

# The Reno-Vascular A2B Adenosine Receptor Protects the Kidney from Ischemia

Almut Grenz<sup>1,2</sup>, Hartmut Osswald<sup>1</sup>, Tobias Eckle<sup>2,3</sup>, Dan Yang<sup>4</sup>, Hua Zhang<sup>1</sup>, Zung Vu Tran<sup>5</sup>, Karin Klingel<sup>6</sup>, Katya Ravid<sup>4</sup>, Holger K. Eltzschig<sup>2,3\*</sup>

**1** Department of Pharmacology and Toxicology, Tübingen University Hospital, Tübingen, Germany, **2** Mucosal Inflammation Program, Department of Anesthesiology and Perioperative Medicine, University of Colorado Health Sciences Center, Denver, Colorado, United States of America, **3** Department of Anesthesiology and Intensive Care Medicine, Tübingen University Hospital, Tübingen, Germany, **4** Department of Biochemistry and Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, Massachusetts, United States of America, **5** Department of Biostatistics, University of Colorado, Denver, Colorado, United States of America, **6** Department of Molecular Pathology, Tübingen University Hospital, Tübingen, Germany

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**Abbreviations:** AR, adenosine receptor; df, degree of freedom; GFR, glomerular filtration rate; HE, hematoxylin and eosin; IL, interleukin; iNOS, inducible nitric oxide synthase; IP, ischemic preconditioning; KC, keratinocyte-derived chemokine (mouse ortholog of human IL-8); KO, knockout; RT, reverse transcription; TNF- $\alpha$ , tumor necrosis factor-alpha; WT, wild type

\* To whom correspondence should be addressed. E-mail: holger.eltzschig@uchsc.edu

## ABSTRACT

### Background

Acute renal failure from ischemia significantly contributes to morbidity and mortality in clinical settings, and strategies to improve renal resistance to ischemia are urgently needed. Here, we identified a novel pathway of renal protection from ischemia using ischemic preconditioning (IP).

### Methods and Findings

For this purpose, we utilized a recently developed model of renal ischemia and IP via a hanging weight system that allows repeated and atraumatic occlusion of the renal artery in mice, followed by measurements of specific parameters or renal functions. Studies in gene-targeted mice for each individual adenosine receptor (AR) confirmed renal protection by IP in  $A1^{-/-}$ ,  $A2A^{-/-}$ , or  $A3AR^{-/-}$  mice. In contrast, protection from ischemia was abolished in  $A2BAR^{-/-}$  mice. This protection was associated with corresponding changes in tissue inflammation and nitric oxide production. In accordance, the A2BAR-antagonist PSB1115 blocked renal protection by IP, while treatment with the selective A2BAR-agonist BAY 60–6583 dramatically improved renal function and histology following ischemia alone. Using an A2BAR-reporter model, we found exclusive expression of A2BARs within the reno-vasculature. Studies using A2BAR bone-marrow chimera conferred kidney protection selectively to renal A2BARs.

### Conclusions

These results identify the A2BAR as a novel therapeutic target for providing potent protection from renal ischemia.

*The Editors' Summary of this article follows the references.*

## Introduction

Acute renal failure is defined as a rapid decrease in the glomerular filtration rate (GFR), occurring over a period of minutes to days [1]. The causes of acute renal failure are classically divided into three categories, prerenal, postrenal, or intrinsic [1]. However, over 50% of hospitalized patients with acute renal failure suffer from renal ischemia leading to prerenal azotemia or “intrinsic” acute tubular necrosis [2]. In fact, a recent study of hospitalized patients revealed that only a mild increase in the serum creatinine level (0.3–0.4 mg/dl) is associated with a 70% greater risk of death than in persons without any increase [3]. Along these lines, surgical procedures requiring cross-clamping of the aorta and renal vessels are associated with a renal failure rate of up to 30% [4]. Similarly, acute renal failure after cardiac surgery occurs in up to 10% of patients under normal circumstances and is associated with dramatic increases in mortality [5]. Taken together, these data highlight the urgent need for additional therapeutic modalities to prevent renal injury from ischemia [6–8].

Extracellular adenosine has been implicated as a critical mediator of reno-vascular functions [9,10]. In most vascular beds adenosine causes vasodilatation (e.g., in the coronary circulation), thereby contributing to the metabolic control of organ perfusion [11]. In contrast, adenosine can cause vasoconstriction in the kidneys, a response that has been suggested to be an organ-specific version of metabolic control designed to restrict organ perfusion when transport work increases [9,10]. In addition, several studies of tissue protection from limited oxygen availability have suggested an anti-inflammatory and tissue protective role of extracellular adenosine, particularly during conditions of hypoxia [12–18]. Extracellular adenosine is derived mainly via enzymatic phosphohydrolysis of precursor nucleotides to adenosine. In fact, recent studies of mice with genetic deletion of enzymatic steps in extracellular adenosine generation revealed that these mice are more prone to renal ischemia (*cd39*<sup>-/-</sup> or *cd73*<sup>-/-</sup> mice) compared to their littermate controls [19,20]. Once released into the extracellular space, adenosine can activate four individual G-protein coupled receptors (A1AR, A2AAR, A2BAR, and A3AR). As such, previous studies have shown a critical role of extracellular adenosine generation and signaling in tissue protection from hypoxia [21–25] or during preconditioning [19,20,26–28], where pretreatment with short and repeated episodes of nonlethal ischemia (ischemic preconditioning [IP]) result in a robust protection from subsequent ischemic injury. At present, it remains unclear which ARs mediate renal protection from ischemia. Some studies suggest a role of signaling through myeloid A2AAR in renal protection [29], other studies found signaling through the A1AR in inhibiting renal inflammation and necrosis [30].

Similarly to studies of renal protection by IP, previous studies in the heart have shown a critical role of extracellular adenosine generation in IP-dependent cardioprotection. For example, gene-targeted mice for *cd39* [27] or *cd73* [28] are not protected by cardiac IP-treatment. Moreover, a recent study compared myocardial infarct sizes in gene-targeted mice for each individual adenosine receptor (AR) and found a pivotal role of the A2BAR in attenuating ischemia-induced myocardial cell death [28]. In addition to its tissue-protective effects,

extracellular adenosine signaling has been implicated in several cardio-specific functions including A1AR-dependent changes in cardiac conduction and heart rhythm (e.g., changes in P-R interval, atrio-ventricular blockade) [31], release of natriuretic peptide by cardiac myocytes [32], or the regulation of coronary blood flow by A2A or A2BARs [11]. In contrast, different functions of extracellular adenosine have been identified in the kidneys. For example, extracellular adenosine generation and signaling has been shown as a critical control point in the regulation of the tubuloglomerular-feedback mechanisms, particularly through activation of the A1AR [10,33,34]. While adaptation of cardiac myocytes triggered by adenosine, bradykinin, and opioids—leading to activation of protein kinase C (PKC)—is central to cardiac IP [35,36], ischemic injury of the kidneys is characterized by vascular and renal-tubular injury [37,38], suggesting that preconditioning phenomena of the kidneys represent a form of vascular or reno-tubular adaptation. Therefore, previous studies of adenosine in IP-dependent cardioprotection have focused on histological assessment of myocardial cell death or leakage of myocardial specific enzymes into the systemic circulation [27,28,39], while studies of extracellular adenosine in reno-protection by IP require assessment of specific renal functions (e.g., glomerular filtration, tubular absorption, or secretion). Therefore, we recently developed a murine model of in situ IP and ischemia of the kidneys using a hanging weight system that allows repeated and atraumatic occlusion of the renal artery in mice, followed by measurements of specific parameters or renal function (e.g., GFR, creatinine clearance, urine production) to study molecular pathways of innate renal protection from ischemia [40].

In the present studies, we utilized renal IP as an experimental strategy to identify adenosine signaling pathways to increase renal resistance to ischemia. Here, we studied gene-targeted mice for each individual AR for renal protection by IP.

## Methods

### Mice

All animal protocols were in accordance with the German guidelines for use of living animals and were approved by the Institutional Animal Care and Use Committee of the Tübingen University Hospital and the Regierungspräsidium Tübingen or the University of Colorado guidelines for animal care. In pharmacological studies, C57BL/6J mice obtained from Charles River Laboratories were used. Mice deficient in *A1AR*, *A2BAR*, or *A3AR* on the C57BL/6 strain, or deficient in *A2AAR* on the CD1 strain were generated, validated, and characterized as described previously [28,41–43]. For bone marrow chimeric studies, mice deficient in the *A2AAR* were backcrossed over six generations to C57BL/6. In all control experiments, age-, gender-, and weight-matched littermate controls were used. In subsets of experiments, a previously described *A2BAR* reporter mouse on the C57BL/6 strain was used (*A2BAR*-knock-out [KO]/ $\beta$ -gal-knock-in mice) [44].

### Murine Model for Renal IP

Mice underwent right nephrectomy followed by left renal artery ischemia with or without prior IP treatment (four cycles of 4 min ischemia and 4 min reperfusion prior to 45

min of ischemia) using a hanging weight system, as previously described [40]. At the end of surgery, mice received 0.3 ml normal saline IP and were allowed to recover for 2 h under a heating lamp. Thereafter mice were placed into metabolic cages (Tecniplast Deutschland) for evaluation of renal function for 24 h, or mice were kept in standard cages for performing inulin clearance on the next day.

### Assessment of Renal Function

Plasma and urine creatinine were measured 24 h after renal ischemia using a commercially available colorimetric method (LT-SYS, Labor + Technik). From these data we calculated the creatinine clearance. Plasma and urine concentrations of  $\text{Na}^+$  and  $\text{K}^+$  were determined with a flame-emission photometer (ELEX 6361, Eppendorf AG). Renal excretory and hemodynamic values were calculated using standard formulas. Kidneys were harvested after 24 h and stored at  $-80^\circ\text{C}$  until further analysis.

### Inulin Clearance

Inulin clearance was measured 24 h after renal ischemia (45 min, with or without prior IP treatment) as described previously [33,45]. Briefly, mice were anaesthetized using 50 mg/kg IP pentobarbital. Animals were then placed on a temperature-controlled operating table to keep rectal temperature at  $37^\circ\text{C}$ . Tracheotomy was performed as described previously [39], and the right jugular vein was cannulated for continuous infusion. Blood samples were taken via a catheter inserted into the left femoral artery. A catheter was placed in the urinary bladder for timed urine collection after removal of the right kidney. After the surgery, all mice received a bolus of 0.85% sodium chloride solution in an amount equal to 20% of body weight. Continuous infusion was maintained at a rate of 600  $\mu\text{l/h}/30\text{g}$  body weight, and  $^3\text{H}$ -inulin was added to the infusion for evaluation of whole kidney GFR. After stabilization of the animals for 20 min, 20-min timed urine collections were performed for determination of urinary flow rate and  $^3\text{H}$ -inulin. Blood was obtained in the middle of every urine collection period for measurement of  $^3\text{H}$ -inulin. Concentration of  $^3\text{H}$ -inulin in plasma and urine were performed by liquid phase scintillation counting, and GFR was calculated by standard formulas.

### Adenosine Measurement

Whole kidneys from *cd73<sup>-/-</sup>* mice or age-, gender-, and weight-matched littermates were removed and immediately snap-frozen with clamps precooled to the temperature of liquid nitrogen with or without IP treatment prior to ischemia. The frozen kidneys were pulverized under liquid nitrogen, and the tissue protein was precipitated with 0.6 N ice-cold perchloric acid. Tissue adenosine levels were determined via HPLC as described previously [46].

### Renal Histology

Kidneys were excised and harvested 24 h following 45 min of ischemia with or without IP in A2BAR<sup>-/-</sup> and their wild-type (WT) controls. Renal tissues were fixed in 4.5% buffered formalin, dehydrated, and embedded in paraffin. Sections (3  $\mu\text{m}$ ) were stained with hematoxylin and eosin (HE). For evaluation of granulocytic infiltrates, sections were stained by histochemistry using chloracetate esterase (CAE) as previously described [40]. Examination and scoring of three representative sections of each kidney ( $n = 4-6$  for each

condition) were carried out blinded to the experimental group as described previously [47,48].

