Kidney International, Vol. 59 (2001), pp. 2114–2125

Enhanced protection from renal ischemia: Reperfusion injury with A_{2A}-adenosine receptor activation and PDE 4 inhibition

Mark D. Okusa, Joel Linden, Liping Huang, Diane L. Rosin, David F. Smith, and Gail Sullivan

Departments of Medicine, Pharmacology, and Molecular Physiology and Biological Physics, University of Virginia Health Sciences Center, Charlottesville, Virginia, USA

Enhanced protection from renal ischemia: Reperfusion injury with A_{2A}-adenosine receptor activation and PDE 4 inhibition.

Background. We previously demonstrated in rats and mice that agonists of A_{2A}-adenosine receptors (A_{2A}-ARs) reduce renal injury following ischemia-reperfusion. We now extend these studies and examine the effects of ATL-146e (formerly DWH-146e), an A_{2A}-AR agonist, and rolipram, a type IV phosphodiesterase (PDE 4) inhibitor, on murine renal injury following ischemia-reperfusion.

Methods. C57BL/6 mice were treated with rolipram, ATL-146e, or both compounds combined and were subjected to renal ischemia for 32 minutes and reperfusion for 24 to 48 hours. In vitro studies were performed on suspended and adhering human neutrophils.

Results. Continuous delivery of rolipram or ATL-146e during reperfusion reduced renal injury in a dose-dependent manner. Maximal protection was observed when ATL-146e was infused for six hours during reperfusion. Elevated plasma creatinine and myeloperoxidase activity produced by ischemia-reperfusion were reduced by rolipram (0.1 ng/kg/min) and ATL-146e (10 ng/kg/min) by up to approximately 60% and 70%, respectively. Co-infusion of both compounds produced a maximum reduction of plasma creatinine of approximately 90% and myeloperoxidase activity. In vitro studies on suspended and adhering human neutrophils demonstrated that selective stimulation of A_{2A}-ARs by ATL-146e increased cAMP accumulation, reduced oxidative activity of activated neutrophils, and decreased activated neutrophil adherence. These responses were potentiated by rolipram.

Conclusions. We conclude that the combined infusion of ATL-146e and rolipram leads to enhanced renal tissue protection from ischemia-reperfusion by mechanisms that may include reduced neutrophil adherence/recruitment and release of reactive oxygen species.

Key words: rolipram, ATL-146e, neutrophil adherence, reactive oxygen species, acute renal failure, oxidative burst.

Received for publication October 4, 2000
and in revised form December 22, 2000
Accepted for publication January 8, 2001

Novel therapeutic interventions for preventing or reducing renal tissue injury following ischemia-reperfusion remain a focus of intense research. One potential approach in reducing injury from ischemia-reperfusion is through the activation of A_{2A}-adenosine receptors (A_{2A}-ARs). ATL-146e (previously referred to as DWH-146e), an A_{2A}-AR agonist, has a higher affinity and selectivity for human and rat A_{2A}-ARs than does CGS21680, a widely used selective A_{2A} agonist [1–3]. ATL-146e binds selectively to human A_{2A}-ARs with 50 times higher affinity than CGS21680. A similar relative potency was noted in studies of cAMP accumulation in transfected HEK-293 cells expressing canine A_{2A}-ARs (ED_{50} 4 nmol/L) [2].

We previously showed that A_{2A} agonists reduce renal tissue injury when administered prior to and immediately after the onset of reperfusion following ischemia [2–4]. A_{2A} agonists may inhibit inflammation by effects on many different cell types that are thought to contribute to ischemia-reperfusion injury. We demonstrated that A_{2A}-AR activation following renal ischemia-reperfusion reduced neutrophil accumulation in renal tissue [3]. This effect appeared to be correlated with a decrease in the expression of P-selectin and intercellular adhesion molecule-1 (ICAM-1) [3], which are endothelial adhesion molecules that participate in renal ischemia-reperfusion injury [5–9]. Such effects suggested that A_{2A} agonists reduced endothelial adhesion molecule expression by binding to A_{2A} receptors expressed on endothelial cells. Alternatively, A_{2A} agonists may have reduced expression of endothelial cell adhesion molecule indirectly by binding to and stabilizing tissue-resident inflammatory cells. Activation of A_{2A}-ARs on neutrophils leads to potent anti-inflammatory effects [10–14].

Type IV phosphodiesterase (PDE 4) inhibitors such as rolipram are being developed as anti-inflammatory compounds [15]. These inhibitors are known to reduce inflammation by decreasing release of cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) and increasing the release of IL-10 [15]. When

infused in rats subjected to endotoxin-mediated acute renal failure, PDE 4 inhibitors stabilize renal function [16, 17].

One cellular mechanism by which A_{2A} agonists may reduce renal injury is through an increase in cAMP in inflammatory cells. A_{2A}-ARs are coupled to adenylyl cyclase via stimulatory G proteins (G_s protein), and when activated, they increase cellular cAMP levels. PDE 4 inhibitors reduce inflammation by increasing cAMP accumulation by inhibiting cAMP degradation [18]. Thus, we hypothesized that A_{2A}-agonists reduce inflammation through an increase in cAMP, and that the coadministration of an A_{2A}-agonist and a PDE 4 inhibitor, such as rolipram, would produce greater tissue protection from ischemia-reperfusion injury than either compound alone. Our results demonstrate that coadministration of rolipram and ATL-146e is more effective than either agent alone in inhibiting neutrophil oxidative activity of human neutrophils and in inhibiting ischemia-reperfusion injury in mouse kidneys.

METHODS

Surgery and experimental protocol

Surgical methods employed in the current study have been described previously [2]. Briefly, C57BL/6 mice (7 to 8 weeks of age, 18 to 28 g; Hilltop Laboratory Animals, Scottsdale, PA, USA) were used for all experiments. Alzet® osmotic minipumps (model 1003D; Alza Corp., Palo Alto, CA, USA) containing vehicle, ATL-146e, or rolipram (prepared in phosphate-buffered saline containing <0.01% dimethylsulfoxide) were inserted subcutaneously under vaporized halothane anesthesia five hours prior to reperfusion (Halothan Vapor 19.1, Germany). Following pump implantation, mice were anesthetized with ketamine [100 mg/kg, intraperitoneally (IP)], xylazine (10 mg/kg, IP), and acepromazine [1 mg/kg, intramuscularly (IM)] and were subjected to bilateral flank incisions. Renal pedicles were identified and cross-clamped for 32 minutes. Surgical wounds were closed with metal staples, and mice were returned to cages for 24 hours. Following 24 hours of reperfusion, animals were re-anesthetized. Blood was obtained by cardiac puncture, and kidneys were removed for various analyses. Our previous studies demonstrated similar degrees of renal protection when ATL-146e treatment was initiated five hours prior to ischemia or immediately after the onset of reperfusion [2]. Table 1 summarizes the control and experimental groups.

In some experiments, the duration of ATL-146e infusion was varied by delivery of the compound via a subcutaneously placed catheter. PE 10 catheters were inserted subcutaneously with the animals under anesthesia, attached to a 3 cc syringe, and sutured in place. Mouse kidneys were then subjected to the ischemia protocol, and mice were then cradled in a plexiglass animal restrainer to maintain the position of the catheter during reperfusion. Beginning immediately after onset of reperfusion, mice were given a bolus of vehicle or ATL-146e (60 ng/kg IP), and vehicle or ATL-146e was infused continuously at a rate of 1 μL/min (10 ng/kg/min) for 2, 4, 6, or 24 hours via syringe pump (model 55-5920; South Natick, MA, USA).

Table 1. Control and experimental groups

Group	Compound administration *ng/kg/min*
1	Vehicle
2	ROL (0.1)
3	ROL (1)
4	ROL (10)
5	ATL-146e (0.1)
6	ATL-146e (0.5)
7	ATL-146e (1)
8	ATL-146e (10)
9	ROL (0.001)
10	ROL (0.005)
11	ROL (0.01)
12	ATL-146e (0.1) + ROL (0.001)
13	ATL-146e (0.5) + ROL (0.005)
14	ATL-146e (1) + ROL (0.01)
15	ATL-146e (10) + ROL (0.1)

Compounds were administered 5 hours prior to the ischemic period in all groups except as noted in the **Results** section. All kidneys were subjected to 32 minutes of ischemia and 24 hours of reperfusion. ROL is rolipram.

Plasma creatinine

From blood samples obtained by cardiac puncture, plasma creatinine concentration was determined using a colorimetric assay according to the manufacturer's protocol (Sigma Chemical, St. Louis, MO, USA).

