Selective A\textsubscript{2A} adenosine receptor activation reduces ischemia-reperfusion injury in rat kidney

**Mark D. Okusa, Joel Linden, Timothy Macdonald, and Liping Huang.** Selective A\textsubscript{2A} adenosine receptor activation reduces ischemia-reperfusion injury in rat kidney. Am. J. Physiol. 277 (Renal Physiol. 46): F404–F412, 1999.—A\textsubscript{2A} adenosine receptors (A\textsubscript{2A}-ARs) are known modulators of renal hemodynamics and potent inhibitors of inflammation. We sought to determine whether selective activation of A\textsubscript{2A}-ARs protects kidneys from ischemia-reperfusion injury. The ester derivative of DWH-146 (DWH-146e), a selective A\textsubscript{2A} agonist, was found to be more potent and selective for A\textsubscript{2A}-ARs than the prototype compound CGS-21680. Osmotic minipumps were implanted subcutaneously to infuse into rats either vehicle or DWH-146e (0.004 µg·kg\textsuperscript{-1}·min\textsuperscript{-1}), during and after ischemia-reperfusion injury. Following 24 and 48 h of reperfusion, the rise in serum creatinine and blood urea nitrogen for vehicle-treated rats was substantially elevated compared with DWH-146e-treated rats. Histological examination revealed widespread tubular epithelial necrosis and vascular congestion in the outer medulla of vehicle-treated compared with DWH-146e-treated animals. ZM-241385, a selective A\textsubscript{2A} antagonist, blocked the protective effect of DWH-146e. Delaying administration of DWH-146e until the initiation of reperfusion also decreased serum creatinine. We conclude that 1) selective A\textsubscript{2A}-AR activation by DWH-146e reduces ischemia-reperfusion injury in rat kidneys, 2) the effect of DWH-146e is A\textsubscript{2A} receptor mediated, and 3) the protective effects are mediated by preventing injury during the reperfusion period.

**Acute renal failure; ischemia; inflammation; DWH-146 ester; ZM-241385**

**Acute Renal Failure** is a common disorder affecting ~5% of all hospitalized patients and is associated with a mortality rate of ~50% (2). In ischemic acute renal failure, loss of renal blood supply results in tissue hypoxia and leads to a complex cascade of events resulting in renal injury (for a review see Ref. 35). Classically, acute renal injury from ischemia has been thought to result from unfavorable changes in renal blood flow as a consequence of vasospasm, alterations in ultrafiltration coefficient, tubular obstruction, and/or backleak. The renal medulla is particularly susceptible to renal ischemia because of the low oxygen tension in this region (P\textsubscript{O\textsubscript{2}} of 10–20 mmHg). With renal ischemia, oxygen content is reduced even farther. Vascular occlusion secondary to hypoxic injury or reperfusion injury results in endothelial cell dysfunction that alters the balance of vascular tone of vasoactive agents such as endothelin and nitric oxide (35) and triggers the release of cytokines. Released cytokines trigger the adhesion and infiltration of inflammatory cells and the release from these cells of toxic reactive oxygen intermediates. This process leads to vascular congestion, tubule injury, and obstruction (28, 35).

Adenosine receptors (ARs) are thought to contribute to renal injury. Adenosine binds to adenosine receptors, members of the G protein-coupled receptor family that include four subtypes: A\textsubscript{1}, A\textsubscript{2A}, A\textsubscript{2B}, and A\textsubscript{3}-ARs (for a review see Ref. 24). Nonselective antagonists of adenosine receptors (3) and selective A\textsubscript{1} antagonists (22) reduce renal injury in models of acute renal failure. The mechanism of A\textsubscript{1}-AR antagonist action has been attributed to a decrease in adenosine-mediated vasoconstriction caused by A\textsubscript{1}-ARs that are expressed on afferent arterioles (33).

In addition to the renal effects of adenosine, studies over the last 10 years have demonstrated that adenosine has a direct effect on neutrophils and on endothelial cells to reduce inflammation (for a review, see Ref. 9). A novel physiological role of endogenous adenosine became apparent following the demonstration that activated neutrophils or endothelial cells release and respond to adenosine (9). Several groups of investigators have demonstrated that adenosine binds to A\textsubscript{2A}-ARs expressed on activated neutrophils to reduce the release of reactive oxygen species, such as superoxide anion or hydrogen peroxide (9) and to reduce neutrophil adherence to endothelial cells (7). These studies have provided evidence that the effects of adenosine to inhibit leukocyte adherence to endothelial cells may contribute to adenosine’s protective effects in ischemia-reperfusion injury in lung (15), heart (14), and brain (25). Given the potential beneficial effects of selective A\textsubscript{2A}-AR activation, we sought to determine whether selective activation of A\textsubscript{2A}-ARs reduces renal ischemia-reperfusion injury. The results indicate that an A\textsubscript{2A} agonist affords substantial protection from injury, even when administered only during the reperfusion period.

**Methods**

DWH-146e. We synthesized an adenosine analog highly selective for A\textsubscript{2A}-ARs by making substitutions at the C-2 and 5' positions. The selectivity of the ester derivative of DWH-146 (DWH-146e) for adenosine receptor subtypes was determined in radioligand studies to membranes derived from HEK-293 cells transfected with recombinant human A\textsubscript{2A}-ARs and to membranes from rat striatum, a rich source of A\textsubscript{2A}-ARs (30).