### Immunofluorescence Histochemistry

Kidneys were excised and harvested 24 h following 45 min of ischemia with or without IP in A2BAR<sup>-/-</sup> and their WT controls. The samples were cut in 5- $\mu\text{m}$  slices, mounted on superfrost glass slides for immunohistochemistry, and stored at  $-20^\circ\text{C}$ . Immunofluorescence histochemistry was carried out using primary polyclonal rabbit anti-inducible nitric oxide synthase (iNOS) antibody (anti macNOS, Transduction Lab) at a 1:50 dilution in 1% bovine serum albumin (BSA) in PBS. The slides were pretreated with 10% normal goat serum for 1 h and then incubated in anti-iNOS antiserum at room temperature for 1 h. As secondary antibodies, FITC-labeled goat anti-rabbit antibodies (Dianova) were applied at 1:50 dilution in PBS at room temperature for 1 h. The primary antibody was omitted on the slides that served as negative controls. After mounting with Dako fluorescent mounting medium containing 15 Mm Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (Dako-Patts), the slides were assessed for fluorescent microscopy. Expression of iNOS in inflammatory cells was assessed in ten randomly selected areas per sample, observed at 600 $\times$  magnification using a scale system iNOS score: 0, no iNOS signal; 1, rare iNOS expression in macrophages; 2, moderate iNOS expression in three to five areas; 3, iNOS expression in five to eight areas; 4, strong iNOS expression in macrophages in all selected areas.

### Measurement of Nitrite/Nitrate Concentrations

Plasma and renal tissue concentrations of nitrite ( $\text{NO}_2$ ) and nitrate ( $\text{NO}_3$ ) were measured using a colorimetric-assay kit (Cayman Chemical Company).

### Renal Tissue Nitric Oxide Synthase Activity

We measured nitric oxide synthase (NOS) activity in the renal tissue with a commercially available kit (Calbiochem, EMD Biosciences). The assay is based on the enzymatic conversion of nitrite to nitrate reductase, followed by the spectrophotometric quantification of nitrite levels using the Griess reagent.

### Myeloperoxidase Activity

To quantify neutrophil infiltration, we performed myeloperoxidase (MPO) measurements as previously described [40].

### ELISA (NF $\kappa$ B, I $\kappa$ B $\alpha$ ) from Renal Tissue

The snap-frozen kidneys were thawed, weighed, and transferred to different tubes on ice containing 1 ml of T-PER (Tissue Protein Extraction Reagent, Pierce Biotechnology). The renal tissues were homogenized at  $4^\circ\text{C}$ . Renal homogenates were centrifuged at  $9,000 \times g$  for 10 min at  $4^\circ\text{C}$ . Supernatants were transferred to clean microcentrifuge tubes, frozen on dry ice, and thawed on ice. Total protein concentrations in the renal tissue homogenates were determined using a BCA (bicinchoninic acid) kit (Pierce Biotechnology). Renal tissue homogenates were diluted with 50% assay diluent and 50% T-PER reagent to a final protein concentration of 400  $\mu\text{g}/\text{ml}$ . Activated NF $\kappa$ B (TransAM, Active Motif North America) and I $\kappa$ B $\alpha$  (TransAM, Active Motif North America) levels were measured in homogenates from whole renal tissues using a mouse ELISA kit according to the manufacturer's instructions.

## Compounds

For specific inhibition of the A2BAR, PSB1115 (Tocris, Biotrend-Chemikalien) was administered IV via the jugular vein (5 mg/kg/h). This dosing regimen was previously shown to effectively inhibit A2BAR signaling [28]. In other experiments, a recently described A2BAR agonist (BAY 60-6583, Bayer HealthCare AG, 60 µg/kg IV via the jugular vein) was used [28].

## Transcriptional Analysis

For assessment of A1AR, A2AAR, A2BAR, and A3AR transcript levels as well as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-6, keratinocyte-derived chemokine (KC, the mouse ortholog of human IL-8), and IL-10 transcript levels, total RNA was isolated and quantification of transcript levels were measured by real-time reverse transcription (RT)-PCR (iCycler, Bio-Rad Laboratories Inc.). In short, total RNA was isolated from renal tissue using the total RNA isolation NucleoSpin RNA II Kit according to the manufacturer's instructions (Macherey & Nagel). For this purpose, liquid nitrogen frozen tissue was homogenized in the presence of RA1 lysis buffer (Micra D8 homogenizer, ART-Labortechnik), and after filtration, lysates were loaded on NucleoSpin RNA II columns, followed by desalting and DNaseI digestion (Macherey & Nagel). RNA was washed, and the concentration was quantified. The PCR reactions contained 1 µM sense and 1 µM antisense oligonucleotides with SYBR Green I (Molecular Probes Inc.). Primer sets (sense sequence, antisense sequence, and transcript size, respectively) for the following genes were: A1AR (5'-AGG GAG GGG TCA AGA ACT GT-3', 5'-TCC CAG TCT CTG CCT CTG TT-3', 109 bp); A2AAR (5'-GAA GAC CAT GAG GCT GTT CC-3', 5'-GAG TAT GGG CCA ATG GGA GT-3', 253 bp); A2BAR (5'-GGA AGG ACT TCG TCT CTC CA-3', 5'-GGG CAG CAA CTC AGA AAA CT-3', 322 bp); A3AR (5'-CAA TTC GCT CCT TCT GTT CC-3', 5'-TCC CTG ATT ACC ACG GAC TC-3', 334 bp); IL-6 (ACCGTAT-GAAGTTCCTCTC, CTCTCCGACTTGGAAGTA); TNF- $\alpha$  (ACTCCAGGCGGTGCCTATGT, TCCAGCTGCTCCTC-CACTTG); IL-10 (CTTACTGACTGGCATGAGGA, GCAT-TAAGGAGTCGGTTAGC); KC (CCAAGCCTTGA-fj;GTCACAGAG, GGACAGCATCTGGCAGAATA). Each target sequence was amplified using increasing numbers of cycles of 94 °C for 1 min, 58 °C (for cytokines 60 °C) for 0.5 min, 72 °C for 1 min. Murine  $\beta$ -actin mRNA (sense primer, 5'-ACA TTG GCA TGG CTT TGT TT-3' and antisense primer, 5'-GTT TGC TCC AAC CAA CTG CT-3') was amplified in identical reactions to control for the amount of starting template.

## Immunoblotting Experiments

In subsets of experiments, we determined renal A2BAR protein content. For this purpose, C57BL/6J mice were humanely killed following renal IP as above, and the kidney was excised at indicated time points (60, 120, and 240 min after IP) and immediately frozen at -80 °C. Tissues were homogenized and lysed for 10 min in ice-cold lysis buffer (150 mM NaCl, 25 mM Tris [pH 8.0], 5 mM EDTA, 2% Triton X-100, and 10% mammalian tissue protease inhibitor cocktail, Sigma-Aldrich), and collected in microfuge tubes. After spinning at 14,000  $\times$  g to remove cell debris, the pellet was resuspended in reducing Laemmli sample buffer and heated to 90 °C for 5 min. Samples were resolved on a 12% polyacrylamide gel and transferred to nitrocellulose mem-

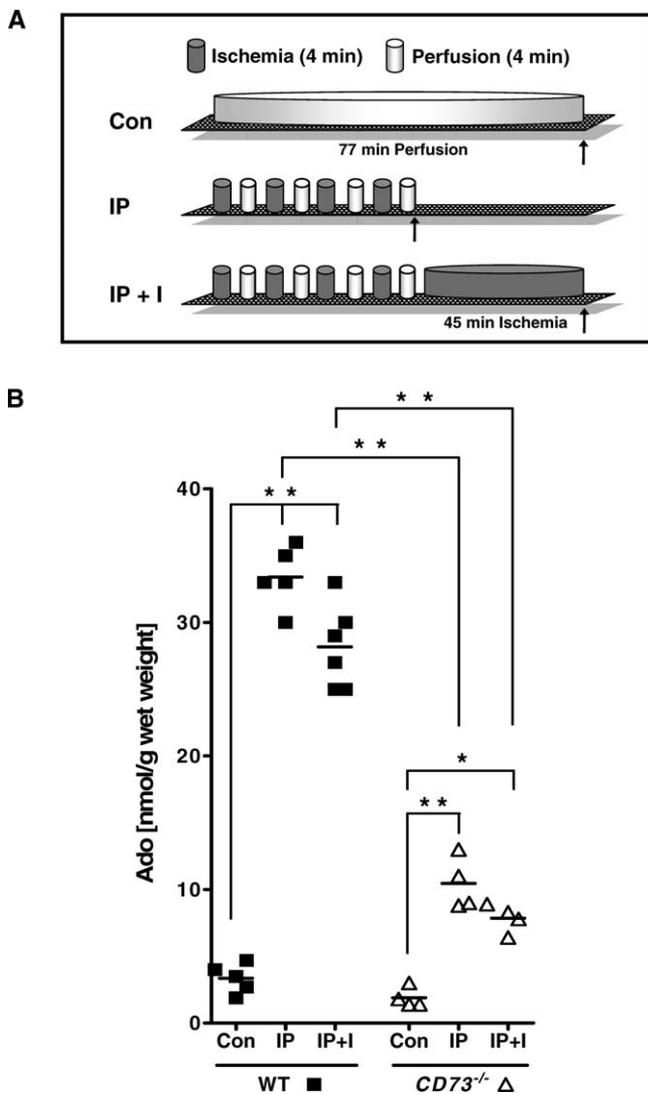
branes. The membranes were blocked for 1 h at room temperature in PBS supplemented with 0.2% Tween 20 (PBS-T) and 4% BSA. The membranes were incubated in 10 µg/ml A2BAR goat polyclonal antibody raised against the C terminus (Santa Cruz) for 1 h at room temperature, followed by 10 min washes in PBS. The membranes were then incubated in 1:3,000 HRP-donkey anti-goat Ig for A2BAR detection (Santa Cruz). The wash was repeated, and proteins were detected by enhanced chemiluminescence (Applied Biosystems). To control for protein loading, blots were stripped in stripping buffer for 15 min, washed ones for 10 min with TBST, and membrane was blocked for 1 h at room temperature in PBS supplemented with 0.2% Tween 20 (PBS-T) and 4% BSA. Thereafter the membrane was incubated with  $\beta$ -actin using a mouse monoclonal anti-human  $\beta$ -actin antibody (Abcam Inc.). The protein levels of  $\beta$ -actin were detected by enhanced chemiluminescence.

## Analysis of $\beta$ -Galactosidase Expression in Renal Sections of A2BAR Reporter Mice

To localize the A2BAR in renal tissues, we analyzed  $\beta$ -galactosidase expression in renal sections in A2BAR-KO/ $\beta$ -gal-knock-in mice. Mice were anesthetized with isoflurane and perfused through the left heart ventricle with 20 ml of PBS (pH 7.4) at a rate of 4 ml/min. Perfusion for fixation with 30 ml of freshly made 2% paraformaldehyde in PBS (pH 7.4) was continued for 15 min at 2 ml/min and was followed by perfusion with PBS for 10 min. Kidneys were dissected from the perfused mouse and stored in ice-cold PBS, prior to staining for  $\beta$ -galactosidase activity. Kidneys were cut into 1–2-mm thick slices, and stained with X-gal staining solution (5 mM  $K_3Fe[CN]_6$ , 5 mM  $K_4Fe[CN]_6 \times 3H_2O$ , [Sigma Aldrich, catalogue numbers P-8131 and P-9287, respectively], 2 mM  $MgCl_2$ , in PBS) containing a final concentration of 1 mg/ml 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal, American Bioanalytical, catalogue number AB02400-1000). Samples were incubated at 37 °C for 2–4 h on a rocking platform. After staining, samples were rinsed with PBS and stored in 4% paraformaldehyde at 4 °C. Samples were embedded in paraffin and cut at a thickness of 5 µm. Thereafter sections were stained with HE.

## Generation of Bone Marrow Chimeric Mice

This animal protocol was in accordance with the German guidelines for use of living animals and was approved by the Institutional Animal Care and Use Committee of the Tübingen University Hospital and the Regierungspräsidium Tübingen or the University of Colorado guidelines for animal care. To define the contribution of renal or hematopoietic A2BARs during renal ischemia or IP, we generated bone marrow chimeric mice in which bone marrow was ablated by radiation in WT mice (C57BL/6) followed by reconstitution with bone marrow derived from previously characterized mice gene-targeted for the A2BAR [28] and vice versa ( $A2BAR^{-/-} \rightarrow A2BAR^{+/+}$ ,  $A2BAR^{+/+} \rightarrow A2BAR^{-/-}$ ). Experiments with  $A2BAR^{+/+} \rightarrow A2BAR^{+/+}$  and  $A2BAR^{-/-} \rightarrow A2BAR^{-/-}$  mice served as controls. Since myeloid A2AAR signaling has been suggested in the attenuation of renal ischemia injury [29], we also performed IP in  $A2BAR^{+/+}$  or  $A2BAR^{-/-}$  transplanted with bone marrow from  $A2AAR^{+/+}$  or  $A2AAR^{-/-}$  mice. In short, male donor mice (8–10 wk old, 20–25 g) were humanely killed, and the marrow from the tibia and femur were harvested by



**Figure 1.** Renal Adenosine Tissue Content with IP Treatment with or without Subsequent Ischemia

Age-, gender-, and weight-matched *cd73*<sup>-/-</sup> mice or littermate controls (WT) were subjected to IP alone (IP) or IP followed by 45 min of ischemia (IP + I) using a hanging weight system for atraumatic occlusion of the left renal artery. The IP protocol consisted of four cycles of ischemia and reperfusion (4 min each).