Myeloperoxidase activity

Mouse kidneys were removed and immediately homogenized in 10 volumes of ice-cold 50 mmol/L KPO_4 buffer, pH 7.4, using a Tekmar tissue grinder. The homogenate was centrifuged at 15,000 × *g* for 15 minutes at 4°C, and the resultant supernatant was discarded. The pellet was washed twice, resuspended in 10 volumes of ice-cold 50 mmol/L KPO_4 buffer with 0.5% hexadecyltrimethylammonium bromide and sonicated. The suspension was subjected three times to freeze/thaw, sonicated for 10 seconds, and centrifuged at 15,000 × *g* for 15 minutes at 4°C. The supernatant was added to an equal volume of a solution consisting of *o*-dianisidine (10 mg/mL), 0.3% H_2O_2, and 45 mmol/L KPO_4, pH 6.0. Absorbance was measured at 460 nm over a period of five minutes [19].

Isolation of neutrophils

Human polymorphoneutrophils (PMNs) were isolated from heparinized (10 U/mL) venous blood by a one-step Ficoll-hypaque separation [20]. Neutrophil isolation

medium was purchased from ICN Biochemicals (Aurora, OH, USA). The resulting PMN preparation contained approximately 98% PMNs, and >95% were viable as determined by trypan blue exclusion. Following separation, PMNs were washed three times with Hank's balanced salt solution (HBSS) and resuspended in HBSS.

Cyclic AMP

Cyclic AMP (cAMP) was measured in PMNs adhering to 24-well tissue culture plates coated with human fibrinogen (5 mg/mL in 1.5% sodium bicarbonate, 0.5 mL/well; Sigma) overnight at 37°C in 5% CO_2 [14]. The wells were washed two times at 37°C with 200 μL of normal saline solution immediately before the addition of unstimulated human PMNs. PMNs (3 to 4 × 10^6/0.5 mL) were incubated within a well of the coated plate for 45 minutes in 0.5 mL of HBSS containing 0.1% human serum albumin and 1 U/mL adenosine deaminase (Sigma) ± 10 U/mL TNF-α (specific activity = 600 pg/U; a gift from Dainippon Pharmaceutical Co. Ltd., Osaka, Japan), ± 3 to 300 nmol/L ATL-146e, and ± 300 nmol/L rolipram (Berlex Laboratories, Cedar Knolls, NJ, USA). Following incubation, 0.5 mL 0.2 N HCl was added to the wells, and the incubation continued for 45 minutes more at room temperature to extract the cAMP. The samples were then centrifuged at 2000 × *g* for 10 minutes to remove cell debris. Half milliliter cell-free samples were frozen for cAMP analysis in the University of Virginia Radioimmunoassay Core Laboratory (Charlottesville, VA, USA).

Suspended neutrophil oxidative activity as measured by chemiluminescence

We evaluated the bioactivity of ATL-146e on the oxidative activity of neutrophils primed with TNF-α and stimulated with f-met-leu-phe (fMLP) as measured by luminol-enhanced chemiluminescence. Chemiluminescence is dependent on both superoxide production and mobilization of the granule enzyme myeloperoxidase (MPO). Light is emitted from unstable high-energy oxygen species generated by activated neutrophils. Purified PMNs were incubated in HBSS containing 0.1% 1 mL human serum albumin, 1 U/mL adenosine deaminase (Sigma), and 10 U/mL TNF-α for 30 minutes at 37°C in a shaking water bath. Some PMNs were incubated during TNF-α priming ± 0.01 to 100 nmol/L ATL-146e, ± 100 nmol/L to 1 μmol/L rolipram, ± the protein kinase A (PKA) inhibitor H-89 (0.1 to 10 μmol/L; Calbiochemical, La Jolla, CA, USA) and ± 10 nmol/L ZM241385. Luminol-enhanced (1 × 10^4 mol/L; Sigma) fMLP-stimulated (1 μmol/L; Sigma) chemiluminescence was read with a Chronolog Photometer (Chronolog Corp., Havertown, PA, USA) at 37°C for eight minutes. Chemiluminescence is reported as the relative peak light emitted (height of the curve) in the presence of a compound compared with TNF-α–primed fMLP-stimulated control samples.

Neutrophil adhesion assay

Adhesion assays were performed with fibrinogen-coated Costar 96-well flat bottomed cell culture cluster plates (Corning, NY, USA) as described previously in this article ("Cyclic AMP"). Human PMNs were incubated with the fluorescent dye calcein-AM (0.1 mg/mL; Molecular Probes, Inc., Eugene, OR, USA) for 30 minutes at 37°C under 5% CO_2. Calcein-loaded PMNs were incubated for 15 minutes with or without 0.001 to 100 nmol/L ATL-146e, 10 nmol/L ZM241385, and 100 nmol/L rolipram. Just prior to addition to the plate, 10 U/mL TNF-α was added to the calcein-loaded PMNs (100 μL/well; 5 × 10^6 cells/mL), and then the treated PMNs were placed into the fibrinogen-coated wells [21]. Plates were covered with aluminum foil and incubated for 30 minutes under 5% CO_2 at 37°C [21]. Nonadherent cells were removed with four washes of 200 μL warm saline and resuspended in 200 μL of HBSS–0.1% human serum albumin. The fluorescence was measured using emission and excitation wavelengths of 538 and 485 nm, respectively (Victor 1420 Multilabel Counter using the Wallac Workstation software). A standard curve of fluorescence was determined for the PMN concentrations between 5 × 10^5 and 1 × 10^4 PMNs per sample with each experiment. The percentage of TNF-activated adherent PMNs was calculated as: (relative fluorescence after washing procedure/relative fluorescence of TNF activated cells before the washing procedure) × 100%.

Superoxide release from PMNs adhering to a biological surface

Superoxide release was measured as described previously but with modifications [22]. PMNs (2 × 10^6/mL) were preincubated for 15 minutes at 37°C in 0.45 mL of HBSS containing 0.1% human serum albumin and 1 U/mL adenosine deaminase, ± 100 nmol/L rolipram, ± 0.1 to 100 nmol/L ATL-146e, and ± 100 nmol/L ZM 241385.

Following incubation, 120 μmol/L cytochrome C (Sigma) and 0.062 mg/mL catalase (Sigma) were added ± 10 U/mL TNF-α. Aliquots of cell suspensions (200 μL) were immediately transferred to duplicate wells of a 96-well flat-bottomed tissue culture plate that had been coated with human fibrinogen as described before. An enzyme-linked immunosorbent assay (ELISA) plate reader (Titertek, McLean, VA, USA) was used to measure the optical density at 550 nm of the samples relative to matched samples before and after two hours of incubation (37°C, 5% CO_2) with superoxide dismutase (200 U/mL; Sigma). Superoxide dismutase-inhibitable superoxide (nmol) released in 0 to 120 minutes was calculated [23].

Statistical analysis

A randomized block design was used to analyze the data. In this design, the day of the procedure was considered as a block factor. Analysis of variance (ANOVA) for the randomized block design and post hoc analysis (Bonferroni) were performed. In some analyses, paired and unpaired Student *t*-tests were used. In the in vitro PMN assays data values (mean, SEM, EC_{50}) were determined using Graph Pad Prism (San Diego, CA, USA). $P < 0.05$ was used to determine significance.

RESULTS

Dose- and time-dependent reduction of renal injury following ischemia-reperfusion using ATL-146e

Previously, we demonstrated that ATL-146e reduced renal ischemia-reperfusion injury when administered either before or immediately after the ischemic period [2]. The optimal dose and duration of treatment, however, are unknown. Beginning with a treatment regimen known to be effective, we sought to determine the dose of ATL-146e necessary for optimal tissue protection following ischemia-reperfusion injury. Vehicle (group 1) or ATL-146e (groups 5 through 8) was administered beginning five hours prior to the 32-minute ischemic period, and was continued for 24 hours during the reperfusion period. There was a dose-dependent effect of ATL-146e. As observed previously, ischemia-reperfusion injury produced an increase in plasma creatinine that was reduced maximally by 60 to 70% by the A_{2A}-agonist treatment (Fig. 1A and Table 2) [2]. The greatest effect was observed with a 1 to 10 ng/kg/min dose. Using the maximally effective dose of ATL-146e (10 ng/kg/min), a 77% reduction in plasma creatinine was observed at 48 hours (Table 2).

