilmington, MA) and were approved by the University of Virginia Animal Research Committee. Preliminary studies were conducted to determine the optimal dosage of PAN (Sigma Chemical Co., St. Louis, MO) to induce and time point to assess for proteinuria. PAN (50, 75, or 100 mg/kg) was administered by a single intravenous injection in the mouse tail vein, and urine was collected for 24 h under water-equilibrated oil at baseline and 3, 6, and 10 d to determine the rate of UAE. After determination of the optimal dosage and time for PAN treatment, mice were treated with saline or with ATL313 (1 ng/kg per min), a selective A2AR agonist, with or without the selective A2AR antagonist ZM241385 (5 ng/kg per min) via osmotic minipumps inserted at the time of injury (n = 6 per group). Urine was collected for 24 h at day 6 to determine UAE, mice were killed, and kidneys were removed for various analyses.

**Transmission EM**

Kidneys of C57BL/6 mice were perfused through the heart with 4% (wt/vol) paraformaldehyde and 2.5% (wt/vol) glutaraldehyde in 0.1 M phosphate bufer. Renal cortices were cut (1 to 2 mm3) and fixed overnight (4°C), postfixed (24°C) in 1.0% OsO4, dehydrated, and embedded in epoxy resin according to routine procedures. Sections (approximately 70-nm thickness) were contrast stained with uranyl acetate and examined in a JEOL 1230 transmission electron microscope. Digital images were acquired using an SIA L3-C digital camera (Scientific Instruments and Applications, Duluth, GA). Sections (n = 3 per group, four different fields per section) were analyzed for density of open slit diaphragms (NIH Image J software version 1.37). Data were expressed as the number of opened slit diaphragms per micrometer of GBM length.

**Cell Line and Culture Conditions**

A conditionally immortalized mouse podocyte cell line was cultured as described previously. All podocytes used in these studies expressed WT-1, and podocyte differentiation was confirmed by synaptopodin.

**Induction of Podocyte Injury by PAN Treatment In Vitro**

All experiments were done on differentiated podocytes (1 × 105 cells/well) in six-well culture dishes cultured for 10 d on collagen-coated coverslips before treatment. Four hours before each experiment, we added adenosine deaminase (1 U/ml; Sigma) to the culture medium to exclude any effect of endogenous adenosine. Differentiated podo-
cytes were then exposed to 100 μg/ml PAN (Sigma) or vehicle for 24 h at 37°C. Podocytes were incubated in medium containing vehicle or ATL313 (50 nM) with or without ZM 241385 (150 nM) for 1 h before PAN treatment, and cells were analyzed using confocal microscopy.

**Immunofluorescence and Confocal Microscopy**

Differentially podocytes grown on collagen-coated glass coverslips were fixed with 3% formaldehyde, permeabilized in PIPES-buffered PBS with 0.05% saponin, and incubated in quenching buffer; blocking solution and cells were then incubated with the primary antibodies. The following primary antibodies were used: (1) Antibodies reacting with podocyte-specific antigens: rabbit anti–WT-1 protein (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-synaptopodin mAb (Research Diagnostics, Concord, MA); (2) FITC-conjugated phalloidin (Sigma); and (3) mouse anti-A2AR (clone 7F6-G5-A2). All specimens’ fields were selected randomly and examined using a Zeiss LSM 510-UV confocal microscope.

**Podocyte BSA Filter Assay**

Collagen-coated Transwell-Col PTFE filters (3 μm pore; Corning, New York, NY) were seeded with 1 × 10⁶ podocytes per filter and cultured under differentiating conditions for 10 d. Cells were then incubated in the presence of adenosine deaminase (1 U/ml) with vehicle or ATL313 (50 nM) with or without ZM 241385 (150 nM) for 1 h followed by 100 μg/ml PAN treatment for 24 h at 37°C. Cells were washed twice with PBS supplemented with 1 mM MgCl₂ and 1 mM CaCl₂ to preserve the cadherin-based junctions. The upper compartment was then refilled with 0.5 ml of RPMI 1640 and the lower compartment with 1 ml of BSA medium (RPMI 1640 supplemented with 40 mg/ml BSA). Total protein concentration in the upper compartment was then determined at 1, 3, and 6 h using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) as described previously.²¹

**siRNA Technique**

Four ready-to-use validated double-stranded 21-nucleotide siRNA for A₂₃₃R (GeneBank accession no. NM_009630) were synthesized and purified (Dharmacon, LaFayette, CO; Table 1). A scrambled or irrelevant siRNA (Ambion, Austin, TX) was used as a negative control. Differentially podocyte were maintained in RPMI 1640 medium for 10 d at 37°C in a collagen-coated plate at 70 to 80% confluence. Transfection of siRNA and controls were carried out using Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol.

**RT-PCR**

Total RNA was extracted, and single-stranded cDNA was synthesized as described previously.²⁸ Gene-specific primers were designed using Beacon Designer Software (Premier Biosoft Int., Palo Alto, CA); the sense primer for A₂₃₃R was TGCCGTGGTACGCGGTATG, whereas the antisense primer was GCGAGGCTTTGTGGAATT. Amplification products were verified by melting curves, agarose gel electrophoresis, and direct sequencing. RT-PCR was performed on the cDNA using the ThermoScript RT-PCR System with Platinum Taq DNA polymerase as described previously,²⁵ and samples were normalized to glyceraldehyde-3-phosphate dehydrogenase. For siRNA validation experiments, we designed primers for A₂₃₃R specific for the different types of siRNA positions (Table 1) and cyclophilin as control gene as described previously. The following PCR protocol was used: Initial denaturation (95°C for 3 min); denaturation, annealing, and elongation program repeated 35 times (95°C for 45 s, 56°C for 60 s, and 72°C for 60 s); final elongation (72°C for 7 min); and finally a holding step at 4°C as described previously.²⁵

**ELISA**

UAE was measured by ELISA using Albunwell M kit (Exocell, Philadelphia, PA).³⁵ cAMP was measured to assess the efficacy of A₂₃₃R agonist in differentiated podocytes (Cayman Chemical, Ann Arbor, MI) in the presence of adenosine deaminase (1 U/ml) to exclude any effect of endogenous adenosine and rolipram (300 nM), a type IV phosphodiesterase inhibitor, to minimize cAMP degradation.

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**Table 1. siRNA oligonucleotides and their corresponding primer pairs for A₂₃₃Rᵃ**

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ᵃF, forward; R, reverse.
Statistical Analyses
Comparisons between groups were examined by one-way ANOVA using SPSS 14.0 software for Windows (SPSS, Chicago, IL). Multiple comparisons of individual pairs of effect means were conducted by using the least squares methods of pooled variance. Data are expressed as means ± SEM. Statistical significance was identified at P < 0.05.

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DISCLOSURES
M.D.O. and J.L. own equity in Adenosine Therapeutics, which provided ATL313 for this study. All other authors have no financial conflict of interest.

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