Cell culture and expression of human A\textsubscript{2A}-ARs in HEK-293 cells. HEK-293 cells were maintained in DMEM-F12 (GIBCO-BRL; Life Technologies, Rockville, MD) supplemented with 10% (vol/vol) fetal bovine serum (GIBCO-BRL) and penicillin

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(100 U/ml)/streptomycin (100 µg/ml) (GIBCO-BRL) under a 5% CO₂ atmosphere at 37°C. Subculturing was performed every 4–5 days. Human A₂A-AR cDNA previously subcloned into pDT was used to transfect HEK-293 cells as previously described (29) by means of Lipofectin (12). Colonies were selected by growth in G418 (0.5 g/l) (GIBCO-BRL). G418 was reduced to 0.25 g/l in stock flasks and eliminated when expanding cells for harvest.

Radioligand binding. Competitive binding assays were performed in HEK-293 cells transfected with human recombinant A₂A-ARs as previously described (29). We compared binding affinities of DWH-146e, DWH-146a (the acid derivative of DWH-146), and (2-p-carboxyethyl)phenethylamine-5'-ethylcarboxamidoadenosine HCl (CGS-21680; Research Biochemicals International, Natick, MA), a well-characterized potent and selective agonist of A₂A-ARs (13). To measure binding to A₂A-ARs, membranes were incubated with an A₂A-selective antagonist, ¹²⁵I-ZM-241385 (0.5 nM) (27) in the presence of competing concentrations of (0.01 nM to 10 µM) DWH-146e, DWH-146a, or CGS-21680 for 2 h at 21°C. Additional experiments were performed to determine the selectivity of DWH-146e to adenosine receptor subtypes. Membranes from HEK-293 cells transfected with human recombinant A₁, A₂A, A₂B, and A₃ were subjected to competitive binding studies. HEK-293 membranes expressing A₁, A₂A, A₂B, or A₃ were incubated with 0.5–1 nM of ¹²⁵I-(3-iodo-4-aminobenzyl)adenosine (¹²⁵I-ABA), ¹²⁵I-ZM-241385, ¹²⁵I-1-propyl-3-(3-iodo-4-aminobenzyl)-8-4-oxycacetatephenylxanthine (¹²⁵I-ABOPX), or ¹²⁵I-ABA, respectively (gift from Dr. Susan Deluge, Galazo-Welcome, Research Triangle, NC). These radioligands exhibit high affinity for adenosine receptor subtypes (20). Radioligand binding to membranes was competed with DWH-146e, CGS-21680, and ZM-241385. Four milliliters of ice-cold wash buffer (25 mM Tris HCl, pH 7.2) was added to terminate the reaction, and the membranes were filtered through Whatman GF/C filters. Filters were washed twice with 4 ml of wash buffer. Bound radioactivity was determined by liquid gamma counting. Data were analyzed by nonlinear regression (GraphPad Prism version 2.0; GraphPad Programs, San Diego, CA). IC₅₀ values were converted to Kᵢ values by the following equation: Kᵢ = IC₅₀ × [L]/(L + Kₐ), where L is the concentration of the radioligand and Kₐ is the equilibrium dissociation constant of the ligand.

cAMP assays. Camp accumulation was measured to assess the effect of DWH-146e on recombinant A₂A-ARs expressed in HEK cells. A₂A-transfected HEK-293 cells were harvested with PBS/5 mM EDTA. DMEM without serum was added to harvested cells, and these were subjected to centrifugation at 1,000 rpm for 10 min. Cells were resuspended in DMEM-F12 with 1 U/ml of adenosine deaminase and incubated with varying concentrations of DWH-146e (0.001 nM to 1 µM). Cells were incubated with DWH-146e for 10 min, after which 0.5 ml of 0.1 N HCl was added, and an aliquot was then removed and frozen until cAMP was assayed. Camp accumulation was measured by an automated gammainflow radioimmunoassay system (RIA Core Facility, University of Virginia) as described by Brooker et. al. (6).

Surgery and experimental protocol. Adult male Sprague-Dawley rats (200–280 g; Hilltop LabAnimals, Scottsdale, PA) were allowed free access to tap water and a regular diet. The experimental groups are summarized in Table 1. Osmotic minipumps (model 1003D; ALZA, Palo Alto, CA) containing vehicle or drug (see below) were inserted subcutaneously under brief vaporized halothane anesthesia (Halothan Vapor 19.1). Five hours after insertion of minipumps, animals were anesthetized with pentobarbital sodium (100 mg/kg) and warmed with a warming blanket maintained at 37°C. Both kidneys were identified and dissected free of surrounding tissue. The right renal artery and vein were ligated, and the right kidney was removed. The left renal artery and vein were cross-clamped for 45 min. Preliminary data in our laboratory indicated that cross-clamping of the renal artery and vein for 45 min produced consistent injury. The abdomen was closed temporarily to maintain thermoregulation. After 45 min of ischemia, the clamp was released and the kidney was observed for immediate reperfusion. The abdomen was closed with sutures and surgical clips, and the rat was transferred to a metabolic cage for 24 or 48 h. The rat was then reanesthe- tized with pentobarbital sodium, blood was obtained by cardiac puncture, and the kidney was removed for histological analysis. Plasma and urine were obtained, and Na, K, and Cl concentrations were measured by using a NOVA analyzer (NOVA Biomedical, Waltham, MA). Urine osmolality was measured by freezing point depression (NOVA Biomedical).