The time course of the ATL-146e effect was evaluated. Following the onset of reperfusion, mice were administered vehicle or 10 ng/kg/min ATL-146e for 2, 4, 6, or 24 hours. As shown in Figure 1B, the magnitude of protection, as assessed by a reduction in plasma creatinine, correlated with the duration of treatment. The greatest degree of protection was observed when the compound was administered during the first six hours of reperfusion. No further significant benefit was observed when treatment was extended to 24 hours.

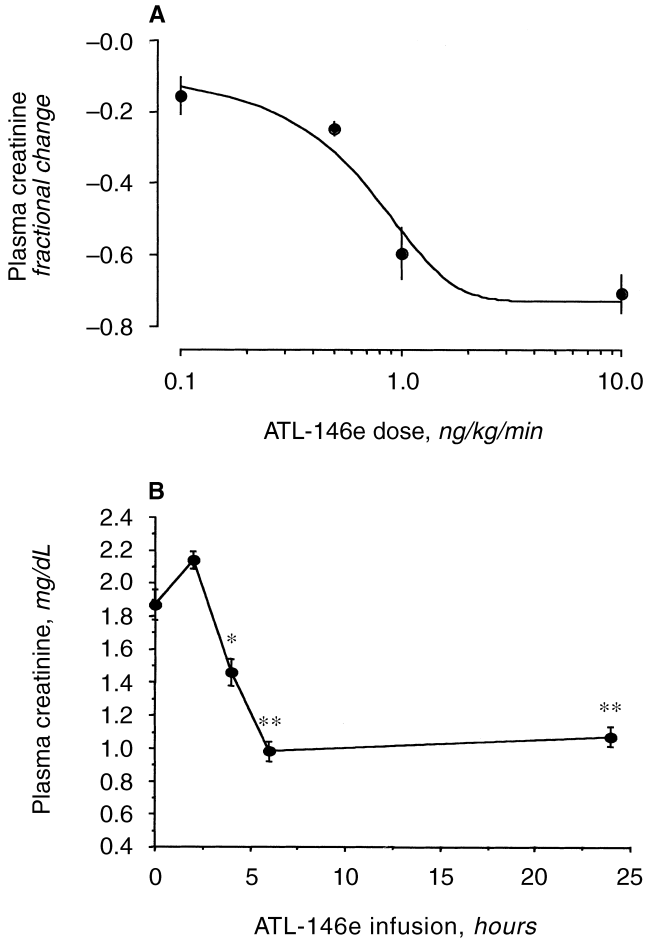

Fig. 1. Dose- and time-dependent reduction in renal injury in mice infused with ATL-146e subjected to ischemia-reperfusion. (*A*) Mouse kidneys were subjected to 32 minutes of ischemia and 24 hours of reperfusion. Vehicle or ATL-146e (0.1, 0.5, 1, and 10 ng/kg/min) was administered continuously via osmotic minipump beginning 5 hours prior to ischemia and continuing through the 24-hour period of reperfusion. Plasma creatinine was measured following 24 hours of reperfusion. (*B*) Kidneys were subjected to ischemia-reperfusion (32 minutes of ischemia/24 hours of reperfusion), and vehicle or 10 ng/kg/min ATL-146e was infused continuously (via catheter inserted subcutaneously) beginning with the onset of reperfusion and continuing for 2, 4, 6, or 24 hours during the reperfusion period. Plasma creatinine was measured following 24 hours of reperfusion. Plasma creatinine levels are expressed as means ± SE. $*P < 0.05$, $**P < 0.001$ when compared with vehicle ($N = 4$ to 5 per group).

PDE 4 inhibition reduces renal injury following ischemia-reperfusion and potentiates the renal protective effect of A_{2A}-agonist treatment

The cellular mechanism underlying the anti-inflammatory effect of A_{2A}-agonists may involve signaling through cAMP/PKA. We reasoned that by inhibiting degradation of cAMP, a more pronounced effect of A_{2A} activation might be achieved. Thus, in initial studies, we first sought to determine whether rolipram, a PDE 4 inhibitor, reduces renal injury when administered prior to and during ischemia-reperfusion injury. Vehicle (group 1) or rolipram at 0.1 (group 2), 1.0 (group 3), or 10 ng/kg/min (group 4) was administered via osmotic minipumps beginning five hours prior to ischemia and continuing through 24 hours of reperfusion. Rolipram alone reduced renal injury, as assessed by a reduction in elevated plasma creatinine levels in a dose-dependent manner. Plasma creatinine values for vehicle and rolipram at 0.1, 1.0, and 10 ng/kg/min were 1.30 ± 0.10, 0.78 ±

Table 2. Effect of combined A_{2A}-agonist and Type IV phosphodiesterase inhibitor on plasma creatinine following ischemia/reperfusion injury

Groups[a]	A Vehicle	B Rolipram	C ATL-146e	D ATL-146e + rolipram	*P* value B vs. D	*P* value C vs. D
ATL 10/ROL 0.1						
24 hours ($N = 9$)	1.39 ± 0.16	0.68 ± 0.13 (0.60)	0.53 ± 0.10 (0.71)	0.28 ± 0.04 (0.88)	< 0.001	< 0.001
48 hours ($N = 7$)	1.56 ± 0.23	0.63 ± 0.11 (0.65)	0.45 ± 0.07 (0.77)	0.28 ± 0.04 (0.89)	< 0.001	< 0.05
ATL 1/ROL 0.01						
24 hours ($N = 5$)	1.30 ± 0.10	0.72 ± 0.10 (0.50)	0.59 ± 0.08 (0.60)	0.28 ± 0.08 (0.84)	< 0.01	< 0.05
ATL 0.5/ROL 0.005						
24 hours ($N = 5$)	1.58 ± 0.08	1.47 ± 0.09 (0.08)	1.22 ± 0.07 (0.25)	1.02 ± 0.05 (0.38)	< 0.001	< 0.01
ATL 0.1/ROL 0.001						
24 hours ($N = 5$)	1.53 ± 0.13	1.42 ± 0.12 (0.08)	1.31 ± 0.13 (0.16)	1.23 ± 0.15 (0.21)	NS	NS

Values are means ± SE of plasma creatinine (mg/dL). Values in parentheses are fractional reduction in plasma creatinine compared to vehicle. Data were analyzed by ANOVA followed by Bonferroni correction for multiple comparisons. Abbreviations are: ATL, ATL-146e; ROL, rolipram.

[a] Groups are identified by dose of ATL and ROL in ng/kg/min and number of hours of reperfusion following ischemia at which time blood samples were taken

0.12, 0.51 ± 0.14, and 1.13 ± 0.18 mg/dL, respectively ($N = 5$ per group, $P = 0.002$, one-way ANOVA). Compared with vehicle-treated mice, treatment with rolipram at doses of 0.1, 1.0, and 10 ng/kg/min ($N = 5$ per group) led to reductions in plasma creatinine of 58, 77, and 43%, respectively. Upon further examination of these data in 5 of 5 mice, rolipram infused at 1.0 ng/kg/min led to a greater reduction in creatinine than the highest dose of 10 ng/kg/min ($P < 0.05$, one-way ANOVA).

Next, a series of experiments was performed using a range of ATL-146e and rolipram at doses starting well below the effective renal protective dose of each compound. These compounds were administered beginning five hours prior to ischemia and continued through 24 hours of reperfusion. The purpose was to determine whether the combined effect of treatment with ATL-146e and rolipram on reducing renal injury was greater than either compound alone (Fig. 2 and Table 2). The administration of rolipram (ROL; 0.001 ng/kg/min; group 9) or ATL-146e (ATL; 0.1 ng/kg/min; group 5) alone or in combination (group 12) in mice subjected to renal ischemia-reperfusion had no significant effect on plasma creatinine when compared with vehicle (group 1). Higher doses of ROL, ATL, and the combination of ROL + ATL led to a dose-dependent reduction in injury as compared with vehicle treatment. Fractional reduction of plasma creatinine for ATL 0.5/ROL 0.005 (group 13, 0.38 ± 0.03 mg/dL) was greater than in 5/5 mice treated with ATL 0.5 alone (group 6, 0.25 ± 0.02 mg/dL, $P < 0.01$) and 5/5 mice treated with ROL 0.005 alone (group 10, 0.08 ± 0.01 mg/dL, $P < 0.001$). Higher doses of the combination of ATL and ROL (groups 14 and 15) produced quantitatively similar results, and the effect was greater than either compound alone (groups 2, 7, 8, and 11). Combining maximally effective doses of ATL and ROL demonstrates enhanced efficacy in reducing renal ischemia-reperfusion injury.