Statistics: One-way ANOVA was used to perform comparisons among groups. The *F*-test results are  $F(5,22) = 210.34$ ,  $p < 0.001$ . All statistically significant differences within groups were compared by Tukey's post-hoc test ( $p < 0.05$ ) and were indicated with a bracket (\*,  $p < 0.01$ ; \*\*,  $p < 0.001$ ).

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flushing the marrow cavity with sterile isotonic sodium chloride solution. The bone marrow cells were then centrifuged at 400g for 5 min, resuspended, and counted. Recipient mice (8–10 wk of age, 20–25 g) were irradiated with a total dose of 12 Gy from a <sup>137</sup>Cs source. Immediately after irradiation,  $10 \times 10^6$  BM cells/recipient were injected in 0.3 ml 0.9% sodium chloride into the jugular vein. The resulting chimeric mice were housed in microisolators for at least 7 wk before experimentation and fed with water containing tetracycline (100 mg/l) in the first 2 wk following BM transplantation. Consistent with other studies using a similar approach [49], preliminary experiments using the same

conditioning regimen and transplanting CD45.1<sup>+</sup> bone marrow into irradiated CD45.1<sup>-</sup> mice resulted in >95% chimerism in B cells, neutrophils, and monocytic cells, and ~85% chimerism in CD4<sup>+</sup> and CD8<sup>+</sup> T cells of recipient mice (unpublished data).

## Data Analysis

Renal injury score data are given as median and range, all other data are presented as mean  $\pm$  SD. Renal injury was analyzed with the Kruskal-Wallis test, with follow-up pairwise comparisons by Wilcoxon-Mann-Whitney test. For all other outcomes, one-way ANOVA was used to perform comparisons among groups. A significant ( $p < 0.05$ ) overall *F*-statistic was followed by Tukey's post-hoc, pairwise multiple comparison test. For the comparison of the different AR transcripts, pairwise comparisons were made between the control and treatment groups by Dunnett multiple comparison technique. All test are two-sided, with significance set at  $p \leq 0.05$ . SPSS version 16.0.1 was used to perform all calculations.

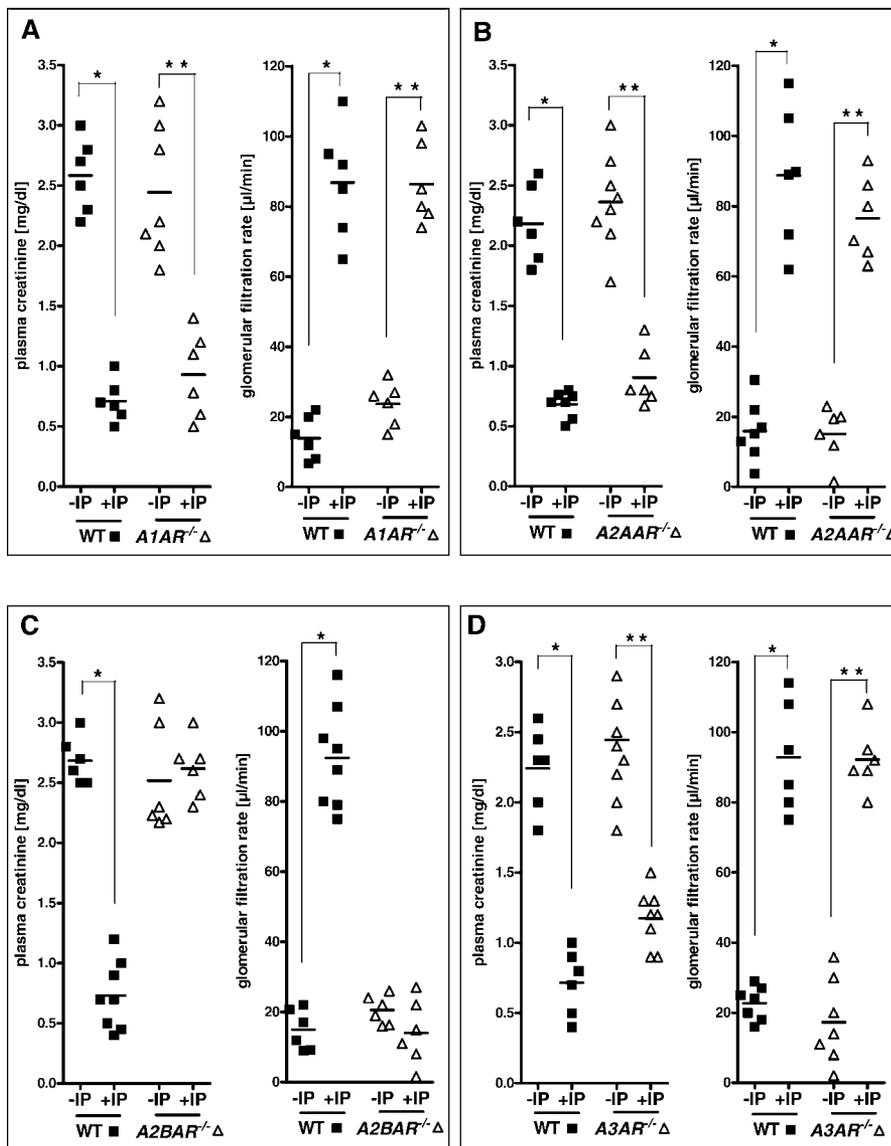
## Results

### Extracellular Adenosine Levels Are Elevated with Renal IP Treatment

Previous studies have suggested extracellular adenosine in renal protection by IP [19,27,50–52]. Therefore, we first measured renal adenosine levels in preconditioned or unpreconditioned renal tissues. As shown in Figure 1, renal adenosine tissue content was approximately 10-fold higher after renal IP as compared to baseline. Similarly, renal adenosine levels were also elevated in IP-treated renal tissues after 45 min of ischemia. To confirm the extracellular nature of these findings, we repeated this experiment in previously characterized mice deficient of *cd73* [23,52]. In fact, CD73 metabolizes the extracellular conversion of adenosine monophosphate (AMP) to adenosine, and thus represents the pacemaker enzyme of extracellular adenosine generation [53]. We found that renal adenosine content in *cd73*<sup>-/-</sup> mice with IP or IP prior to ischemia was significantly lower compared to littermate controls, thereby confirming the extracellular nature of IP-dependent adenosine elevations. Taken together, these studies demonstrate extracellular elevations of adenosine with renal IP treatment.

### IP Is Selectively Abolished in A2BAR<sup>-/-</sup> Mice

To elucidate adenosine signaling pathways through individual ARs, we exposed previously described *A1AR*<sup>-/-</sup> [42], *A2AAR*<sup>-/-</sup> [43], *A2BAR*<sup>-/-</sup> [28], and *A3AR*<sup>-/-</sup> [41], mice to renal IP prior to ischemia. For this purpose, mice were subjected to 45 min of renal artery occlusion with or without prior IP (four cycles, 4 min ischemia, 4 min reperfusion) followed by 24 h of reperfusion until assessment of renal function. To avoid surgical trauma during the procedure, we utilized a recently described model of intermittent renal artery occlusion via a hanging-weight system for isolated renal artery occlusion [40]. As shown in Figure 2A–2D, plasma creatinine and GFR, assessed by inulin clearance, were improved by IP in all genotyped mice for individual ARs to a similar degree as their respective littermate control mice, with the notable exception of *A2BAR*<sup>-/-</sup> mice. In fact, attenuation of plasma creatinine and improved GFR with IP were completely abolished in *A2BAR*<sup>-/-</sup> mice (Figure 2C). In addition, the renal protective



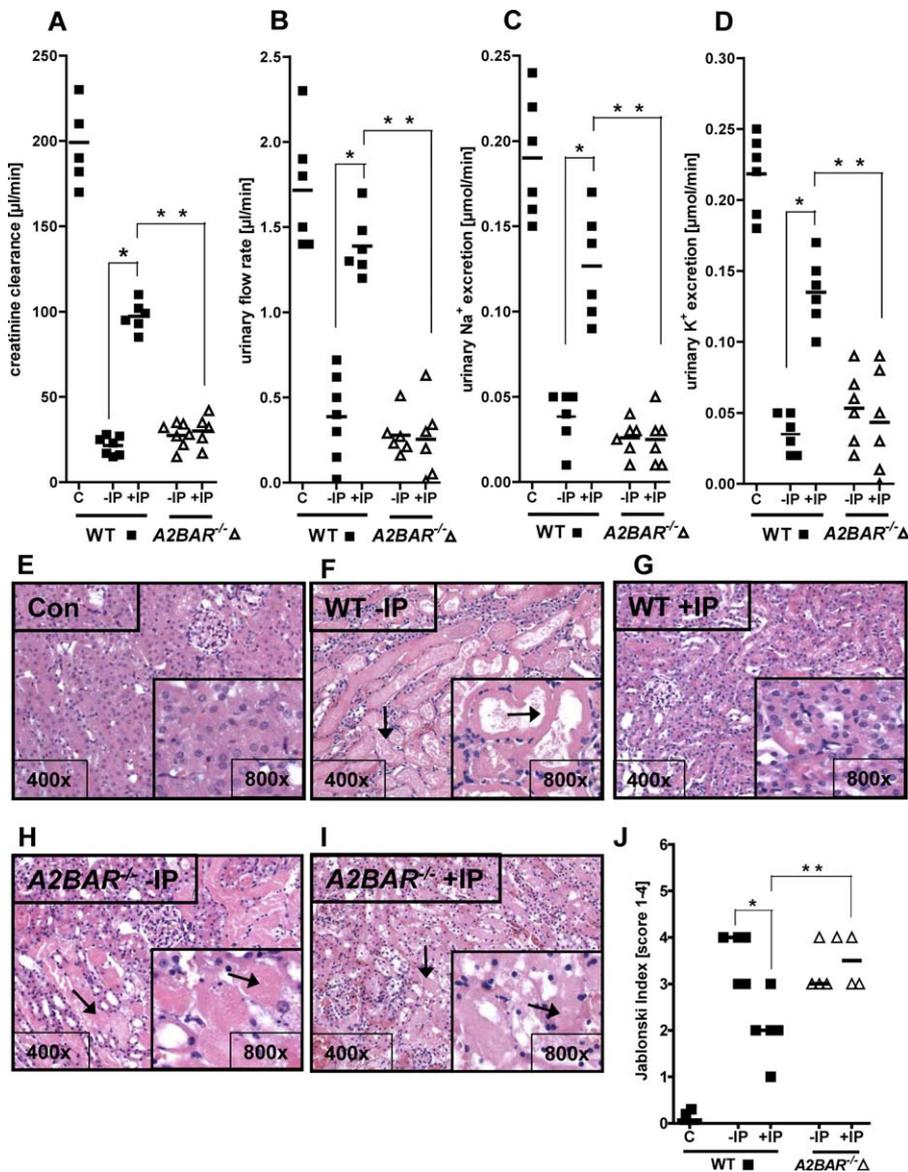
**Figure 2.** Renal Ischemia and Preconditioning in Gene-Targeted Mice for Individual ARs

(A–D) Previously characterized *A1AR*<sup>-/-</sup>, *A2AAR*<sup>-/-</sup>, *A2BAR*<sup>-/-</sup>, and *A3AR*<sup>-/-</sup> mice or their respective age-, weight-, and gender-matched littermate controls (WT) were subjected to right nephrectomy followed by 45 min of left renal artery ischemia with or without prior in situ IP (consisting of four cycles of 4 min ischemia followed by 4 min reperfusion). Plasma creatinine and GFR (as measured by inulin clearance) were obtained after 24 h of reperfusion. Note: Renal protection conferred by IP is abrogated in *A2BAR*<sup>-/-</sup> mice.