Compounds were prepared with a PBS/0.01% DMSO solution and placed in osmotic minipumps. Rats received either 1) DWH-146e (0.004 µg·kg⁻¹·min⁻¹), 2) DWH-146e + ZM-241385, a selective A₂A antagonist (0.003 µg·kg⁻¹·min⁻¹); an amount that was calculated to be a molar equivalent to the delivered amount of DWH-146e, or 3) vehicle. In a separate group of rats (groups 6 and 7) a similar protocol was followed as described above; however, osmotic minipumps containing DWH-146e or vehicle were inserted immediately after cross clamping were removed and an additional priming dose of DWH-146e (0.24 µg/kg) or vehicle was administered intraperitoneally.

Tail-cuff blood pressure measurement. Mean arterial pressure and pulse rate were measured by using a photoelectric sensor for pulse detection in rat tail (model 179; IITC/Life Science Instruments, Woodland Hills, CA). Rats were allowed to rest quietly for 10 min in a chamber with the temperature controlled at 26°C. Blood pressures were measured twice and averaged. Tail-cuff blood pressures were measured in rats prior to surgery and 20 h after reperfusion with either vehicle- or DWH-146e-treated rats (groups 1 and 2).

Histology. Kidneys were fixed in periodate-lysine-paraformaldehyde (4% paraformaldehyde) and embedded in parafﬁn, and 4-µm sections were obtained. Sections were subjected to routine staining with hematoxylin and eosin and viewed by routine light microscopy (Zeiss Axioskop). We quantitated the degree of necrosis in a blinded fashion by scoring the degree of renal injury based on the following scoring system from five vehicle- and DWH-146e-treated rats: 0 = normal, 1 = loss of brush border and/or tubule debris, 2 = loss of nuclei, 3 = partial tubule obstruction, and 4 = tubule obstruction and dilatation. Eight fields from each of cortex, outer medulla, and inner medulla were evaluated, scored, and averaged.

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug Infusion</th>
<th>Time of Drug Administration, h</th>
<th>Reperfusion Time, h</th>
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<tbody>
<tr>
<td>1</td>
<td>Vehicle</td>
<td>–5</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>DWH-146e</td>
<td>–5</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>Vehicle</td>
<td>–5</td>
<td>48</td>
</tr>
<tr>
<td>4</td>
<td>DWH-146e</td>
<td>–5</td>
<td>48</td>
</tr>
<tr>
<td>5</td>
<td>DWH-146e + ZM-241385</td>
<td>–5</td>
<td>48</td>
</tr>
<tr>
<td>6</td>
<td>Vehicle</td>
<td>+0.75</td>
<td>48</td>
</tr>
<tr>
<td>7</td>
<td>DWH-146e</td>
<td>+0.75</td>
<td>48</td>
</tr>
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</table>

Values are in hours, relative to the time that cross-clamping of renal artery was initiated.
were incubated in the presence of 1 nM 125I-ZM-241385. ARs were subjected to radioligand binding. Membranes were 0.82 and 60 nM, respectively. Binding affinity from HEK-293 cells transfected with recombinant A2A-ARs was 45-fold higher affinity for A2A-ARs than CGS-21680 or CGS-21680, a well-characterized, selective A2A agonist (13). Figure IA shows the results of competitive binding experiments in membranes derived from HEK-293 cells transfected with human A2A-ARs. In Fig. 1A, the Kᵢ values for DWH-146e, DWH-146a, and CGS-21680 were 37 nM, 1,170 nM, and 1,680 nM, respectively. These results indicate that DWH-146e binds selectively to A2A-ARs in a dose-dependent manner and has a 40- to 50-fold higher affinity for A2A-ARs than CGS-21680 or the acid derivative of DWH-146. Similar results were obtained from rat striatal membranes (data not shown). Additional experiments were performed to determine the selectivity of A2A ligands, DWH-146e, CGS-21680, and ZM-241385, for A2A-ARs by performing competition experiments on membranes expressing recombinant adenosine receptor subtypes (Table 2). The affinities of DWH-146e and CGS-21680 for high-affinity A2A receptor were 0.82 and 60 nM, respectively. Binding affinities for CGS-21680 for adenosine receptor subtypes are consistent with those reported previously (36). To demonstrate the effect of DWH-146e on cAMP accumulation, we measured cAMP accumulation in the presence of varying concentrations of DWH-146e. As shown in Fig. 1B, DWH-146e produced a dose-depen-

1 A2A-ARs and A3-ARs exist in two conformation states with different affinities for agonists. Receptors that are coupled to G proteins bind agonists with high affinity and receptors that are uncoupled bind agonists with low affinity (21). In striatal membranes, the majority of A2A-ARs are uncoupled and in cells overexpressing A2A; the vast majority of A2A-ARs are uncoupled (21). For this reason, the A2A binding assay detects primarily the G protein-uncoupled, low-affinity agonist binding sites, whereas the A2 binding assay mostly detects the G protein-coupled, high-affinity receptor conformation. Hence, the A2A/A2 binding selectivity is underestimated by a factor of ~63 (21). DWH-146e binds to A2A receptors with 74 times higher affinity than does CGS-21680 (the prototype A2A agonist), but with only 1.8-fold higher affinity to A3-ARs. Hence, DWH-146e is 41-fold more selective for A2A-ARs vs. A3-ARs than is CGS-21680.