Effect of PDE 4 inhibition and A_{2A}-AR activation on myeloperoxidase activity

Our prior study showed that ischemia-reperfusion injury caused a pronounced infiltration of neutrophils in the kidney, and ATL-146e reduced this neutrophil accumulation [3]. The degree of neutrophil accumulation can be assessed by measuring the activity of MPO, an enzyme that is present in neutrophils and in which the activity correlates with the degree of ischemia-reperfusion injury [3]. To extend these previous findings, we sought to determine whether the combination of rolipram and ATL-146e produced a greater reduction in neutrophil accumulation, as measured by MPO activity in kidney homogenates, than either compound alone. Treatment with rolipram alone (group 2), like ATL-146e alone (group 8), significantly reduced the increase in MPO activity produced by ischemia-reperfusion injury when compared with vehicle-treated mice (group 1). Co-administration of maximally effective doses of ATL 10 and ROL 0.1 (group 15) in mice subjected to ischemia-reperfusion injury led to a further decrease in MPO activity that was greater than either compound alone (Fig. 3).

ATL-146e alone or in combination with rolipram inhibits activated human neutrophil adherence

The administration of ATL-146e alone or in combination with rolipram is associated with a reduction of neutrophil accumulation in kidneys subjected to ischemia-reperfusion (Fig. 3). Such an effect may be mediated in part by effects of these compounds on inflammatory cells or endothelial cells. To determine whether ATL-146e and rolipram alone or in combination may have direct effects on neutrophils to regulate neutrophil adherence factors, we studied neutrophil adherence to biological surfaces. This method permits a direct assessment of the independent effect of ATL-146e/rolipram on neutrophil factors that regulate adherence. Therefore, we sought to determine (*1*) whether ATL-146e by binding to A_{2A}-ARs

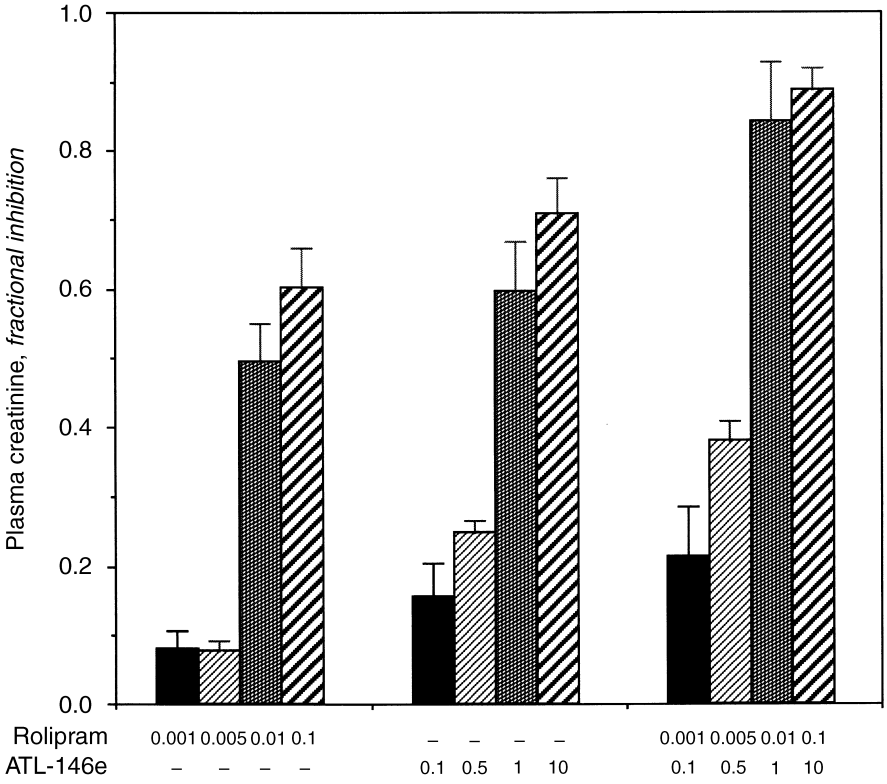

Fig. 2. Enhanced protection from ischemia-reperfusion injury in mice coadministered ATL-146e and rolipram. Kidneys were subjected to ischemia-reperfusion. Vehicle, rolipram alone, ATL-146e alone, or a combination of rolipram and ATL-146e was administered via osmotic minipumps beginning five hours prior to reperfusion and continuing through the reperfusion period in the doses shown. Values are mean ± SE of fractional inhibition in plasma creatinine compared with vehicle treatment. Plasma creatinine values and statistics are shown in Table 2.

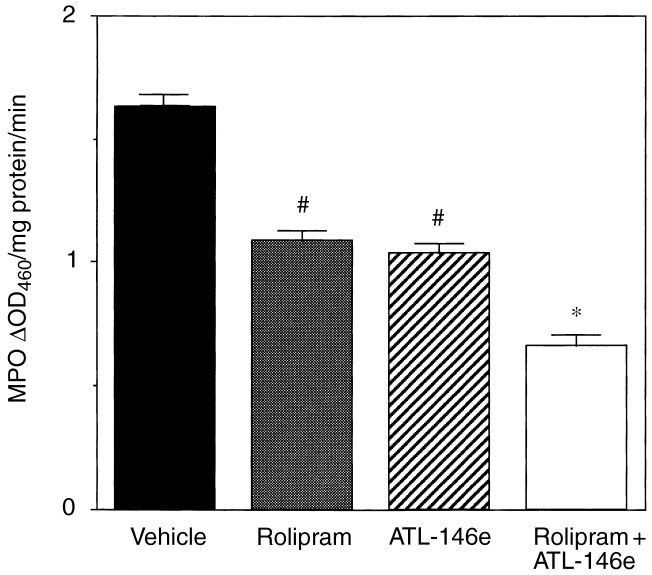

Fig. 3. Enhanced reduction in myeloperoxidase (MPO) activity in mice coadministered ATL-146e and rolipram. Kidneys were subjected to ischemia-reperfusion. Vehicle, 0.1 ng/kg/min rolipram, 10 ng/kg/min ATL-146e, or the combination of 0.1 rolipram and 0.1 and 10 ng/kg/min ATL-146e, respectively, was administered via osmotic minipumps (times of administration as in Fig. 2). MPO activity was measured in kidneys harvested at the end of the reperfusion period. Values are mean ± SE ($N = 7$ for each group). *$P < 0.001$ vs. vehicle, rolipram or ATL-146e; #$P < 0.001$ vs. vehicle.

had a direct effect on neutrophils to decrease neutrophil adhesion to a biological surface and to decrease the resulting oxidative burst of these adhering neutrophils, and (*2*) whether rolipram could potentiate the anti-inflammatory effect of ATL-146e on activated neutrophil adherence and oxidative burst. Calcein-loaded PMNs were activated with 10 U/mL TNF-α, and then the treated PMNs were placed into the fibrinogen-coated wells. The effect of ATL-146e and rolipram on adherence of human PMNs is shown in Figure 4A. In the absence of rolipram, 0.01 to 100 nmol/L ATL-146e reduced activated neutrophil adherence by a maximum of approximately 25%. The addition of rolipram (100 nmol/L), at a concentration that produced relatively little effect alone, potentiated the effect of ATL-146e by reducing neutrophil adherence by approximately 75%. The effect of ATL-146e on the activated neutrophil adherence was blocked in the presence of ZM243185 (100 nmol/L), a selective A_{2A} antagonist (Fig. 4B).