Statistics: One-way ANOVA with Tukey's post-hoc test was performed (\*,  $p < 0.001$  versus WT + IP; \*\*,  $p < 0.001$  versus the respective gene targeted mice with IP). The *F*-test results are for plasma creatinine: (A)  $F(3,21) = 41.2$ ,  $p < 0.001$ ; (B)  $F(3,23) = 60.66$ ,  $p < 0.001$ ; (C)  $F(3,22) = 67.87$ ,  $p < 0.001$ ; (D)  $F(3,25) = 48.45$ ,  $p < 0.001$ ; and for GFR (A)  $F(3,20) = 79.34$ ,  $p < 0.001$ ; (B)  $F(3,21) = 59.12$ ,  $p < 0.001$ ; (C)  $F(3,22) = 109.75$ ,  $p < 0.001$ ; (D)  $F(3,22) = 93.43$ ,  $p < 0.001$ . doi:10.1371/journal.pmed.0050137.g002

effects of IP as assessed by creatinine clearance (Figure 3A), urinary flow rate (Figure 3B), urinary excretion of sodium (Figure 3C), or potassium (Figure 3D) were abrogated following targeted deletion of the *A2BAR*. To confirm the absence of renal protection of IP in *A2BAR*<sup>-/-</sup> mice on a histological evaluation depicts level, we examined kidneys from WT or *A2BAR*<sup>-/-</sup> mice after 45 min of ischemia with and without previous IP (control: Figure 3E, WT without IP; Figure 3F, WT with IP; Figure 3G). Histological intact tubular cell morphology in kidneys with IP prior to ischemia (WT + IP) in contrast to kidneys without IP (WT - IP). Here, severe acute tubular necrosis—obvious from the loss of tubular cell nuclei (arrow in Figure 3F) in the cortex and outer medulla with destruction of

the proximal tubular brush border—was observed (Figure 3F). In addition, hyaline cast formation, intraluminal necrotic cellular debris (arrow in Figure 3F), and casts containing brush border blebs could be seen in the cortex as well as in the outer medulla. In contrast, these signs of tubular damage were only sporadic and quantitatively mild in mice with IP (Figure 3G). Semiquantitative histological analysis demonstrated a reduction in the Jablonski index from 4 (range 3–4) without IP to 2 (range 1–3, Figure 3J,  $p < 0.05$ ) with IP. This obvious improvement of renal histology by IP was completely abolished in *A2BAR*<sup>-/-</sup> mice (Figure 3H and 3I). Taken together, these data provide the first functional evidence for a critical role of the *A2BAR* in renal protection by IP.



**Figure 3.** Renal Function and Histology in  $A2BAR^{-/-}$  Mice Exposed to Renal Ischemia or Preconditioning

$A2BAR^{-/-}$  or age-, weight-, and gender-matched littermate controls (WT) were subjected to right nephrectomy followed by 45 min of left renal artery ischemia with or without prior in situ IP (consisting of four cycles of 4 min ischemia followed by 4 min reperfusion). Renal function tests and renal histology were obtained after 24 h of reperfusion. (A) Creatinine clearance.

(B) Urinary flow rate.

(C) Urinary sodium excretion.

(D) Urinary potassium excretion.

(E–I) Representative HE staining (400 $\times$  and 800 $\times$ ). Tissue damage was characterized by loss of tubular cells, tubular cast, and tubular dilation (arrows). Slice thickness was 3  $\mu\text{m}$ .

(J) Quantification of histological tissue damage assessed by Jablonski index.

Statistics: (A–D) One-way ANOVA with Tukey's post-hoc test was performed (\*,  $p < 0.001$  versus WT + IP; \*\*,  $p < 0.001$  versus the  $A2BAR^{-/-}$  mice with IP).

The  $F$ -test results are (A)  $F(4,28) = 207.93$ ,  $p < 0.001$ ; (B)  $F(4,26) = 50.96$ ,  $p < 0.001$ ; (C)  $F(4,25) = 57.65$ ,  $p < 0.001$ ; (D)  $F(4,25) = 57.65$ ,  $p < 0.001$ .

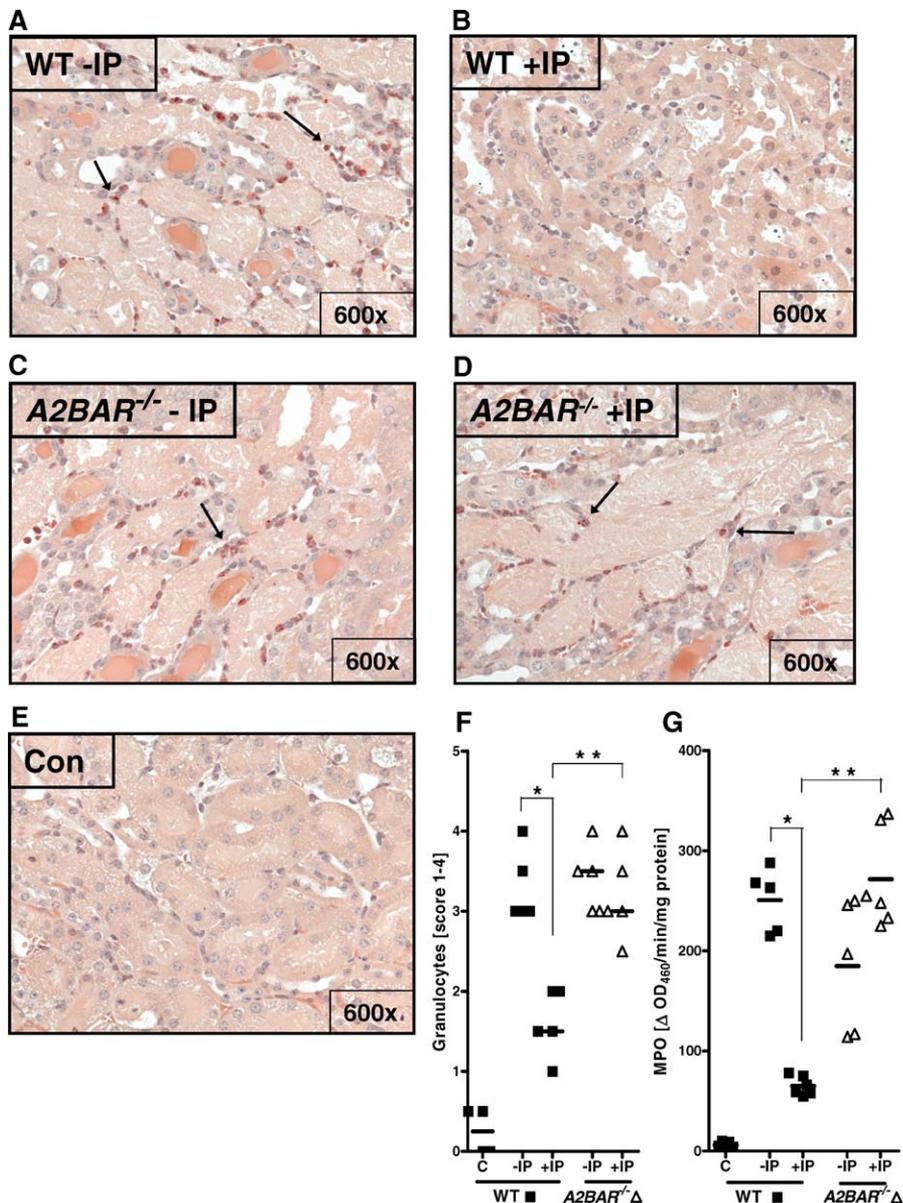
(J) Kruskal-Wallis nonparametric analysis (chi-square = 17.175, degree of freedom (df) = 4,  $p = 0.002$ ) with follow-up pairwise comparisons by Wilcoxon-Mann-Whitney test was performed with \*,  $p < 0.05$  versus WT + IP and \*\*,  $p < 0.05$  versus the  $A2BAR^{-/-}$  mice with IP.

doi:10.1371/journal.pmed.0050137.g003

### Anti-inflammatory Effects of IP Are Abrogated in $A2BAR$ Deficient Mice

After having shown that  $A2BAR^{-/-}$  mice are not protected by renal IP, we next attempted to understand how  $A2BAR$  signaling can affect renal IP. Here, we considered a study showing an anti-inflammatory role of  $A2BAR$  signaling in hypoxic preconditioning [26]. In fact, Khoury et al. demonstrated that adenosine-dependent signaling pathways inhibit

NF $\kappa$ B activation during preconditioning [26]. Based on the hypothesis, that  $A2BAR$  signaling may be critical in mediating anti-inflammatory effects of IP, we assessed renal inflammatory cell accumulation during ischemia. As shown in Figure 4A–4E, 45 min of renal ischemia followed by 24 h of reperfusion was associated with dramatic increases in renal granulocyte accumulation as shown in histological sections (Figure 4A–4E), granulocyte score (Figure 4F), and measurement of the



**Figure 4.** Inflammatory Changes in  $A2BAR^{-/-}$  Mice during Renal Preconditioning

$A2BAR^{-/-}$  mice and their respective age-, weight-, and gender-matched littermate controls (WT) were subjected to right nephrectomy followed by 45 min of left renal artery ischemia with or without prior in situ IP (consisting of four cycles of 4 min ischemia followed by 4 min reperfusion).

(A–E) Chloracetate esterase staining. Arrows indicate granulocytes (magnification 600 $\times$ ).

(F) Quantification of granulocyte accumulation.

(G) Quantification of neutrophil tissue accumulation by measurement of myeloperoxidase (MPO).

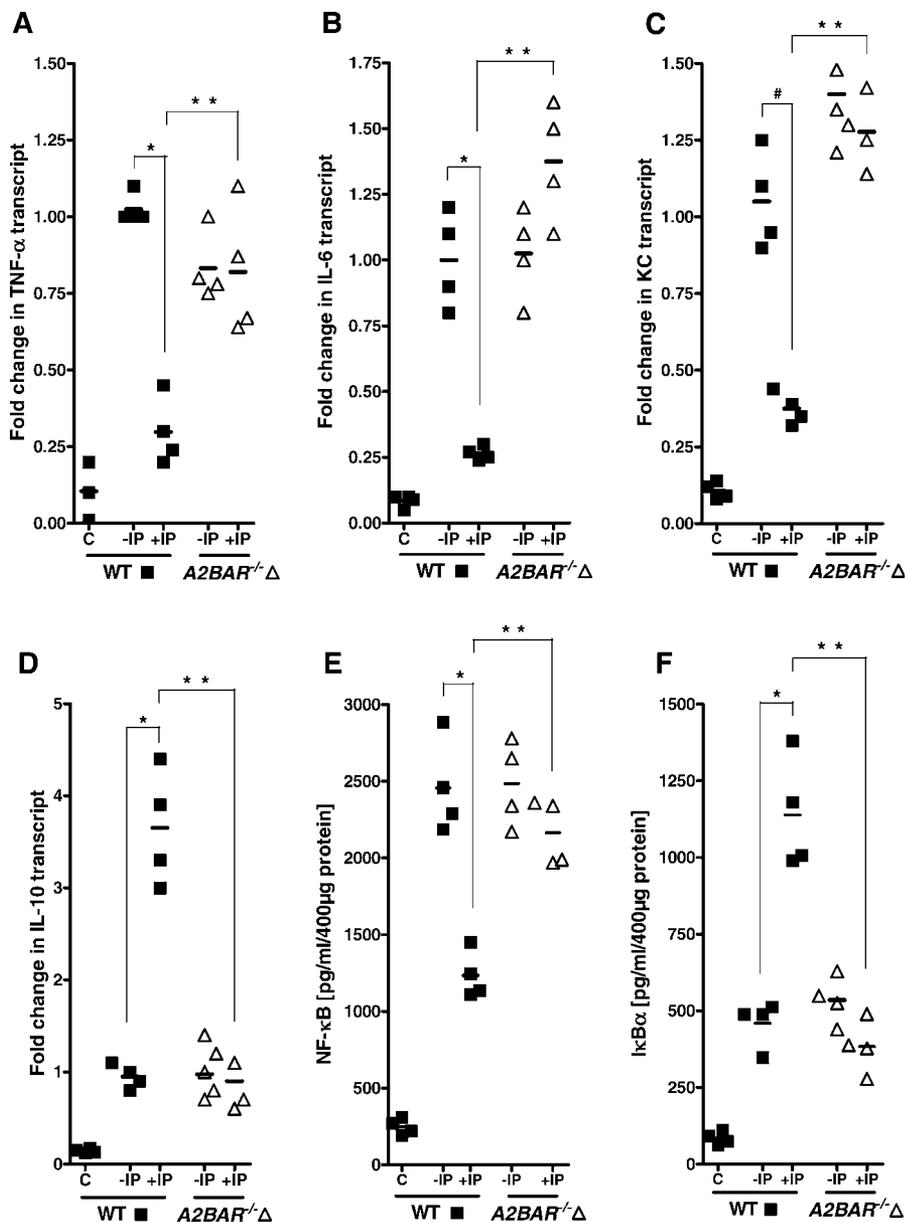
Statistics: (F) Kruskal-Wallis nonparametric analysis (chi-square = 17.86, df = 4,  $p = 0.001$ ) with follow-up pairwise comparisons by Wilcoxon-Mann-Whitney test was performed with \*,  $p < 0.01$  versus WT + IP and \*\*,  $p < 0.01$  versus the  $A2BAR^{-/-}$  mice with IP.