RESULTS

Selective activation of A2A-ARs by DWH-146e in transfected HEK-293 cells. We sought to develop a new A2A agonist with greater adenosine receptor subtype selectivity and potency than CGS-21680. Furthermore, to avoid the accumulation of A2A agonists during renal failure, a strategy was utilized to develop a selective A2A agonist containing an ester that would be cleaved to an inactive product by serum esterases. We reasoned that the half-life of such a compound would not depend on renal function. The ester DWH-146e is stable in saline, but is rapidly inactivated in blood. To determine the selectivity of DWH-146e for A2A-ARs, we performed competitive binding experiments in which membranes from HEK-293 cells transfected with recombinant A2A-ARs were subjected to radioligand binding. Membranes were incubated in the presence of 1 nM 125I-ZM-241385 and 0.01 nM to 10 µM of DWH-146e, DWH-146a, and CGS-21680, a well-characterized, selective A2A agonist (13). Figure IA shows the results of competitive binding experiments in membranes derived from HEK-293 cells transfected with human A2A-ARs. In Fig. 1A, the Kᵢ values for DWH-146e, DWH-146a, and CGS-21680 were 37 nM, 1,170 nM, and 1,680 nM, respectively. These results indicate that DWH-146e binds selectively to A2A-ARs in a dose-dependent manner and has a 40- to 50-fold higher affinity for A2A-ARs than CGS-21680 or the acid derivative of DWH-146. Similar results were obtained from rat striatal membranes (data not shown). Additional experiments were performed to determine the selectivity of A2A ligands, DWH-146e, CGS-21680, and ZM-241385, for A2A-ARs by performing competition experiments on membranes expressing recombinant adenosine receptor subtypes (Table 2). The affinities of DWH-146e and CGS-21680 for high-affinity A2A receptor were 0.82 and 60 nM, respectively. Binding affinities for CGS-21680 for adenosine receptor subtypes are consistent with those reported previously (36). To demonstrate the effect of DWH-146e on cAMP accumulation, we measured cAMP accumulation in the presence of varying concentrations of DWH-146e. As shown in Fig. 1B, DWH-146e produced a dose-depen-

Table 2. Adenosine receptor radioligand binding assays

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<tbody>
<tr>
<td>DWH-146e</td>
<td>0.82*</td>
<td>45</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>CGS-21680</td>
<td>60</td>
<td>82</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>ZM-241385</td>
<td>1.4</td>
<td>269</td>
<td>31</td>
<td>536</td>
</tr>
</tbody>
</table>

Values are Kᵢ (nM, n = 3) for inhibition of radioligand binding to recombinant human adenosine receptors (AR). Assays were performed in side-by-side assay on the same day using membranes derived from transfected HEK-293 cells and various ligands as described in METHODS. *Affinity of DWH-146e and CGS-21680 for A2A-ARs were corrected for binding to A2A, high affinity sites (21).
temic hemodynamics. We found that bolus injections of high doses of DWH-146e produced a dose-dependent decrease in blood pressure. Administration of 0.69, 3.4, and 6.9 µg/kg infused over 2–3 s produced a decrease in blood pressure of 2, 42, and 72 mmHg, respectively. However, when the highest dose, 6.9 µg/kg, was infused continuously over 1 h (0.115 µg·kg⁻¹·min⁻¹), no change in blood pressure was noted (data not shown). In the current study, we used an infusion rate that delivered 30-fold less drug (0.004 µg·kg⁻¹·min⁻¹) and consistently produced a serum concentration of DWH-146e of <1 nM.

Pretreatment with DWH-146e improves renal function following ischemia-reperfusion injury and the effect is receptor mediated. Rats were paired to receive continuous infusion of either vehicle or DWH-146e by osmotic minipump prior to and during ischemia-reperfusion. Rats were subjected to 45 min ischemia, and renal function was assessed by plasma creatinine following 24 (groups 1 and 2) and 48 h (groups 3 and 4) of reperfusion. As shown in Fig. 2A, plasma creatinine rose significantly following ischemia-reperfusion injury in rats treated with vehicle. Plasma creatinine was reduced significantly after 24 and 48 h of reperfusion in rats pretreated with DWH-146e compared with rats treated with vehicle. DWH-146e significantly decreased plasma creatinine in 7/7 rats (3.79 ± 0.34 vs. 2.76 ± 0.47 mg/dl; vehicle vs. DWH-146e) and in 6/6 rats treated with DWH-146e (6.17 ± 0.57 vs. 1.31 ± 0.22 mg/dl, vehicle vs. DWH-146e) at 24 and 48 h of reperfusion, respectively (Fig. 2A). To determine whether the protective effect of DWH-146e was receptor mediated, we also pretreated rats with equimolar amounts of DWH-146e and ZM-241385, a selective A₂A antagonist, followed by 45 min of ischemia and 48 h of reperfusion (group 5). As shown in Fig. 2B, ZM-241385 reduced by 70% the effect of DWH-146e on plasma creatinine. No significant difference was observed between vehicle and DWH-146e + ZM-241385. In vehicle-treated rats, elevated blood urea nitrogen (BUN) concentrations produced by 48 h of reperfusion were reduced by 57% in DWH-146e-treated rats (groups 3 and 4), an effect that was antagonized by ZM-241385 (Table 3).