ATL-146e and rolipram inhibit activated human neutrophil oxidative activity in suspended and adherent human PMNs

Previous studies in neutrophil suspensions and neutrophils adherent to biological surfaces indicated differences in neutrophil oxidative activity in response to TNF-α [24]. Therefore, to study the direct effects of ATL-146e and

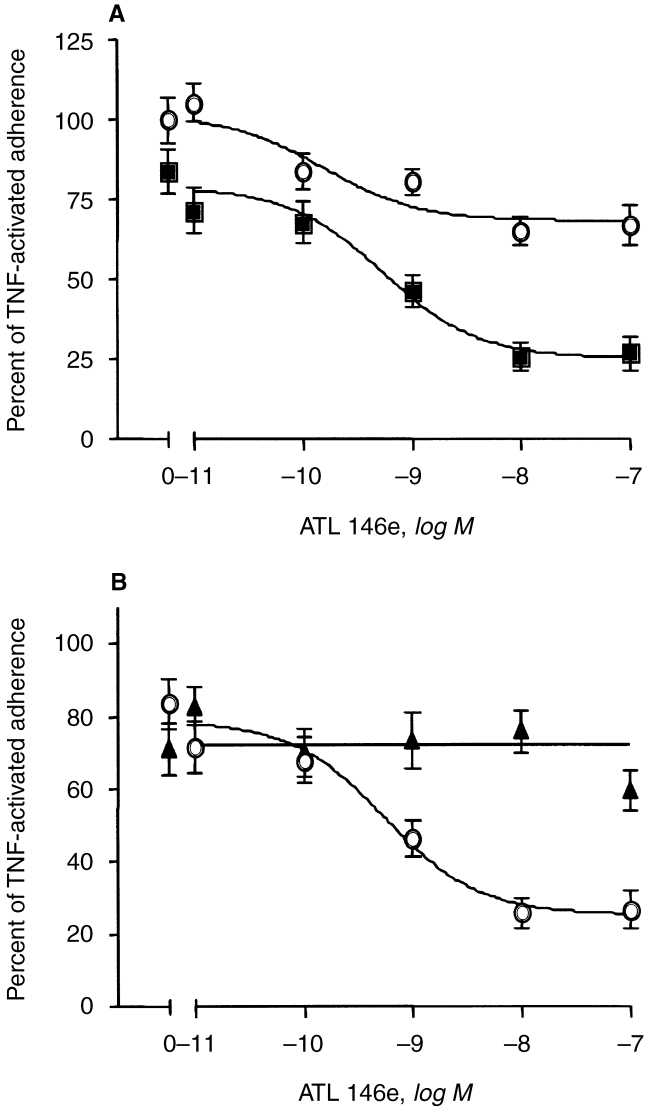

Fig. 4. ATL-146e alone or in combination with rolipram inhibits activated neutrophil adherence of human polymorphoneutrophils (PMNs). The adherence of isolated human neutrophils to biological surfaces was measured as described in the **Methods** section. (*A*) The effects of 0.01 to 100 nmol/L ATL-146e on neutrophil adherence to biological surfaces were assessed in the presence (■) and absence (○) of 100 nmol/L rolipram. The percentage of TNF-activated adherent PMNs was determined by measuring the fluorescence of calcein-loaded neutrophils before and after washing the plates to remove nonadherent cells. (*B*) Specificity of the effects of 0.01 to 100 nmol/L ATL-146e on neutrophil adherence was determined by using 100 nmol/L ZM241385 (▲) or no ZM241385 (○). All studies were done in the presence of 100 nmol/L rolipram (N = 9 to 22). Values are mean ± SE in both panels.

rolipram on neutrophil oxidative activity, the effects of these compounds were examined in two experimental conditions: suspended and adherent PMNs. In suspended human neutrophils, cells were primed with 10 U/mL TNF-α and stimulated with 1 μmol/L fMLP, and luminol-enhanced chemiluminescence was assayed as an index of oxidative activity. Samples of neutrophils incubated with 0.01 to 100 nmol/L ATL-146e had reduced oxidative activity compared with controls. The effect of ATL alone on oxidative activity in suspended cells (Fig. 5A) was much greater than the effect observed on adherent cells (Fig. 5B). However, in both suspended and adhering PMNs, 100 nmol/L rolipram synergistically augmented the inhibitory effect of ATL-146e on the oxidative burst (Fig. 5 A and B, respectively). In suspended cells (Fig. 5A), the dose response curve for ATL-146e was shifted markedly to the left, and the IC_{50} for ATL-146e was reduced sixfold by the addition of rolipram (from 0.451 to 0.073 nmol/L in the absence and presence of rolipram), whereas in adherent cells (Fig. 5B), the IC_{50} was reduced 34-fold (6.974 and 0.209 nmol/L in the absence and presence of rolipram, respectively). To determine whether the effects of ATL-146e on activated neutrophil oxidative burst were mediated by A_{2A} receptors, ZM243185 was used. The effect of ATL-146e on activated neutrophil adherence oxidative burst in suspended (Fig. 5C) or adhering cells (Fig. 5D) was blocked in the presence of 100 nmol/L ZM243185.

ATL-146e and rolipram inhibit activated human neutrophil oxidative activity in adherent and suspended human PMNs via cAMP/PKA

To determine the contribution of the cAMP/PKA pathway as a signaling pathway to neutrophil oxidative activity, the effect of ATL-146e on cAMP accumulation was determined in activated neutrophils. Incubation of TNF-activated human neutrophils with 3 to 300 nmol/L ATL-146e led to a dose-dependent increase in cAMP (Fig. 6A), an effect that was enhanced in the presence of rolipram. To determine whether the effect of ATL-146e on human neutrophil oxidative activity was mediated by an effect on cAMP-dependent PKA activity, the selective PKA inhibitor, H-89 (0.1 to 10 μmol/L) was used [25]. Although H-89 had no effect on neutrophil oxidative activity in the absence of ATL-146e, it effectively blocked inhibition of the oxidative burst by 10 nmol/L ATL-146e (Fig. 6B). These results suggest that the decrease in neutrophil oxidative activity produced by the selective activation of A_{2A}-ARs expressed on neutrophils may be due to increased cAMP and activation of PKA and could provide a mechanism for the reduction of renal injury observed with ATL-146e in ischemia-reperfusion.

DISCUSSION

The purpose of this study was to test the hypothesis that agonists of A_{2A} adenosine receptors and phosphodiesterase inhibitors produce an enhanced reduction of renal tissue injury when administered together. The rationale for these studies stems from (*1*) our previous work demonstrating potent reduction of renal injury

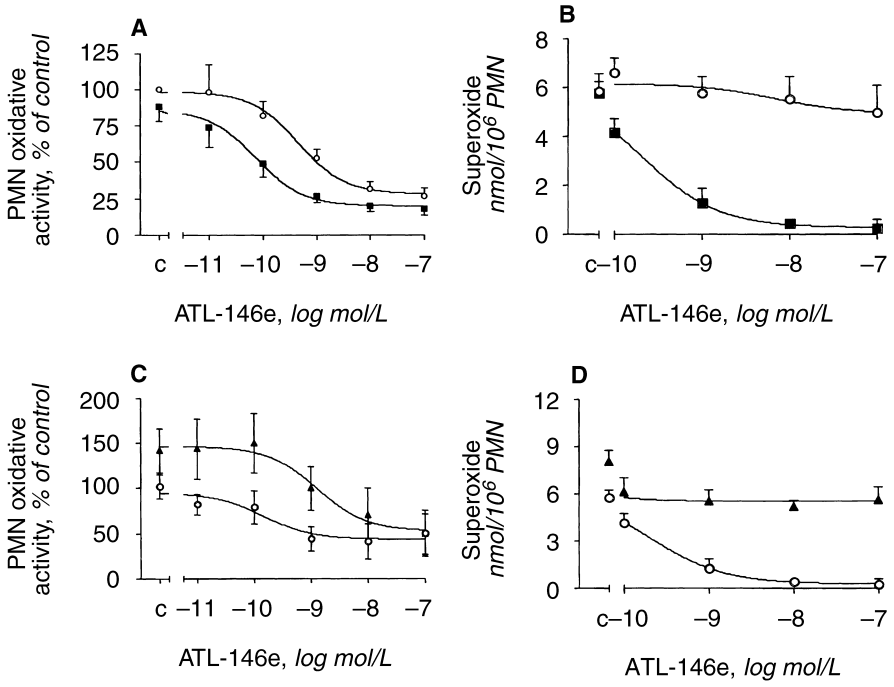

Fig. 5. ATL-146e and rolipram potently reduce oxidative activity in adherent and suspended human PMNs. The effect of ATL-146e on neutrophil oxidative activity on suspensions of PMNs is shown in (*A* and *C*), and the effect on adherent PMNs is shown in (*B* and *D*). The method for measurement of neutrophil oxidative activity is described in the **Methods** section. Symbols in A and B are: (○) no rolipram; (■) rolipram. Symbols in C and D are: (▲) ZM243185; (○) no ZM243185. (A) The bioactivity of ATL-146e on TNF-α–primed, fMLP-stimulated human PMN oxidative activity ± 100 nmol/L rolipram as measured by luminol-enhanced chemiluminescence. Chemiluminescence is reported as relative peak light emitted (= height of the curve), compared with TNFα-primed fMLP-stimulated control samples ($N = 5$). (B) The effect of 0.1 to 100 nmol/L ATL-146e on the oxidative activity of adherent neutrophils was examined ± 100 nmol/L rolipram. Oxidative activity is expressed as the release of superoxide from cells incubated with cytochrome c and stimulated with recombinant human TNF-α ($N = 5$). (C) The specificity of effect of ATL-146e on neutrophil oxidative activity in suspended PMNs was determined ± ZM243185 ($N = 6$). (D) The specificity of effect of ATL-146e on neutrophil oxidative activity in adherent PMNs was determined ± 100 nmol/L ZM243185. All studies were done in the presence of rolipram ($N = 9$ to 22). Values in all panels are given as mean ± SE.

when the novel A_{2A}-agonist ATL-146e is administered either before or immediately after the onset of reperfusion [2], and (*2*) work by others demonstrating a reduction of renal injury following infusion of a PDE 4 inhibitor [16, 17]. Because both types of compounds act along different pathways to increase intracellular cAMP accumulation, we evaluated the effects of combination therapy and found that treatment with both compounds together has a greater effect in reducing renal injury than either compound alone. Furthermore, this reduction in tissue injury may be due, in part, to direct effects of ATL-146e and rolipram on neutrophils to decrease oxidative activity.