(G) One-way ANOVA with Tukey's post-hoc test was performed (\*,  $p < 0.001$  versus WT + IP; \*\*,  $p < 0.001$  versus the  $A2BAR^{-/-}$  mice with IP). The  $F$ -test result is  $F(4,22) = 46.26$ ,  $p < 0.001$ .

doi:10.1371/journal.pmed.0050137.g004

polymorphonuclear leukocyte (PMN) marker myeloperoxidase (MPO) (Figure 4G). Pretreatment with IP resulted in a profound reduction in inflammatory cell accumulation. However, the anti-inflammatory effects of renal IP were completely abolished in  $A2BAR^{-/-}$  mice. Along the same lines, renal transcript of pro-inflammatory cytokines TNF- $\alpha$ , IL-6, and KC were all attenuated following IP treatment of WT mice, whereas no changes with IP occurred in  $A2BAR^{-/-}$  mice (Figure 5A–5C). In contrast, transcript levels of the anti-inflammatory cytokine IL-10 were elevated with IP treatment of WT mice,

whereas IP in gene-targeted mice for the  $A2BAR$  did not alter renal IL-10 transcript levels (Figure 5D). Consistent with previous studies showing adenosine signaling in inhibiting NF $\kappa$ B activity [26], we found that increases of renal NF $\kappa$ B activity with ischemia were attenuated following IP treatment (Figure 5E). In contrast, IP-dependent inhibition of NF $\kappa$ B activation was abrogated in  $A2BAR^{-/-}$  mice (Figure 5E). While a number of mechanisms have been suggested to mediate the endogenous inactivation of NF $\kappa$ B, each of these pathways converges on changes in the inducible degradation of I $\kappa$ B [54].



**Figure 5.** Transcriptional Responses of Inflammatory Cytokines Measured in Renal Tissue

$A2BAR^{-/-}$  mice and their respective age-, weight-, and gender-matched littermate controls (WT) were subjected to right nephrectomy followed by 45 min of left renal artery ischemia with or without prior in situ IP (consisting of four cycles of 4 min ischemia followed by 4 min reperfusion). (A)  $TNF-\alpha$ , (B) IL-6, (C) KC (mouse ortholog of human IL-8), and (D) IL-10 were assessed by real-time RT-PCR from renal tissues. Data were calculated relative to  $\beta$ -actin and are expressed as fold change compared to sham-operated animals without IP treatment.

(E)  $NF-\kappa B$  and (F)  $I\kappa B\alpha$  levels were evaluated in renal tissue homogenates using a mouse enzyme-linked immunosorbent assay (ELISA).

Statistics: One-way ANOVA with Tukey's post-hoc test was performed (\*,  $p < 0.001$  versus WT + IP; #,  $p < 0.05$  versus WT+IP; \*\*,  $p < 0.001$  versus the  $A2BAR^{-/-}$  mice with IP). The  $F$ -test results are (A)  $F(4,15) = 39.28$ ,  $p < 0.001$ ; (B)  $F(4,15) = 53.40$ ,  $p < 0.001$ ; (C)  $F(4,15) = 201.01$ ,  $p < 0.001$ ; (D)  $F(4,15) = 61.46$ ,  $p < 0.001$ ; (E)  $F(4,15) = 75.76$ ,  $p < 0.001$ ; and (F)  $F(4,15) = 56.18$ ,  $p < 0.001$ .

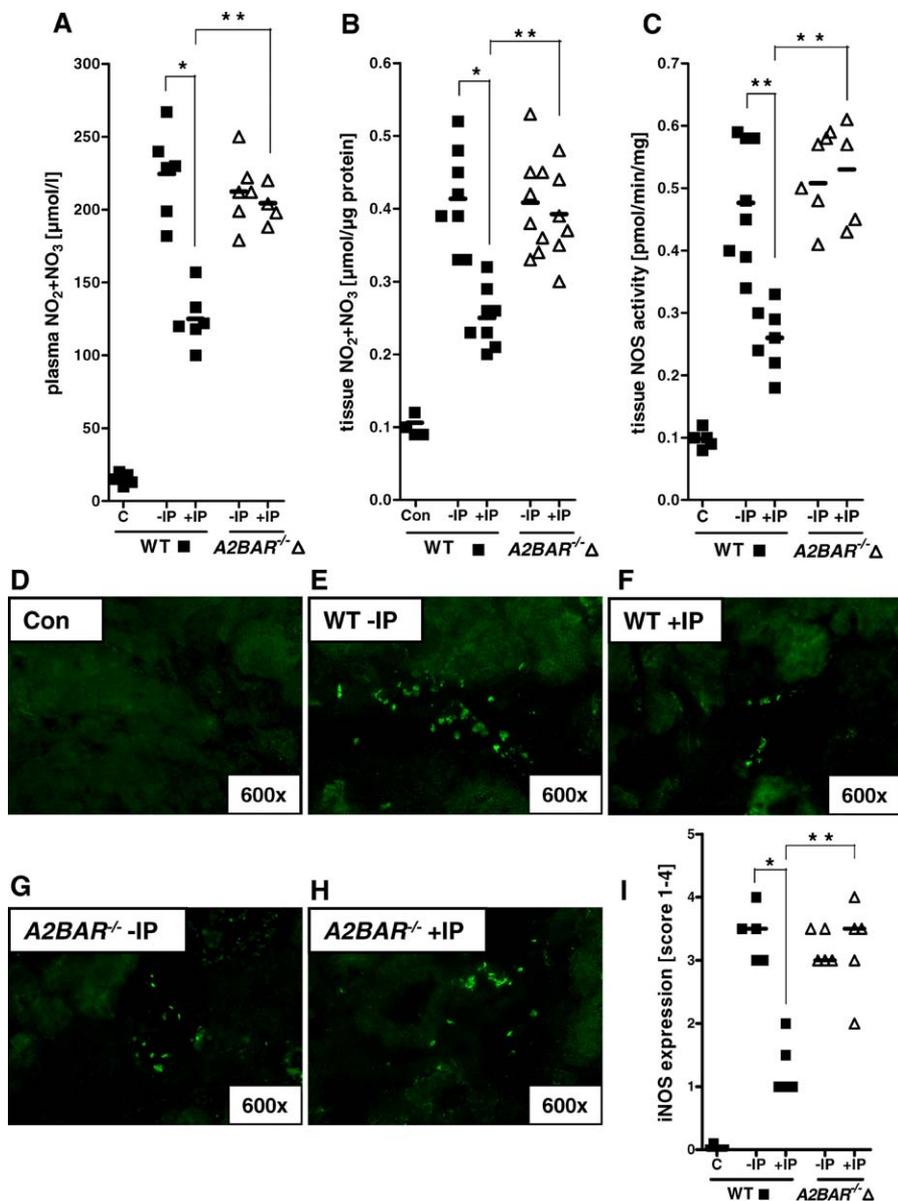
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Thus, we determined whether A2BAR signaling influences  $I\kappa B$  activity during IP. In fact, IP associated increase in renal  $I\kappa B$  activity were attenuated in  $A2BAR^{-/-}$  mice (Figure 5F). Taken together, these studies reveal A2BAR-dependent anti-inflammatory effects of renal IP.

#### IP-Associated Attenuation of Nitric Oxide Pathways Is Abolished in $A2BAR^{-/-}$ Mice

Previous studies of renal ischemia revealed that ischemic tissues and infiltrating inflammatory cells express iNOS and

release reactive oxygen species and N-derived oxidants [37]. As such, inhibition of iNOS attenuates ischemia reperfusion injury [37]. In turn, oxidative stress is involved in the pathogenesis of ischemia-reperfusion injury by adding further insult to renal tissues and inducing the release of pro-inflammatory molecules, which recruit inflammatory cells into the interstitium [55]. Consistent with these studies, we found significant increases in plasma and renal tissue levels of nitrite/nitrate with renal ischemia, which were attenuated by IP-treatment (Figure 6A and 6B). However,



**Figure 6.** Nitrite, Nitrate Plasma Concentrations, and Renal Tissue Content and Nitric Oxide Synthetase Activity in Renal Tissues

*A2BAR*<sup>-/-</sup> mice and their respective age-, weight-, and gender-matched littermate controls (WT) were subjected to right nephrectomy followed by 45 min of left renal artery ischemia with or without prior in situ IP (consisting of four cycles of 4 min ischemia followed by 4 min reperfusion). (A) Plasma or (B) tissue concentrations of nitrite (NO<sub>2</sub>) and nitrate (NO<sub>3</sub>) were measured using colorimetric-assay kits.

(C) Tissue nitric oxide synthetase (NOS) activity.

(D–H) Immunohistochemical staining for iNOS.

(I) iNOS expression score.

Statistics: (A–C) One-way ANOVA with Tukey's post-hoc test was performed (\*,  $p < 0.001$  versus WT +IP; \*\*,  $p < 0.001$  versus the *A2BAR*<sup>-/-</sup> mice with IP). The *F*-test results are (A)  $F(4,22) = 89.90$ ,  $p < 0.001$ ; (B)  $F(4,30) = 32.41$ ,  $p < 0.001$ ; (C)  $F(4,25) = 36.54$ ,  $p < 0.001$ .

(I) Kruskal-Wallis nonparametric analysis (chi-square = 19.1,  $df = 4$ ,  $p = 0.001$ ) with follow-up pairwise comparisons by Wilcoxon-Mann-Whitney test was performed with \*,  $p < 0.01$  versus WT +IP and \*\*,  $p < 0.05$  versus the *A2BAR*<sup>-/-</sup> mice with IP.

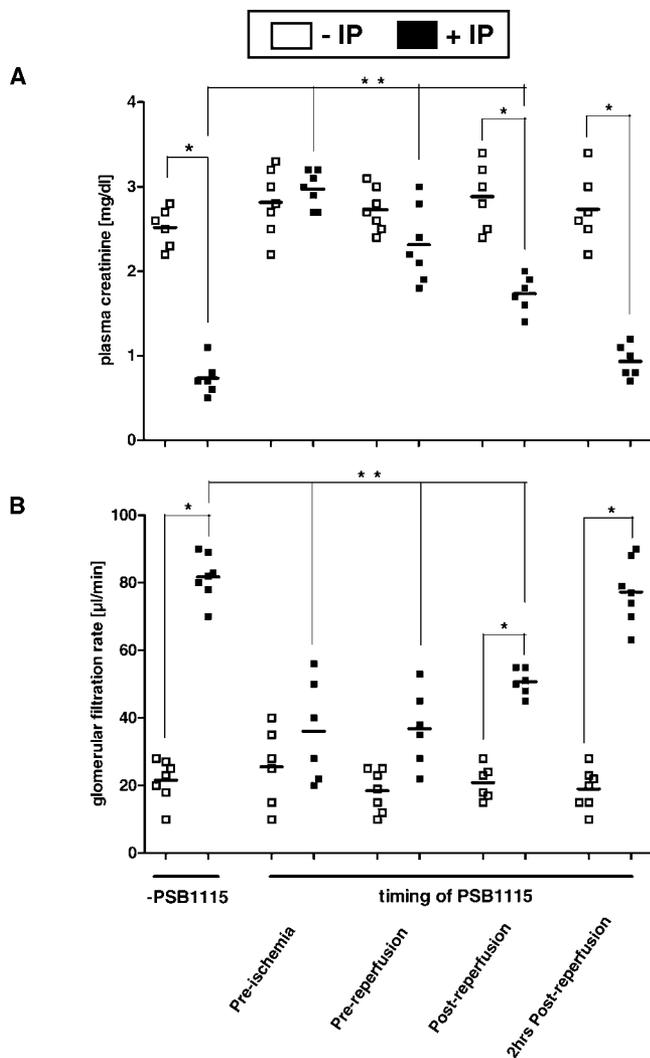
doi:10.1371/journal.pmed.0050137.g006

attenuation by IP was abolished in gene-targeted mice for the *A2BAR* (Figure 6A and 6B). Similarly, iNOS activity was reduced in IP-treated renal tissues of *A2BAR* WT mice but not in *A2BAR*<sup>-/-</sup> mice (Figure 6C). Moreover, immunohistochemistry revealed a strong immune-reactivity for iNOS in macrophages following ischemia. Macrophage infiltration and iNOS expression was reduced following IP treatment of WT but not of *A2BAR*<sup>-/-</sup> mice (Figure 6D–6I). Taken together, these studies suggest that IP-associated attenuation

of nitric oxide production involves signaling through the *A2BAR*.