Effect of DWH-146e on fluid and electrolytes and kidney weight in rats. Tables 3 and 4 summarize the effect of DWH-146e on plasma ion concentrations and kidney weights in rats following ischemia and 48 h of reperfusion. Kidneys from vehicle-treated rats weighed ~30% more than kidneys from control uninjured rats (23). In five of six rats, kidneys weighed less following DWH-146e treatment than with vehicle treatment, although as a group this difference did not reach statistical significance. Plasma concentrations of Na and Cl were similar between the two groups; however, plasma K concentration was higher by 64% in the vehicle- vs. DWH-146e-treated rats. Ion excretion rates were similar in both groups; urine osmolality was significantly greater by 39% in the vehicle-treated than in the DWH-146e-treated rats. The effect of DWH-146e on urine osmolality following ischemia-reperfusion was blocked by ZM-241385.

DWH-146e reduces tubular injury and ischemic necrosis following ischemia-reperfusion injury in rats. Gross histological examinations of coronal sections of kidneys were compared in rats treated with vehicle (group 3) or

Fig. 2. Pretreatment with DWH-146e improves renal function following ischemia-reperfusion injury, and the effect is receptor mediated. A: Rat kidneys were subjected to 45 min of ischemia and 24 (groups 1 and 2) or 48 h (groups 3 and 4) of reperfusion. DWH-146e (DWH; 0.004 µg·kg⁻¹·min⁻¹) or vehicle was administered continuously via minipump beginning 5 h prior to ischemia-reperfusion. Plasma creatinine increased dramatically in vehicle-treated rats after 24 and 48 h of reperfusion. DWH-146e significantly decreased plasma creatinine in 7/7 rats (P < 0.05) and in 6/6 rats treated with DWH-146e (P < 0.001) at 24 and 48 h, respectively. Values are means ± SE. **P < 0.01. *P < 0.05. Values were determined by paired t-test. B: rats were pretreated with equimolar amounts of DWH-146e and ZM-241385 (0.003 µg·kg⁻¹·min⁻¹) followed by 45 min of ischemia and 48 h of reperfusion. ZM-241385 reversed the protective effect of DWH-146e on plasma creatinine (n = 6; P < 0.01, one-way ANOVA). No significant differences (NS) were observed between vehicle and DWH-146e + ZM-241385 (one-way ANOVA). Values in both A and B are means ± SE.
Table 3. Kidney weight and plasma ion concentrations following ischemia-reperfusion injury in rats

<table>
<thead>
<tr>
<th></th>
<th>Group 3 (Vehicle)</th>
<th>Group 4 (DWH)</th>
<th>Group 5 (DWH + ZM)</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td>Kidney wt, g/g</td>
<td>5.85 ± 0.17 (6)</td>
<td>5.51 ± 0.35 (6)</td>
<td>5.83 ± 0.28 (6)</td>
<td>NS</td>
</tr>
<tr>
<td>[Na+]P, mM</td>
<td>133.2 ± 1.21 (6)</td>
<td>140.1 ± 1.08 (6)</td>
<td>139.5 ± 2.63 (6)</td>
<td>NS</td>
</tr>
<tr>
<td>[K+]P, mM</td>
<td>6.76 ± 0.70 (4)</td>
<td>4.12 ± 0.13 (6)</td>
<td>6.10 ± 0.41 (5)</td>
<td>&lt;0.01 NS</td>
</tr>
<tr>
<td>[Cl+]P, mM</td>
<td>104.3 ± 4.33 (6)</td>
<td>107.7 ± 2.75 (6)</td>
<td>101.2 ± 1.83 (6)</td>
<td>NS</td>
</tr>
<tr>
<td>[BUN]P, mg/dl</td>
<td>159.6 ± 18.7 (6)</td>
<td>68.0 ± 11.0 (6)</td>
<td>129.8 ± 25.3 (6)</td>
<td>&lt;0.05 NS</td>
</tr>
<tr>
<td>[Cr+]P, mg/dl</td>
<td>6.17 ± 0.57 (6)</td>
<td>1.31 ± 0.22 (6)</td>
<td>4.67 ± 1.29 (6)</td>
<td>&lt;0.001 NS</td>
</tr>
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Values are means ± SE; numbers of rats are in parentheses. [Na+]P, [K+]P, [Cl+]P, [BUN]P, and [Cr+]P are plasma sodium, potassium, blood urea nitrogen and creatinine concentrations, respectively; values were determined following 48 h of reperfusion. P values were determined by 1-way ANOVA followed by Bonferroni correction for multiple comparisons. NS, not significant; DWH, DWH-146e; ZM, ZM-241385.