We previously demonstrated a profound reduction in injury when ATL-146e administration was initiated before ischemia or immediately after the ischemic period and continued through the reperfusion period [2, 3]. In mice subjected to ischemia-reperfusion, we observed a maximal reduction in plasma creatinine of up to 60% following treatment for 24 to 48 hours. We now extend those studies by examining the dose response characteristic of ATL-146e and the treatment duration in reducing ischemia-reperfusion injury. ATL-146e infusions of 1 and 10 ng/kg/min during the 24-hour reperfusion period produced a similar reduction of plasma creatinine of approximately 70 and 80%, respectively. Lower doses were less effective, however. Our current studies also indicate that infusion of compound during the first six hours of the reperfusion period produces maximal tissue protection. No further benefit was observed when the infusion was continued for 24 hours; however, tissue protection was maintained during the remainder of the 24-hour period. We cannot exclude the possibility that prolonged infusion of 24 hours produced a measurable beneficial effect, if a marker more sensitive than plasma creatinine had been used to assess the extent of injury and function (glomerular filtration rate, urinary tubular enzymes, etc.). It can be concluded from these experiments that maximal protection is observed when 1 to 10 ng/kg/min of ATL-146e is maintained for six hours during the reperfusion period. These findings have important clinical implications. The low dose of ATL-146e and the short duration of treatment will likely minimize any untoward side effects that are potentially associated with the use of this agent.

Cyclic nucleotide phosphodiesterases are responsible for the degradation of cGMP and cAMP [26]. The PDE family includes a list of specific phosphodiesterases that currently include 11 isoforms [15]. The PDE 4 family comprises four genetically different subtypes, and inhibitors of this group of enzymes produce anti-inflammatory effects [15]. In studies of the kidney, inhibitors of PDE 4 have been found to reduce renal injury in models of acute renal failure [16, 17] and glomerulonephritis [27].

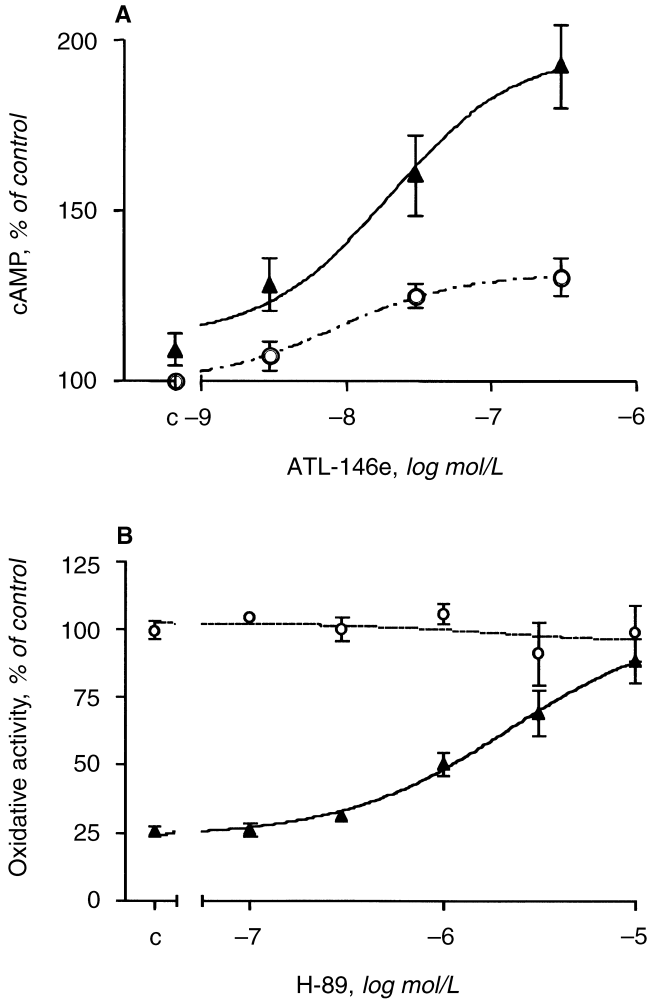

Fig. 6. ATL-146e and rolipram potently reduce oxidative activity in human PMNs via cAMP/PKA. (*A*) The effect of ATL-146e on cAMP accumulation in activated human PMNs is shown. PMNs were incubated (3 to 5 × 10^6 PMNs/0.5 mL/sample) with 3 to 300 nmol/L ATL-146e and without (○) or with (▲) 300 nmol/L rolipram for 45 minutes at 37°C. Reactions were stopped, and cAMP was determined by radioimmunoassay (N = 5). (*B*) The effect of H-89, a PKA inhibitor, on activated human PMNs is shown. Neutrophils were incubated with 0.1 to 10 μmol/L H89 without (○) or with (▲) ATL-146e, and oxidative activity was measured (N = 6). Values are mean ± SE in all panels.

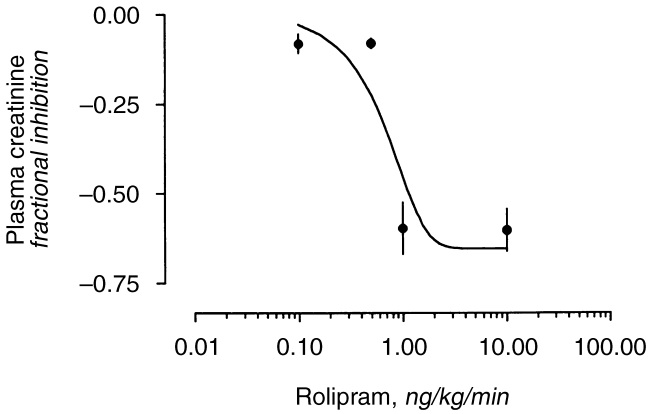

Fig. 7. Dose-dependent reduction in renal injury in mice infused with rolipram and subjected to ischemia/reperfusion. A portion of the data from Table 2 is redrawn to show the dose response relationship between fractional reduction in plasma creatinine and 0.001 to 0.1 ng/kg/min doses of rolipram. Mice were subjected to 32 minutes of ischemia and 24 hours of reperfusion. Vehicle or rolipram was administered via osmotic minipumps beginning five hours prior to reperfusion and continuing through the reperfusion period in the doses shown. Values are mean ± SE of the fractional change in plasma creatinine from vehicle treatment. Numbers and statistics are shown in Table 2.

Infusion of 10 μg/kg/min Ro20-1724, a selective and potent PDE 4 inhibitor, attenuated the observed reduction of renal blood flow and glomerular filtration and improved survival in endotoxin-mediated acute renal failure [16, 17]. Although these observations could represent a direct effect on maintaining favorable renal hemodynamics, they could also reflect the benefits of reducing inflammation within the kidney. By increasing cAMP in inflammatory cells, PDE 4 inhibitors decrease inflammation [15, 22].