#### *A2BAR* Antagonist Treatment Abolishes Renal Protection by IP

After having shown a critical role of *A2BAR*-signaling in renal protection by IP in gene-targeted mice, we next pursued pharmacological approaches. For this purpose, we administered a highly selective, water soluble *A2BAR*



**Figure 7.** Pharmacological Studies of A2BAR Signaling during Renal Ischemia and Preconditioning

A2BAR antagonist (PSB1115): Age-, weight-, and gender-matched C57BL/6J underwent right nephrectomy. Prior to the surgery, immediately before ischemia, 5 min or 2 h after ischemia, they received the selective A2BAR antagonist PSB1115 (5 mg/kg, intravenous) or treatment with a vehicle control. They were exposed to 45 min of left renal artery ischemia with or without prior in situ IP (consisting of four cycles of 4 min ischemia followed by 4 min of reperfusion). (A) Plasma creatinine and (B) GFR as assessed by inulin clearance were obtained after 24 h of reperfusion.

Statistics: One-way ANOVA with Tukey's post-hoc test was performed (\*,  $p < 0.001$  versus the respective group without IP; \*\*,  $p < 0.001$  versus WT mice with IP and with PSB1115 treatment). The  $F$ -test results are (A)  $F(9,54) = 42.20$ ,  $p < 0.001$ ; (B)  $F(9,55) = 51.67$ ,  $p < 0.001$ .

doi:10.1371/journal.pmed.0050137.g007

antagonist PSB1115 [56] and treated WT mice with intravenous PSB1115, 5 mg/kg/h, a dose we had previously shown to be effective in inhibiting the murine A2BAR in vivo [28]. To determine a time-window of PSB1115 in attenuating renal IP-protection, we administered PSB1115 or vehicle prior to the surgery, before ischemia, and 5 min and 2 h after ischemia. As shown in Figure 7, renal protective effects of IP on plasma creatinine (Figure 7A) and on GFR (Figure 7B) were abolished with PSB1115 treatment, when given before or early during reperfusion. These pharmacological studies

confirm the critical role of A2BAR signaling for renal protection by IP.

### A2BAR Agonist (BAY 60–6583) Improves Renal Function during Ischemia

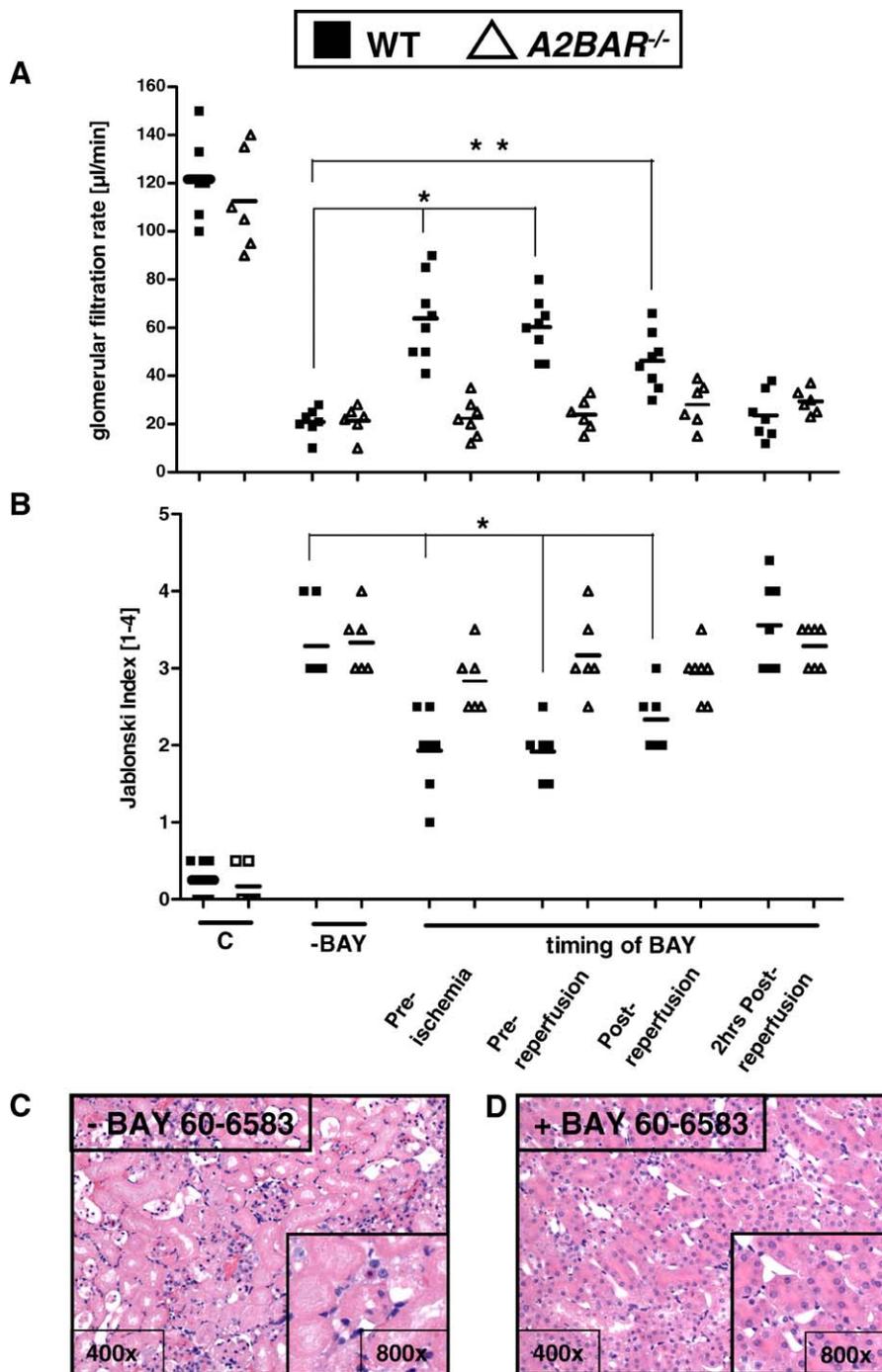
After having shown that selective inhibition of the A2BAR attenuates renal protection by IP, we next pursued a potential therapeutic role of a specific A2BAR agonist (BAY 60–6583). In fact, a recent study demonstrated selectivity and functional in vivo evidence of this compound [28]. Here, we treated C57BL/6J mice with a single intravenous bolus of BAY 60–6583 (60 µg/kg body weight) prior to the surgical procedure, directly before renal ischemia, and 5 min or 2 h after ischemia. BAY 60–6583 treatment resulted in a dramatic improvement of GFR (Figure 8A) and plasma creatinine levels (unpublished data). When this experiment was repeated in  $A2BAR^{-/-}$  mice, no improvement in GFR was found (Figure 8A), confirming the specificity of the renal protective effects of BAY 60–6583 through A2BAR signaling. Semiquantitative histological analysis confirmed severe acute tubular necrosis without BAY 60–6583 treatment in WT mice (Jablonski index of 3, range 3–4, Figure 8B–8D). Treatment with BAY 60–6583 was associated with a significant attenuation of tubular destruction (Jablonski index of 2, range 1–3,  $p < 0.05$ , Figure 8B–8D). In contrast  $A2BAR^{-/-}$  mice showed no reduction in tubular damage with BAY 60–6583 treatment (Figure 8B–8D). Moreover, renal protection from ischemia with BAY 60–6583 treatment was limited to a time window that ended during early reperfusion. Taken together, these studies provide strong rationale for therapeutically targeting the A2BAR during renal ischemia.

### Renal IP Is Associated with Selective Induction of the A2BAR

After having demonstrated A2BAR-dependent signaling pathways in renal protection by IP, we next investigated transcriptional consequences of renal IP on expression levels of all four ARs. For this purpose, renal tissues were harvested at indicated time points following IP-treatment and transcript levels of individual ARs were determined by real-time RT-PCR (Figure 9A–9D). These studies revealed prompt and selective induction of the A2BAR transcript following renal IP (approximately 6-fold induction 60 min after IP,  $p < 0.05$ , Figure 9C). In contrast, mRNA levels for the A1AR, A2AAR, and A3AR remained unchanged following 60, 120, or 240 min of IP (Figure 9A, 9B, and 9D). In addition, Western blot analysis of A2BAR protein from preconditioned renal tissues confirmed A2BAR induction (Figure 9E). Consistent with previous work showing selective A2BAR induction with vascular hypoxia [24] or myocardial IP [28], these studies reveal induction of A2BAR transcript and protein with renal IP treatment.

### Studies in A2BAR Reporter Mice Confer A2BAR Expression to the Reno-Vasculature

On the basis of the above studies showing induction of the renal A2BAR with preconditioning, we were interested to define, which tissues express the A2BAR within the kidneys. Here, we used a previously characterized A2BAR reporter mouse (A2BAR-KO/ $\beta$ -gal-knock-in mice) or their respective littermate control mice [44]. Surprisingly,  $\beta$ -gal staining could only be detected within the reno-vasculature. In contrast,



**Figure 8.** A2BAR Agonist (BAY 60-6583).  $A2BAR^{-/-}$  Mice or Age-, Weight-, and Gender-Matched Littermate Controls Underwent Right Nephrectomy followed by 45 min of Left Renal Artery Ischemia and 24 h of Reperfusion

They underwent treatment with BAY 60-6583 (60  $\mu\text{g}/\text{kg}$  intravenous) or vehicle control given before the surgery, immediately before ischemia, 5 min or 2h after ischemia.

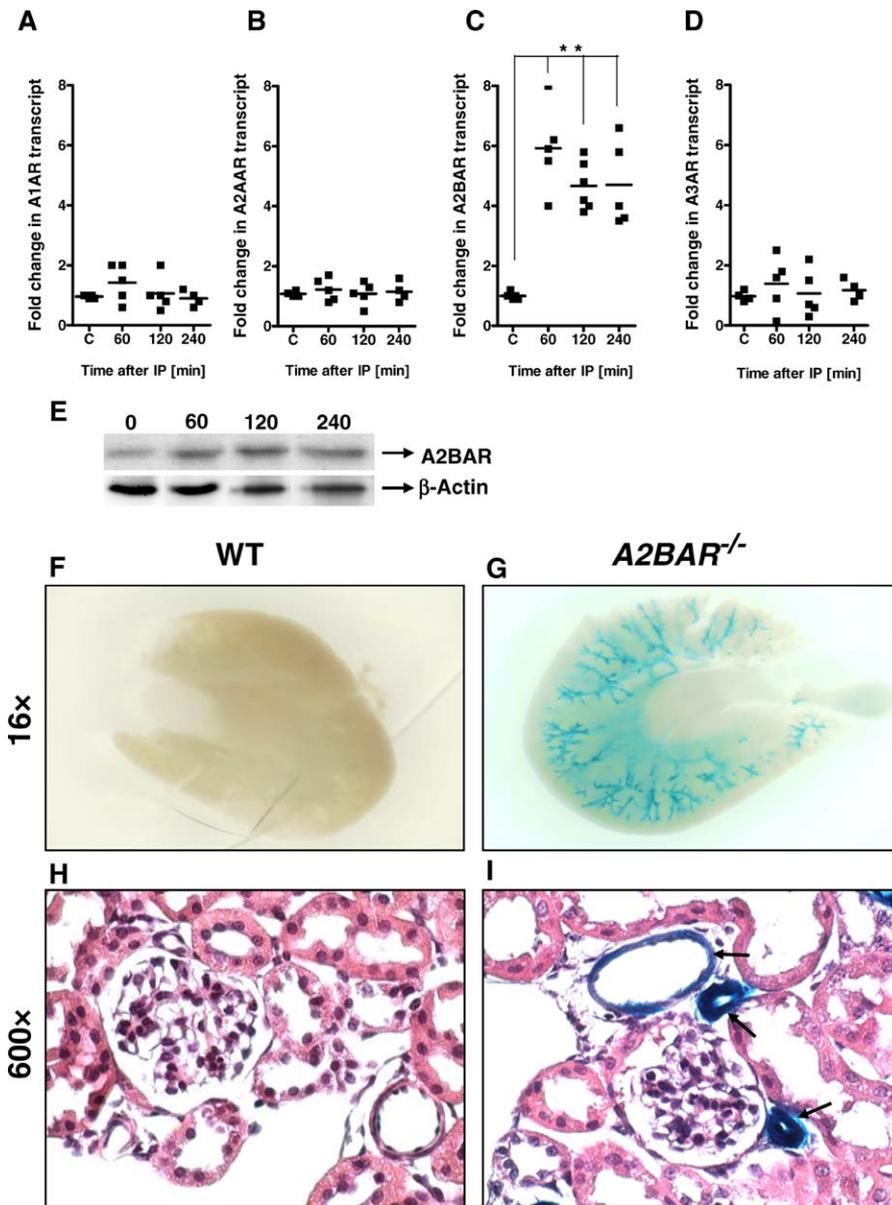
(A) GFR (as measured by inulin clearance and (B) Jablonski index for histological quantification of ischemic injury were obtained after 24 h of reperfusion.