DWH-146e (group 4) 5 h prior to and during ischemia-reperfusion (Fig. 3A). A prominent whitish, pale area consistent with necrosis was observed in the outer medulla and a reddish area consistent with hyperemia was observed in the inner medulla of vehicle-treated rat kidneys. These changes were markedly reduced in kidneys from DWH-146e-treated rats.

Light microscopic analysis of tissue following ischemia-reperfusion injury revealed a loss of brush border villi, tubular necrosis, and obstruction of proximal tubule cells in vehicle-treated animals (Fig. 3B). The severity of the injury was reduced in kidneys from rats treated with DWH-146e (Fig. 3C) and the effect was reversed by ZM-241385 (Fig. 3D). Table 5 demonstrates injury quantitatively in cortex, outer medulla, and inner medulla of kidneys. DWH-146e reduced injury significantly in the outer medulla and the inner medulla. No significant difference was observed in the cortex.

Delayed administration of DWH-146e reduces renal injury following ischemia-reperfusion. To determine whether delayed administration until the reperfusion period can protect kidneys from ischemia-reperfusion injury, we administered vehicle (group 6) or DWH-146e (group 7) immediately after initiation of reperfusion. As shown in Fig. 4, delaying drug administration until the beginning of the 48-h reperfusion period resulted in a significant reduction in plasma creatinine in five of five rats (5.34 ± 0.32 vs. 1.90 ± 0.40 mg/dl, vehicle vs. DWH-146e). This degree of reduction in plasma creatinine was similar to the reduction seen when the drug was administered prior to renal ischemia.

Effect of DWH-146e on blood pressure and heart rate. Tail-cuff blood pressure was measured in rats before and after 20 h of continuous infusion of drug (group 1) or vehicle (group 2) (Table 6). Compared with vehicle-treated rats, no effect on blood pressure or heart rate was observed in DWH-146e-treated rats.

**DISCUSSION**

We have demonstrated that activation of A2A-ARs using a new A2A agonist, DWH-146e, which is more potent and selective than CGS-21680, reduces renal injury in a model of ischemia-reperfusion injury. Radioligand binding experiments demonstrated that the ester derivative of DWH-146 (DWH-146e) has a higher selectivity for human and rat A2A-ARs than the acid derivative (DWH-146a) or CGS-21680, a selective A2A agonist (36). Furthermore, functional studies measuring cAMP accumulation in HEK-293 cells transfected with canine A2A-ARs demonstrated that DWH-146e increases cAMP accumulation and is more potent than CGS-21680 (personal communication, Joel Linden, University of Virginia). The primary mode of metabolism of esters is via serum esterases. Since the compound is inactivated in serum, in vitro, its excretion probably is minimally affected by renal function.

The protection, which occurred at doses of the compound that are below the threshold for systemic hemodynamic responses, is seen if the compound is administered prior to and during ischemia-reperfusion or if the administration of the compound is delayed until the reperfusion period. Histological studies demonstrated a marked reduction in injury to the proximal straight tubule segments of the outer medulla. The observed decrease in BUN and creatinine with DWH-146e treatment provided additional supportive evidence that

Table 4. Effects of drug treatment on fluid balance, urine osmolality, and ion excretion rates

<table>
<thead>
<tr>
<th></th>
<th>Group 3 (Vehicle)</th>
<th>Group 4 (DWH)</th>
<th>Group 5 (DWH + ZM)</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td>Water intake, ml/48 h</td>
<td>30.1 ± 4.3 (6)</td>
<td>42.2 ± 8.4 (6)</td>
<td>35.4 ± 7.3 (6)</td>
<td>NS</td>
</tr>
<tr>
<td>Urine output, ml/48 h</td>
<td>25.4 ± 6.1 (6)</td>
<td>35.6 ± 6.1 (6)</td>
<td>20.2 ± 6.1 (6)</td>
<td>NS</td>
</tr>
<tr>
<td>UNaV, mmol/48 h</td>
<td>1.67 ± 0.36 (6)</td>
<td>1.15 ± 0.19 (6)</td>
<td>2.40 ± 0.75 (6)</td>
<td>NS</td>
</tr>
<tr>
<td>UKV, mmol/48 h</td>
<td>1.02 ± 0.28 (6)</td>
<td>1.93 ± 0.25 (6)</td>
<td>1.44 ± 0.45 (6)</td>
<td>NS</td>
</tr>
<tr>
<td>UClV, mmol/48 h</td>
<td>2.46 ± 0.60 (6)</td>
<td>3.00 ± 0.47 (6)</td>
<td>3.00 ± 0.87 (6)</td>
<td>NS</td>
</tr>
<tr>
<td>Uosm, mosmol/kgH2O</td>
<td>593.5 ± 41.4 (6)</td>
<td>824.5 ± 66.7 (6)</td>
<td>645.5 ± 33.0 (6)</td>
<td>&lt;0.05 NS</td>
</tr>
</tbody>
</table>

Values are means ± SE. UNaV, UKV, and UClV are sodium, potassium, and chloride excretion rates, respectively; Uosm, urine osmolality. Samples were collected by placing the rats in metabolic cages for 48 h of reperfusion. P values were determined by 1-way ANOVA and Bonferroni correction for multiple comparisons. NS, not significant.
DWH-146e reduced renal injury. The protective effects of DWH-146e on renal function were secondary to activation of $A_{2A}$-ARs, as ZM-241385, a selective $A_{2A}$ antagonist, blocked the effects of DWH-146e. $A_{2A}$-AR activation during ischemia-reperfusion also led to an increase in urine osmolality, suggesting a protective effect on tubule responsiveness to vasopressin. The fact that delaying administration of DWH-146e until the reperfusion period still reduced renal injury suggests a potential clinical utility of $A_{2A}$ agonist administration during recovery of kidney from an ischemic insult.