Our studies demonstrate a dose-dependent effect of rolipram to reduce the injury associated with ischemia-reperfusion (Fig. 7 and Table 2). In mice subjected to renal ischemia-reperfusion, infusion of rolipram at 0.01 to 0.1 ng/kg/min reduced plasma creatinine by 50 to 60%. This reduction in renal injury was associated with a significant reduction of inflammatory cell accumulation, as assessed by MPO activity. MPO is an enyzme primarily present in neutrophils as well as in mononuclear cells. Thus, its activity likely represents contributions from both cell types. It is striking that these doses are 10^5 to 10^6 lower than those used previously in studies with Ro20-1724 [16, 17], and therefore, our observations may reflect the effect of doses that have primarily anti-inflammatory rather than hemodynamic properties. Although these two studies used different PDE 4 inhibitors, both Ro20-1724 (IC_{50} 2 μmol/L) and rolipram (IC_{50} 0.8 μmol/L) have similar affinities for PDE 4. It is interesting to note in our preliminary dose-response studies using higher doses of rolipram (0.1, 1.0, and 10 ng/kg/min), the highest dose was associated with a smaller reduction in plasma creatinine than the intermediate dose. This result suggests that higher doses of rolipram may produce other effects in addition to PDE 4 inhibition.

Both ATL-146e and rolipram increase cAMP by different mechanisms. Whereas rolipram reduces cAMP degradation by blocking PDE 4, ATL-146e increases cAMP synthesis through its action on stimulatory guanine nucleotide proteins and activation of adenylyl cyclase. Thus, we hypothesized that the combination of the two compounds would be more potent in reducing renal ischemia-reperfusion when coadministered. The results indicate that the co-administration of ATL-146e and rolipram produced a potent inhibition of renal injury

that was greater than either compound alone (Fig. 2 and Table 2).

There are several potential mechanisms that may contribute to the renal protective effect of the combination of ATL-146e and rolipram. First, both compounds in high doses are vasodilators. A_{2A} agonists, through the activation of A_{2A}-ARs, produce medullary vasodilation [28]. However, at low doses (4 ng/kg/min), ATL-146e has no effect on systemic blood pressure and heart rate [2]. Thus, an even lower dose of ATL-146e (1 ng/kg/min) is unlikely to have significant systemic hemodynamic effects. Ro20-1724 at doses of 10 μg/kg/min produced significant effects on renal blood flow and glomerular filtration rate [16, 17]; however, our doses of rolipram were 10^5 to 10^6 lower. Although systemic hemodynamic parameters were not measured, rolipram is unlikely to produce effects at these doses. Our studies did not include measurement of regional blood flow in the kidney. Although there was no effect on systemic hemodynamics, we cannot exclude a favorable effect to increase medullary circulation. Such an effect could increase oxygenation to an area that is relatively hypoxic. It is possible that these compounds could increase medullary flow by two mechanisms: These compounds alone or in combination may improve medullary circulation by a direct effect on renal vasculature. Additionally, it is possible that by reducing inflammatory cell infiltration, capillary plugging is reduced in the vasa recta in the outer stripe of the outer medulla. Direct microcirculatory studies could provide additional important information on the mechanism of tissue protection by A_{2A} agonists and type IV PDE inhibitors.

Second, both compounds are known to produce robust effects on inflammatory responses. It is well known that activated neutrophils or endothelial cells release and respond to adenosine, an effect that appears to be mediated by A_{2A}-ARs [10–13]. Activation of A_{2A}-ARs expressed on activated neutrophils reduces the release of reactive oxygen metabolites [12, 29–32], and adenosine may also interfere with neutrophil adherence to endothelial cells [33]. PDE 4 inhibitors also have known anti-inflammatory effects, which is due, in part, to the prominent expression of a low K_m cAMP specific member of family of PDE in inflammatory cells [34].

To determine whether the reduction in plasma creatinine, elicited by ATL-146e in mice subjected to ischemia-reperfusion could be due in part to a direct action on neutrophils, we performed in vitro studies on isolated human neutrophils. Our studies focused on the participation of neutrophils as targets of A_{2A}-agonist action. Although a number of studies demonstrated the pathophysiological role of neutrophils in ischemia-reperfusion injury [5], controversy still exists as not all studies support the role of neutrophils in ischemic renal injury [35, 36]. Recent evidence indicates that in addition to neutrophils, T-cells, which have been largely overlooked, may play an important role in ischemia-reperfusion injury [37]. Although our studies focused on neutrophils, ATL-146e could also mediate protective effects, in part through its action on mononuclear cells. Given the obvious limitations of sample size in mice, we chose to use human neutrophils because of the ease in obtaining large enough quantities to perform in vitro studies. This study found that ATL-146e reduced neutrophil oxidative activity, an effect that was potentiated in the presence of rolipram (Fig. 5 A, B). Thus, the pronounced renal protection observed with the combined infusion of ATL-146e and rolipram may be mediated, in part, by a direct effect on neutrophils to decrease oxidative activity. Moreover, the effect was blocked in the presence of H-89, a PKA inhibitor, suggesting that the effect was mediated by cAMP (Fig. 6B).

In kidneys subjected to ischemia-reperfusion, we demonstrated that the combined administration of ATL-146e and rolipram had a greater effect to reduce MPO activity than either compound alone. An effect of ATL-146e and/or rolipram to decrease neutrophil adhesion and accumulation in kidneys subjected to ischemia-reperfusion may be mediated by direct effects on neutrophils or by effects on endothelial cells to regulate the expression of adhesion molecules. Evidence for the latter stems from studies that show that ischemia-reperfusion increases endothelial cell adhesion molecule expression, an effect that is blocked by infusion of ATL-146e [3]. To determine whether ATL-146e and rolipram alone or combined may have direct effects on neutrophils to regulate neutrophil adherence factors, we studied neutrophil adherence to biological surfaces, a method that permits a direct assessment of the independent effect of ATL-146e/rolipram on neutrophil. These studies demonstrate that ATL-146e alone decreased neutrophil adherence. Moreover, this effect by ATL-146e on neutrophil adherence was markedly potentiated by the addition of rolipram. Taken together with our previous study [3], our results suggest that ATL-146e and rolipram decrease adherence by altering both endothelial and neutrophil adherence factors. Such an effect on neutrophils could be explained by ATL-146e's ability to decrease the heightened activated neutrophil expression of CD11b/CD18. However, studies using a high concentration (1 μmol/L) of a less selective A_2 agonist, 5′N-ethylcarboxamidoadenosine (NECA), failed to show regulation of CD11b/CD18 expression [38]. Thus, the mechanism responsible for the regulation of neutrophil adherence by ATL-146e and rolipram is largely unknown.

The therapeutic possibilities for the use of ATL-146e or other highly selective agonists of A_{2A}-ARs are quite apparent from the foregoing discussion. First, ATL-146e is capable of maximally reducing renal injury at extremely low concentrations that are not known to pro-

duce systemic hemodynamic effects [2]. In rats, with infusion rates of 4 ng/kg/min, the plasma level of ATL-146e was less than 1 nmol/L. In our dose response experiments, renal tissue protection from ischemia-reperfusion injury was observed with doses as low as 1 ng/kg/min. The fact that systemic hemodynamic effects are not observed with even higher doses of ATL-146e should make it useful clinically. Second, the duration of therapy can be limited to six hours following ischemia-reperfusion injury. This finding suggests that treatment can be limited to a short period and thus avoid the possibility of any adverse events from prolonged treatment. Third, the addition of a low dose of a type IV phosphodiesterase inhibitor produces additional protection when combined with a low dose of an A_{2A} agonist. The low dose of rolipram will reduce the likelihood of potential side effects of the PDE 4 inhibitor. Fourth, our approach likely involves multiple potential targets, including neutrophils and endothelial cells, to reduce neutrophil-endothelial cell adhesion and function. The advantage of such an approach derives from the fact that many cellular functions are redundant, and disabling one proinflammatory protein may be compensated for by another protein. By targeting several factors involved in inflammation, the use of selective A_{2A} agonists and phosphodiesterase inhibitors is likely to produce a greater protective effect. Thus, the use of A_{2A} agonists alone or in combination with PDE 4 inhibitors, in very low doses, holds promise for future clinical trials in ischemia-reperfusion injury. Moreover, our current study demonstrates the value of combination therapy and suggests that future studies target such strategies [9].

ACKNOWLEDGMENTS

This work was supported in part by funds from National Institutes of Health RO1 DK56223-01, 1R41 DK58413, AHA Grant-in-Aid 0050329N (to M.D.O.) and National Institutes of Health R01 HL37942 (to J.L.). M.D.O. was a recipient of the Clinical Scientist Award from the National Kidney Foundation (CSA-16). The authors gratefully acknowledge Dr. Timothy Macdonald and Jayson Rieger (Department of Chemistry, University of Virginia, Charlottesville, VA, USA) for the generous gift of ATL-146e. A portion of this work was published in preliminary form (*J Am Soc Nephrol* 10:638A, 1999).