(C, D) Representative HE staining (400 $\times$  and 800 $\times$ ) of renal histology following 45 min of ischemia with (+BAY) or without BAY 60-6583 (-BAY) given prior to ischemia are displayed.

Statistics: (A) One-way ANOVA with Tukey's post-hoc test was performed (\*,  $p < 0.01$  versus the WT mice with BAY treatment, \*\*,  $p < 0.001$  versus WT mice with BAY treatment). The  $F$ -test results are (A)  $F(11,69) = 54.84$ ,  $p < 0.001$ .

(B) Kruskal-Wallis nonparametric analysis (chi-square = 61.08,  $df = 11$ ,  $p < 0.001$ ) with follow-up pairwise comparisons by Wilcoxon-Mann-Whitney test was performed with \*,  $p < 0.01$  versus WT mice with BAY treatment.

doi:10.1371/journal.pmed.0050137.g008

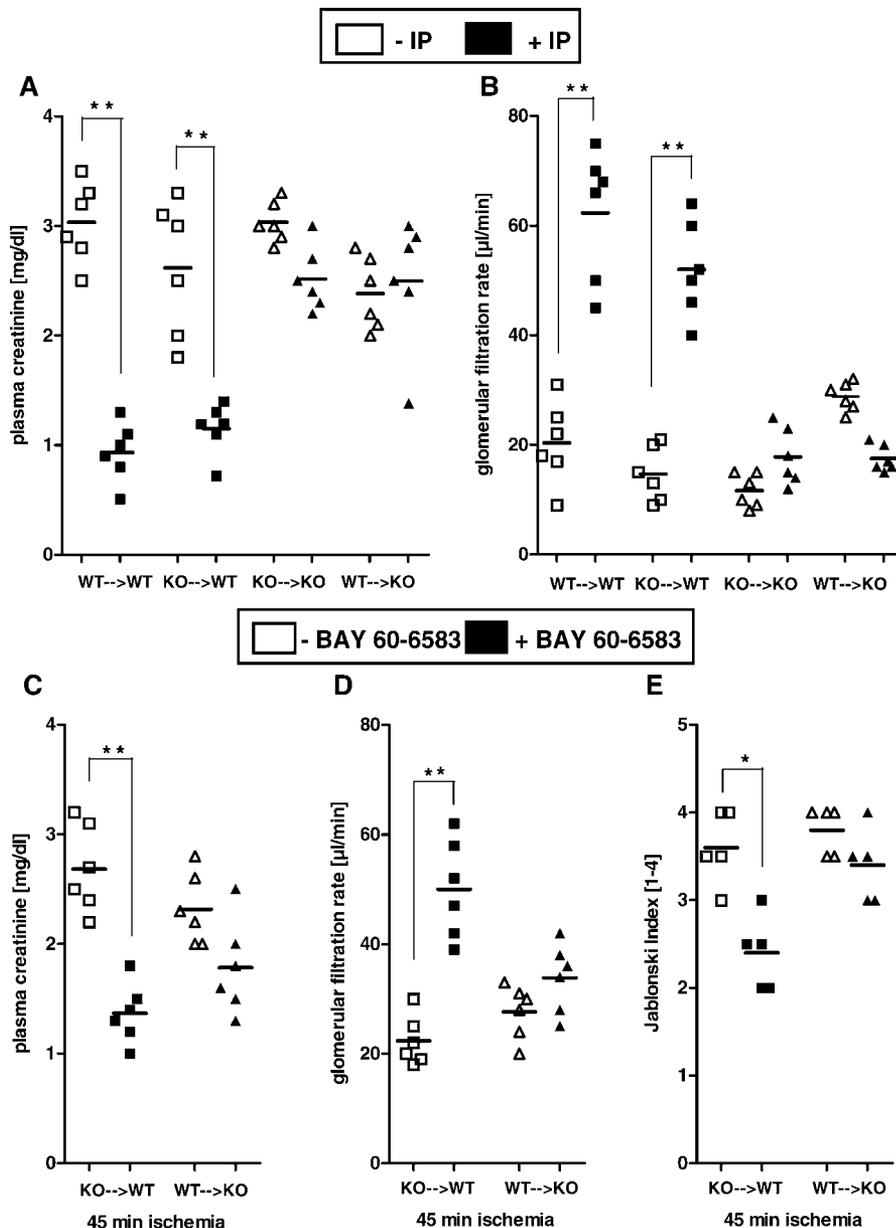


**Figure 9.** Transcriptional Responses to Renal IP and Expression of the A2BAR within the Kidneys (A–D) Age-, gender-, and weight-matched C57BL/6J mice were subjected to right nephrectomy followed by in situ IP with a hanging weight system for atraumatic occlusion of the left renal artery. The IP protocol consisted of four cycles of ischemia/reperfusion (4 min each), followed by indicated times of reperfusion. Transcriptional responses of the A1AR, A2AAR, and A2BAR of the A3AR were assessed by real-time RT-PCR. Data were calculated relative to  $\beta$ -actin and are expressed as fold change compared to sham-operated animals without IP treatment. (E) Western blotting for renal A2BAR following IP treatment. Kidneys were excised at indicated times, flash frozen, and lysed; proteins were resolved by SDS PAGE and transferred to nitrocellulose. Membranes were probed with anti-A2BAR antibody. The same blot was reprobed for  $\beta$ -actin as a control for protein loading. One representative experiment of three is shown. (F–I) A previously described A2BAR reporter mouse was used (A2BAR-KO/ $\beta$ -gal-knock-in mice). Renal tissues were prepared and stained for  $\beta$ -galactosidase ( $\beta$ -Gal) as indicative of the A2BAR gene promoter, which drives the expression of this reporter gene. Procedures are as detailed under Methods. (F, G)  $\beta$ -Gal staining of kidney derived from littermate controls (WT) or A2BAR<sup>-/-</sup> mice, and captured at the indicated magnifications (16 $\times$ ) with an Olympus IX70 microscope combined with Hamamatsu charge-coupled device camera C4742–95. (H, I) HE-staining histological examination of renal sections derived from the kidney shown in upper panel. Arrows point to  $\beta$ -Gal stained blood vessels. Fold magnification of captured images are 600 $\times$ . WT mice were subjected to similar staining showing no expression.  $\beta$ -Gal expression in kidney is clear and localized only in blood vessels. Statistics: (A–D) One-way ANOVA with Dunn's multiple comparison test (\*\*,  $p < 0.001$  versus control WT mice without IP [C]). The  $F$ -test results are (A)  $F(3,17) = 19.82$ ,  $p < 0.001$ . doi:10.1371/journal.pmed.0050137.g009

renal epithelia stained negative for the A2BAR (Figure 9F–9I). Taken together, these studies reveal for the first time, to our knowledge, a selective reno-vascular expression of the A2BAR within the kidneys.

#### Renal IP in A2BAR Bone-Marrow Chimeric Mice

On the basis of these studies showing reno-vascular expression of the A2BAR and other studies showing myeloid A2BAR expression, particularly on macrophages [44], we



**Figure 10.** Renal Ischemia and Preconditioning in A2BAR Bone Marrow Chimeric Mice

A2BAR bone marrow chimeric mice [ $A2BAR^{+/+} \rightarrow A2BAR^{+/+}$  [WT $\rightarrow$ WT],  $A2BAR^{-/-} \rightarrow A2BAR^{-/-}$  [KO $\rightarrow$ KO],  $A2BAR^{-/-} \rightarrow A2BAR^{+/+}$  [KO $\rightarrow$ WT] and  $A2BAR^{+/+} \rightarrow A2BAR^{-/-}$  [WT $\rightarrow$ KO)] were generated. Experiments were performed 8 wk following transplantation. Mice underwent right nephrectomy and underwent 45 min of left renal artery ischemia with or without prior IP (consisting of four cycles of 4 min ischemia followed by 4 min reperfusion). Following 24 h of reperfusion time, (A) plasma creatinine and (B) GFRs were measured.

Influence of A2BAR agonist (BAY 60-6583) on renal function in bone marrow chimeric mice exposed to ischemia. A2BAR bone marrow chimeric mice were subjected to 45 min of ischemia with or without treatment with A2BAR agonist BAY 60-6583 (60 μg/kg intravenous). Following 24 h of reperfusion, (C) plasma creatinine, (D) GFR, and (E) histological tissue injury (Jablonski index) were obtained.

Statistics: (A–D) One-way ANOVA with Tukey's post-hoc test was performed (\*\*,  $p < 0.001$  versus the respective group without IP. The  $F$ -test results are (A)  $F(7,40) = 24.82$ ,  $p < 0.001$ ; (B)  $F(7,40) = 48.04$ ,  $p < 0.001$ ; (C)  $F(3,20) = 15.54$ ,  $p < 0.001$ ; (D)  $F(3,20) = 20.88$ ,  $p < 0.001$ .

(E) Kruskal-Wallis nonparametric analysis (chi-square = 12,  $df = 3$ ,  $p = 0.007$ ) with follow-up pairwise comparisons by Wilcoxon-Mann-Whitney test was performed with \*,  $p < 0.05$  versus WT mice with BAY treatment.

doi:10.1371/journal.pmed.0050137.g010

studied the contribution of renal or hematopoietic A2BAR signaling to renal protection during ischemia or IP. For this purpose, we generated A2BAR bone marrow chimeric mice. As expected,  $A2BAR^{+/+} \rightarrow A2BAR^{+/+}$  chimeric mice showed a similar degree of renal protection (attenuated plasma creatinine and improved GFR) following IP treatment as WT mice (Figure 10A and 10B). Moreover, renal protection

by IP was abrogated in  $A2BAR^{-/-} \rightarrow A2BAR^{-/-}$  mice, similar to  $A2BAR^{-/-}$  mice (Figure 10A and 10B). Surprisingly, and in contrast to previous studies showing myeloid A2BAR signaling in vascular inflammation [44], renal protection by IP was conferred solely to renal A2BAR. In fact,  $A2BAR^{-/-} \rightarrow A2BAR^{+/+}$  chimeric mice showed a similar degree of renal protection by IP as  $A2BAR^{+/+}$  mice (Figure 10A and 10B). In

contrast, renal resistance to ischemia was not increased by IP treatment of  $A2BAR^{+/+} \rightarrow A2BAR^{-/-}$  chimeric mice. Taken together, these studies suggest a critical role of renal A2BARs (as opposed to hematopoietic) in mediating the protective effects of IP.

### Increased Resistance to Renal Ischemia with A2BAR Agonist BAY 60–6853 Treatment Requires Renal A2BARs

To further define the contribution of renal or hematopoietic A2BARs to renal protection from ischemia, we next performed studies of renal ischemia in A2BAR bone-marrow chimeric mice pretreated with the selective A2BAR agonist BAY 60–6853. Bone marrow chimeric A2BAR mice expressing the A2BAR on the tissues but not on their hematopoietic cells ( $A2BAR^{-/-} \rightarrow A2BAR^{+/+}$  mice) showed dramatic improvement in renal function with BAY 60–6853 pretreatment (60 µg/kg) as assessed by plasma creatinine, GFR, and renal histology—similar to  $A2BAR^{+/+}$  mice (Figure 10C–10E). In contrast, BAY 60–6853 pretreatment of A2BAR bone marrow chimeric mice with the A2BAR expressed exclusively on hematopoietic cells ( $A2BAR^{+/+} \rightarrow A2BAR^{-/-}$  mice) lacked any measurable improvement of renal function during ischemia (Figure 10C–10E). Together with our studies in A2BAR reporter mice showing exclusive expression of the renal A2BAR within the vasculature, these data suggest that signaling through the reno-vascular A2BAR is a critical control point for attenuating acute renal failure from ischemia.