Because of known effects of adenosine on control of renal circulation and metabolic cellular activity, endogenous adenosine is thought to play a role in ischemia-reperfusion injury. Normally, the renal tissue adenosine concentration is less than 1 µM; however, within 2 min of renal ischemia, adenosine concentration increases 6-fold and its metabolite hypoxanthine increases 300-fold (26). Within 10 min of reversal of ischemia, adenosine levels return to baseline. It is likely that tissue adenosine levels return to normal after reperfusion due to washout and the short half-life of adenosine. The effects of endogenous adenosine can be both detrimental and beneficial. Because adenosine binds nonselectively to all four adenosine receptor subtypes, the net effect of increased adenosine levels will then depend upon the balance of effects on multiple receptor subtypes and duration of action. Adenosine binds to $A_1$-ARs, an effect likely to produce vasoconstriction and worsen kidney function. Binding of adenosine to $A_{2A}$-ARs could produce a beneficial effect by improv-

**Table 5. Renal injury following ischemia reperfusion**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Group 3 (Vehicle)</th>
<th>Group 4 (DWH-146e)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>1.72 ± 0.33</td>
<td>0.83 ± 0.30</td>
<td>NS</td>
</tr>
<tr>
<td>Outer medulla</td>
<td>3.98 ± 0.02</td>
<td>2.17 ± 0.33</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Inner medulla</td>
<td>2.02 ± 0.62</td>
<td>0.48 ± 0.22</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 vehicle-treated rats and 5 DWH-146e-treated rats. Rat kidneys were subjected to 45 min ischemia/48 h reperfusion. Vehicle or DWH-146e was administered continuously beginning 5 h prior to ischemia-reperfusion. We quantitated the degree of necrosis by assigning a value to the degree of renal injury based on the following scoring system from 5 vehicle- and DWH-146e-treated rats: 0, normal; 1, loss of brush border and/or tubule debris; 2, loss of nuclei; 3, partial tubule obstruction; and 4, tubule obstruction and dilatation. Kidney tissue was viewed under x200 magnification. Eight fields from each of cortex, outer medulla, and inner medulla were evaluated, scored, and averaged. P values given for comparison of vehicle- to DWH-treated rats were determined from unpaired t-tests.
ing renal blood flow or by reducing neutrophil endothelial cell interaction and subsequent cascade of inflammatory events. Whatever the effect of endogenous adenosine, it is transient. Thus strategies to protect kidneys from ischemia-reperfusion injury have focused primarily on delivering continuously compounds that bind selectively to adenosine receptor subtypes.

A number of studies have demonstrated the protective effect of nonselective adenosine antagonists and selective A1-AR antagonist in different models of acute renal failure in both animals (4) and humans (11, 16, 18). Blockade of A1-ARs that are expressed on afferent arterioles (33) is presumed to be responsible for the protective effect of A1 antagonists. Additionally, A2A agonists increase medullary renal blood flow and can reduce injury by increasing medullary oxygenation (1). Intrarenal infusion of adenosine produces a decrease in glomerular filtration rate with little or minimal change in total blood flow to the kidney (33). These effects are thought to be secondary to an increase in afferent and decrease in efferent resistance.

It is now thought that the effects of adenosine in kidney could be mediated through different receptor subtypes. Northern blot analysis has demonstrated the presence of both A1- and A2A-ARs in rat kidney (37). Infusions of subtype-selective analogs have demonstrated contrasting hemodynamic effects (1). Intravenous (17) or intra-arterial (1) infusion of N6-cyclopentyladenosine revealed that adenosine-mediated reduction in renal blood flow was due to activation of A1-ARs. In contrast, intravenous (17) and intrarenal arterial (1) infusion of CGS-21680 confirmed the role of A2A-ARs in mediating the vasodilation observed following adenosine infusion. Regional blood flow assessed by laser-Doppler flow indicated that A1-AR activation produced a decrease in both cortical and medullary blood flow and that A2A-AR activation increased medullary flow (1). The effect of A1-selective agonists in mediating vasoconstriction is likely to be due to the presence of A1-ARs on afferent arterioles (38). In contrast, selective A2A-AR activation does not produce significant effects on microrvascular dynamics (38). In vitro micropерfusion of outer medullary descending vasa recta demonstrated that A1- and A2A-ARs mediated vasoconstriction and vasodilation, respectively (32). The resultant effect of modulation of intrarenal blood flow by adenosine may alter renal oxygenation. Adenosine infusion resulted in an increase in medullary PO2 and a decrease in cortical PO2 (10). Thus the infusion of adenosine may produce an increase in medullary blood flow leading to an increase in oxygenation and renal tissue protection.