Reprint requests to Mark D. Okusa, M.D., Division of Nephrology, Box 133, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908, USA.
E-mail: mdo7y@virginia.edu

APPENDIX

Abbreviations used in this article are: ATL, ATL-146e; cAMP, cyclic adenosine 3′,5′ monophosphate; fMLP, f-met-leu-phe; G_s-protein, stimulatory G-protein; HBSS, Hank's balanced salt solution; ICAM, intercellular adhesion molecule; MPO, myeloperoxidase; NECA, 5′-N-ethylcarboxamidoadenosine; PDE 4, Type IV phosphodiesterase; PKA, protein kinase A; PMNs, polymorphoneutrophils; ROL, rolipram; TNF-α, tumor necrosis factor-α.

REFERENCES

1. Jarvis MF, Schulz R, Hutchison AJ: [^{3}H]CGS 21680, a selective A_2 adenosine receptor agonist directly labels A2 receptors in rat brain. *J Pharmacol Exp Ther* 251:888–893, 1989
2. Okusa MD, Linden J, Macdonald T, Huang L: Selective A_{2A}-adenosine receptor activation during reperfusion reduces ischemia-reperfusion injury in rat kidney. *Am J Physiol* 277:F404–F412, 1999
3. Okusa MD, Linden J, Huang L, *et al:* A_{2A}-adenosine receptor mediated inhibition of ischemia reperfusion injury and neutrophil adhesion in rat and mouse kidneys. *Am J Physiol* 279:F809–F818. 2000
4. Thadhani R, Pascual M, Bonventre JV: Acute renal failure. *N Engl J Med* 334:1448–1460, 1996
5. Rabb H, O'Meara YM, Maderna P, *et al:* Leukocytes, cell adhesion molecules and ischemic acute renal failure. *Kidney Int* 51:1463–1468, 1997
6. Kelly KJ, Williams WW, Colvin RB, Bonventre JV: Antibody to intercellular adhesion molecule 1 protects the kidney against ischemic injury. *Proc Natl Acad Sci USA* 91:812–816, 1994
7. Kelly KJ, Williams WW, Colvin RB, *et al:* Intercellular adhesion molecule-1-deficient mice are protected against ischemic renal injury. *J Clin Invest* 97:1056–1063, 1996
8. Molitoris BA, Marrs J: The role of cell adhesion molecules in ischemic acute renal failure. *Am J Med* 10:583, 1999
9. Kelly KJ, Molitoris BA: Acute renal failure in the new millennium: Time to consider combination therapy. *Semin Nephrol* 20:4–19, 2000
10. Basford RE, Clark RL, Stiller RA, *et al:* Endothelial cells inhibit receptor-mediated superoxide anion production by human polymorphonuclear leukocytes via soluble inhibitor. *Am J Respir Cell Mol Biol* 2:235–243, 1990
11. Gunther GR, Herring MB: Inhibition of neutrophil superoxide production by adenosine released from vascular endothelial cells. *Ann Vasc Surg* 5:325–330, 1991
12. Cronstein BN, Kramer SB, Weissman G, Hirschhorn R: Adenosine: A physiological regulator of superoxide anion generation by human neutrophils. *J Exp Med* 158:1160–1177, 1983
13. Cronstein BN, Levin RI, Belanofff J, *et al:* Adenosine: An endogenous inhibitor of neutrophil-mediated injury to endothelial cells. *J Clin Invest* 78:760–770, 1986
14. Sullivan GW, Linden J, Buster BL, *et al:* A_{2A} adenosine receptor inhibits inflammation in a rat model of meningitis: Synergy with the type IV phosphodiesterase inhibitor. *Rolipram J Infect Dis* 180:1550–1560, 1999
15. Torphy TJ, Page C: Phosphodiesterases: The journey towards therapeutics. *Trends Pharmacol Sci* 21:157–259, 2000
16. Carcillo JA, Herzer WA, Thomas NJ, Jackson EK: Treatment with type IV phosphodiesterase inhibitor RO20-1724 protects renal and mesenteric blood flow in endotoxemic rats treated with norepinephrine. *J Pharmacol Exp Ther* 279:1197–1204, 1996
17. Begany DP, Carcillo JA, Herzer WA, *et al:* Inhibition of type IV phosphodiesterase by Ro-01724 attenuates endotoxin-induced acute renal failure. *J Pharmacol Exp Ther* 278:37–41, 1996
18. Dongmin M, Ping W, Egan RW, *et al:* Phosphodiesterase 4B gene transcription is activated by lipopolysaccharide and inhibited by interleukin 19 in human monocytes. *Mol Pharmacol* 55:50–57, 1999
19. Bradley PP, Priebat DA, Christensen RD, Rothstein G: Measurement of cutaneous inflammation: Estimation of neutrophil content with enzyme marker. *J Invest Dermatol* 78:206–209, 1982
20. Ferrante A, Thong YH: Optimal conditions for simultaneous purification of mononuclear and polymorphonuclear leukocytes from human blood by hypaque-ficoll method. *J Immunol Methods* 36:109–117, 1980
21. Petersen TK, Britta VB, Jensen AL: Determination of the adhesive capability of canine polymorphonuclear neutrophil granulocytes using a fluorometric microtiter plate cellular adhesion assay. *Vet Immunol Pathol* 68:283–291, 1999
22. Sullivan GW, Carper HT, Mandell GL: The specific type IV phosphodiesterase inhibitor rolipram combined with adenosine reduces tumor necrosis factor-α (TNF-α)-primed neutrophil oxidative activity. *Int J Immunopharmacol* 17:793–803, 1995

23. Van Gelder BF, Slate EC: The extinction coefficient of cytochrome c. *Biochim Biophys Acta* 58:593–596, 1962
24. Nathan CF: Neutrophil activation on biological surfaces: Massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. *J Clin Invest* 80:1550–1560, 1987
25. Chijiwa T, Mishima A, Hagiwara M, *et al:* Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *J Biol Chem* 265:5267–5272, 1990
26. Beavo JA: Cyclic nucleotide phosphodiesterases: Functional implications of multiple isoforms. *Physiol Rev* 75:725–748, 1995
27. Tsuboi Y, Shankland SJ, Grande JP, *et al:* Suppression of mesangial proliferative glomerulonephritis development in rats by inhibitors of cAMP phosphodiesterase isozymes types III and IV. *J Clin Invest* 98:262–270, 1996
28. Silldorff EP, Kreisberg MS, Pallone TL: Adenosine modulates vasomotor tone in outer medullary descending vasa recta of the rat. *J Clin Invest* 98:18–23, 1996
29. Cronstein BN, Rosenstein ED, Kramer SB, *et al:* Adenosine; a physiological modulator of superoxide anion generation by human neutrophils. Adenosine acts via an A2 receptor on human neutrophils. *J Immunol* 135:1366–1371, 1985
30. Roberts PA, Newby AC, Hallett MB, Cambell AK: Inhibition by adenosine of reactive oxygen metabolite production by human polymorphonuclear leukocytes. *Biochem J* 227:669–674, 1985
31. Schmeichel CJ, Thomas LL: Methylxanthine bronchodilators potentiate multiple human neutrophil functions. *J Immunol* 138:1896–1903, 1987
32. Schrier DJ, Imre KM: The effects of adenosine agonists on human neutrophil function. *J Immunol* 137:3284–3289, 1986
33. Bullough DA, Magill MJ, Firestein GS, Mullane KM: Adenosine activates A_2 receptors to inhibit neutrophil adhesion and injury to isolated cardiac myocytes. *J Immunol* 155:2579–2596, 1995
34. Dent G, Giembycz MA: Interaction of PDE4 inhibitors with enzymes an cell function, in *Phosphodiesterase Inhibitors*, London, Academic Press, 1996, pp 115–119
35. Rabb H, Ramirez G, Saba SR, *et al:* Renal ischemic-reperfusion injury in l-selectin-deficient mice. *Am J Physiol* 271(2 Pt 2):F408–F413, 1996
36. Paller MS: Effect of neutrophil depletion on ischemic renal injury in the rat. *J Lab Clin Med* 113:379–386, 1989
37. Rabb H, Daniels F, O'Donnell M, *et al:* Pathophysiological role of T lymphocytes in renal ischemia-reperfusion injury. *Am J Physiol* 279:F525–F531, 2000
38. Cronstein BN, Levin RI, Philips M, *et al:* Neutrohil adherence to endothelium is enhances via adenosine A_1 receptors and inhibited via adenosine A receptors. *J Immunol* 148:2201–2206, 1992