Whether DWH-146e has any effects on renal hemodynamics at the dose administered in the study is not known. For our experiment, we chose to administer DWH-146e continuously via osmotic minipumps to avoid changes in systemic or intrarenal hemodynamics. In this study, a ~30-fold lower rate of drug delivery (0.004 µg·kg−1·min−1) was found to produce no effect on blood pressure or heart rate. An infusion rate that was ~30-fold higher also did not result in any change in systemic or intrarenal hemodynamics. At the rate of agonist infusion used in the study (0.004 µg·kg−1·min−1), DWH-146e plasma levels were determined to be <1 nM, far too low to affect blood pressure or heart rate (Table 6). Nevertheless, we cannot rule out the possibility that A2A agonist infusion produced favorable effects on local renal hemodynamics that contributed to renal protection. It is interesting to note that the concentration of DWH-146e necessary to produce a protective effect from ischemia-reperfusion injury in vivo was lower than the concentration predicted from in vitro binding studies. This finding is consistent with the phenomenon described previously relating to “spare receptors” (31). This concept describes the disproportionately greater functional response to the increment of receptor occupancy by an agonist. In the heart, it was estimated that activation by CGS-21680 of 5% of the total number of A2A-ARs can cause half-maximal response (31). This phenomenon could explain our findings.

Alternatively, the reduction in renal injury afforded by A2A-AR agonist infusion could be due to attenuation of the inflammatory response that accompanies ischemia-reperfusion injury. Adenosine also has been shown to reduce inflammation in in vitro and in vivo studies (9, 34). A number of studies have now documented the protective effect of A2A-AR activation in heart, lung, and brain (14, 15, 25). The mechanism by which protection is achieved is in part due to a favorable hemodynamic effect, but equally important is the effect of A2A-ARs in reducing inflammation. In human endothelial cells adenosine reduces cytokine release and E-selectin and VCAM-1 expression (5). Although locally produced adenosine appears to protect tissue by decreasing the production of toxic reactive oxygen products by neutrophils (9), it also paradoxically mediates neutrophil chemotaxis via A1-ARs (9). Such a dual effect might protect endothelial cells from the deleterious effects of activated neutrophils yet allow chemotaxis to the site of

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug Treatment</th>
<th>Baseline MAP (6)</th>
<th>HR (6)</th>
<th>Treatment MAP (6)</th>
<th>HR (6)</th>
<th>ΔMAP (6)</th>
<th>ΔHR (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle</td>
<td>101.8 ± 2.7</td>
<td>376 ± 9.3</td>
<td>94.0 ± 3.13</td>
<td>350 ± 5.8</td>
<td>-7.8 ± 5.0</td>
<td>-25.3 ± 9.3</td>
</tr>
<tr>
<td>2</td>
<td>DWH-146e</td>
<td>94.7 ± 2.5</td>
<td>362 ± 10.2</td>
<td>93.9 ± 4.2</td>
<td>364 ± 13.6</td>
<td>-0.9 ± 3.6</td>
<td>1.6 ± 16.4</td>
</tr>
</tbody>
</table>

Values are means ± SE; numbers of rats are in parentheses. MAP, mean arterial pressure (mmHg); HR, heart rate (beats/min). Baseline tail-cuff blood pressure was measured prior to surgery and after 20 h of reperfusion. Significance was determined by unpaired t-test. NS, not significant.
infection (9). In vitro studies demonstrated that two highly selective A2a agonists, WRC-0470 and DWH-146e, inhibited superoxide release from human neutrophils following activation on fibrinogen-coated plates (personal communication, Gail Sullivan, University of Virginia). Furthermore, this effect was synergistic with rolipram, a type IV phosphodiesterase inhibitor, and was inhibited by a selective A2a antagonist, ZM-241385. These data provide compelling evidence that A2a-AR activation decreases neutrophil oxidative activity and formed the basis for ischemia-reperfusion studies that have demonstrated the protective effect in lung, brain, and heart following A2a-AR activation (14, 15, 25). No prior studies to our knowledge have been directed at the potential protective effect of A3a activation in renal injury, despite the abundant data suggesting that neutrophils contribute to renal injury (8, 19, 28, 39).

In summary, our data demonstrate for the first time that continuous administration during reperfusion of a selective A2a agonist, DWH-146e, starting before or immediately after ischemia, reduces reperfusion injury. We believe that the protective effect of DWH-146e is mediated by selective activation of A2a-ARs. Pharmacological studies demonstrated that DWH-146e displayed a higher affinity for A2a-ARs than did the well-characterized A2a agonist, CGS-21680. Furthermore, the protection afforded by DWH-146e was blocked by a selective A2a antagonist, ZM-241385. These results suggest possible therapeutic uses of DWH-146e or similar compounds as treatments of or prophylaxis for acute renal failure.

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Address for reprint requests and other correspondence: M. D. Okusa, Division of Nephrology, Box 133, Univ. of Virginia Health Sciences Center, Charlottesville, VA 22908 (E-mail: mdo7@virginia.edu).

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