### Renal Ischemia Reperfusion Injury Involves Myeloid A2AAR Signaling, While Preconditioning Is Conferred to Renal A2BARs

On the basis of previous studies showing a critical role of myeloid A2AAR signaling in attenuating renal ischemia reperfusion injury [29] and the above results, we pursued the hypothesis that myeloid A2AAR signaling attenuates renal ischemia-reperfusion injury, while renal A2BAR signaling is important for preconditioning phenomena. For this purpose, we transplanted  $A2BAR^{-/-}$  or  $A2BAR^{+/+}$  mice with bone marrow from  $A2AAR^{-/-}$  or  $A2AAR^{+/+}$  mice. Consistent with the above hypothesis, these studies revealed that reno-protection by IP required renal A2BAR signaling, while mice without a myeloid A2AAR had more severe ischemia reperfusion injury (Figure 11A and 11B).

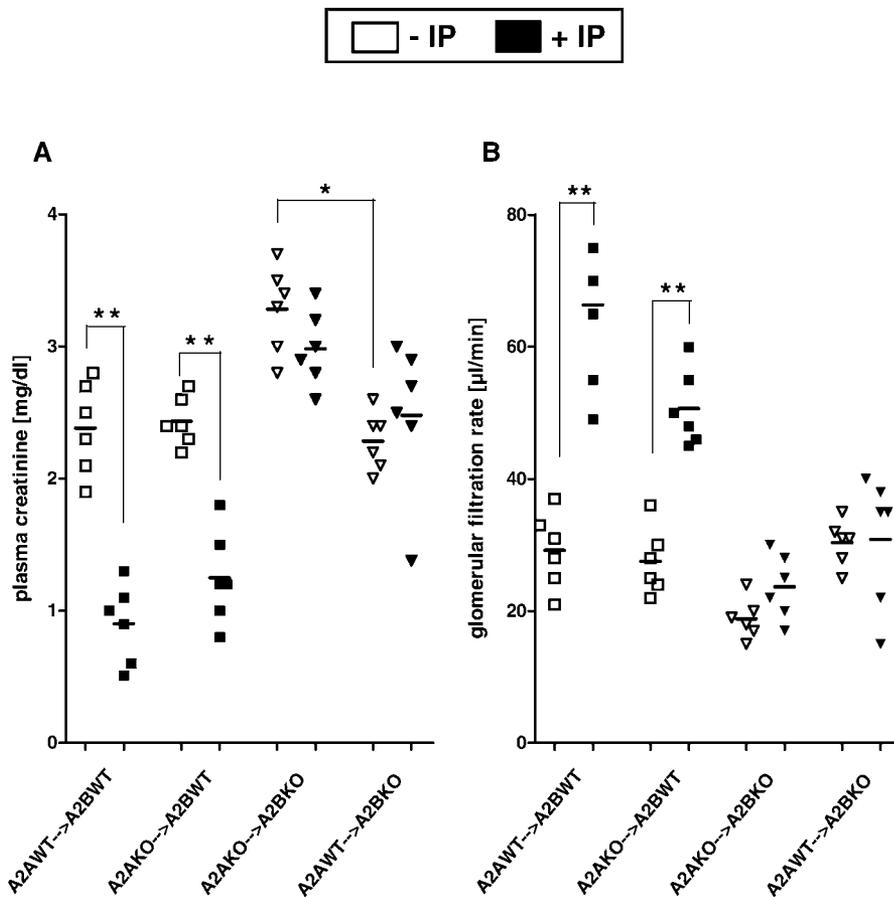
## Discussion

In the present study, we investigated endogenous pathways for renal protection from ischemia. For this purpose, we utilized a recently developed model of renal IP via isolated renal artery occlusion by use of a hanging weight system, which allows for highly reproducible renal injury [40]. While previous studies on IP in the heart have focused on histological measurements of myocardial cell death or leakage of myocardial specific enzymes into the systemic circulation, this model allowed us to assess the consequences of IP-treatment on the preservation of specific renal functions, such as GFR, urinary sodium or potassium excretion, or urine production. On the basis of previous work showing extracellular adenosine generation in renal protection from ischemia [19,20], we tested the contribution of signaling through individual ARs to renal protection from ischemia by IP. Using genetic and pharmacological ap-

proaches, these studies revealed a pivotal role of the A2BAR. In fact, selective A2BAR agonist treatment provided a similar degree of renal protection from ischemia as IP treatment itself, suggesting that A2BAR agonists “precondition” the kidneys. Finally, the use of a reporter mouse model and studies in bone marrow A2BAR chimera helped to pinpoint the source of A2BAR protection to the reno-vasculature. This vascular phenotype was a surprising finding, as previous studies in the heart have conferred preconditioning phenomena mainly towards a metabolic adaptation of the myocardium [27,28,35,36,57]. In contrast, the present studies have revealed a critical contribution of the reno-vascular A2BAR to renal IP protection. Taken together, these data provide what we believe to be a previously not appreciated role of reno-vascular A2BAR signaling in enhancing renal resistance to ischemia and provide strong rationale for therapeutically targeting the A2BAR during renal ischemia.

Presently, the molecular mechanism of how renal A2BAR signaling attenuates renal injury from ischemia remains unknown. As previous studies have shown an important role of erythropoietin in tissue adaptation to hypoxia [58] or preconditioning [59], it would be tempting to speculate that extracellular adenosine generation and signaling (e.g., through the A2BAR) could influence renal erythropoietin secretion, e.g., as part of an autocrine feedback loop. However, recent work tested the influence of adenosine generation and signaling on renal erythropoietin release using genetic and pharmacological approaches [60,61]. These studies revealed that targeting extracellular adenosine generation ( $cd3^{-/-}$  mice) [60,61] or individual ARs ( $AR^{-/-}$  mice) [61] did not influence stimulated erythropoietin release. Taken together these studies rule out a contribution of extracellular adenosine generation or signaling on renal erythropoietin release. On the other hand, a study on extracellular adenosine in hypoxic preconditioning uncovered a novel molecular pathway of how adenosine signaling through the A2BAR can promote tissue adaptation to limited oxygen availability [26]. In this study, the authors investigated hypoxic precondition as a form of endogenous protection that renders cells tolerant to subsequent and more severe challenges of hypoxia. In order to define anti-inflammatory properties of preconditioning, the authors identified a cluster of NFκB-regulated genes whose expression was attenuated by preconditioning. Subsequent studies revealed that preconditioning-elicited protection was conferrable, as a soluble supernatant from preconditioned cells, and the active fraction identified as adenosine. Guided by recent studies demonstrating bacterial inhibition of NFκB through cullin-1 (Cul-1) deneddylation, they found a dose-dependent deneddylation of Cul-1 mediated by signaling through the A2BAR, suggesting that extracellular accumulation of adenosine and signaling through the A2BAR suppresses NFκB activity via deneddylation of Cul-1. These results are consistent with our present study showing that A2BAR-dependent signaling pathways are important for the anti-inflammatory properties of renal IP.

However, renal A2BAR signaling could also involve other (and also faster) mechanisms of renal protection other than modulating inflammation. Based on the fact that dead tissues may themselves cause the induction of inflammation, the observed inflammatory infiltrates may well be a result of dead and dying cells rather than the cause of cell death. Moreover,



**Figure 11.** Renal Ischemia and Preconditioning in A2A/A2B AR Bone Marrow Chimeric Mice

A2BAR<sup>+/+</sup> (A2BWT) or A2BAR<sup>-/-</sup> (A2BKO) mice underwent lethal radiation followed by bone marrow transplantation with marrow derived from A2AAR<sup>+/+</sup> (A2AWT) or A2AAR<sup>-/-</sup> (A2AKO) mice. Experiments were performed 8 wk following transplantation. Mice underwent right nephrectomy and underwent 45 min of left renal artery ischemia with or without prior IP (consisting of four cycles of 4 min ischemia followed by 4 min reperfusion). Following 24 h of reperfusion time, (A) plasma creatinine and (B) GFRs were measured.

Statistics: One-way ANOVA with Tukey's post-hoc test was performed (\*\*,  $p < 0.001$  versus the respective group with IP; \*,  $p < 0.001$  versus A2AWT→A2BKO). The  $F$ -test results are (A)  $F(7,40) = 32.45$ ,  $p < 0.001$ ; (B)  $F(7,40) = 29.67$ ,  $p < 0.001$ .

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the studies of bone-marrow chimeric A2BAR mice also tended to support inflammation as an effect rather than a cause as the receptors responsible for the protection were found not to be on the marrow-derived cells but on the reno-vasculature. As such, considerable evidence now indicates that IP-protection in the heart may be the result of preservation of the cardiac mitochondria by preventing permeability transition pore formation, which normally occurs in the first minutes of reperfusion [36,57,62]. Therefore, similar mechanisms could be accounting for the reno-protective effects observed in the kidneys. Moreover, the fact that abolished renal protection with A2BAR antagonist treatment and reno-protective effects with A2BAR agonist treatment are limited to a time window that ends during early reperfusion (Figures 6 and 7) speaks in favor for such mechanisms.

The present study is consistent with previous work showing an anti-inflammatory role of vascular A2BAR signaling. In fact, a study by Yang et al. used an “A2BAR-knockout/reporter gene-knock-in” approach to study the role of the A2BAR in inflammatory models [44]. The authors found highest expressional levels of the A2BAR in the vasculature and on macrophages. In addition, genetic deletion of the

A2BAR was associated with low-grade vascular inflammation, augmentation of pro-inflammatory cytokines such as TNF- $\alpha$ , and a consequent down-regulation of I $\kappa$ B $\alpha$  in addition to up-regulation of adhesion molecules of the vasculature. Consistent with the present study on renal protection by IP, vascular inflammation was significantly increased in mice gene-targeted for the A2BAR [44]. Moreover, the authors showed that exposure to endotoxin resulted in augmented pro-inflammatory cytokine levels in gene-targeted mice. However, and in contrast to the present studies showing a critical role of the reno-vascular A2BAR in providing renal protection from ischemia, Yang et al. found that bone marrow (and to a lesser extent vascular) A2BARs regulated the low grade inflammation observed in their studies [44].

The present study on A2BAR signaling during renal ischemia is consistent with recent work on cardiac generation and signaling of adenosine [63,64]. For example, two recent studies provided genetic evidence for extracellular adenosine generation in cardio-protection by IP [27,28]. In extension of these findings, the authors compared preconditioning responses of gene-targeted mice for each individual AR. Consistent with the present studies and other studies on

myocardial pre- or postconditioning [63,64], the authors found a pivotal role of the A2BAR, revealing that A2BAR agonist treatment preconditions the myocardium. Other investigations of renal ischemia and reperfusion injury have found contributions of the A2AAR [29,51,65]. As such, a series of studies of renal ischemia and reperfusion in chimeric mice found that bone marrow-derived cells play an important role in A2AAR-mediated tissue protection [29,65,66]. Further work using adoptive transfers into *Rag-1<sup>-/-</sup>* mice revealed that IFN $\gamma$  produced by CD4<sup>+</sup> T cells appears to be an important mediator for this complex interplay [66]. Taken together with results from the present studies, these findings suggest a combined role of myeloid A2AARs—predominantly expressed on T-lymphocytes—and reno-vascular A2BARs in attenuation of renal ischemia.

In conclusion, the present studies revealed induction and signaling through the reno-vascular A2BAR as part of an endogenous pathway to increase renal resistance to ischemia and attenuate ischemia reperfusion injury. Extensions of these findings provide evidence for targeting the A2BAR therapeutically during renal ischemia. Based on the fact that the presented data are all derived from murine studies, further challenges will include the translation from mice to human. In addition, it will be important to address pharmacokinetics and additional effects of A2BAR agonist treatment on coagulation, blood pressure, or pulmonary function, before such studies can be implemented and tested in a clinical setting.

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**Author contributions.** AG, HO, TE, KR, and HKE designed the experiments/the study. AG, TE, DY, HZ, KK, and KR collected data or did experiments for the study. AG, ZVT, KK, KR, and HKE analyzed the data. AG, HO, ZVT, KR, and HKE contributed to writing the paper.

**Competing Interests:** Bayer HealthCare has patented the use of the A2BAR agonist BAY 60-6583 in the treatment of different medical conditions. The authors are not associated with Bayer HealthCare and are not involved in the patent application. The authors report no competing interests.

## References

- Abuelo JG (2007) Normotensive ischemic acute renal failure. *N Engl J Med* 357: 797–805.
- Thadhani R, Pascual M, Bonventre JV (1996) Acute renal failure. *N Engl J Med* 334: 1448–1460.
- Chertow GM, Burdick E, Honour M, Bonventre JV, Bates DW (2005) Acute kidney injury, mortality, length of stay, and costs in hospitalized patients. *J Am Soc Nephrol* 16: 3365–3370.
- Gelman S (1995) The pathophysiology of aortic cross-clamping and unclamping. *Anesthesiology* 82: 1026–1060.
- Mehta RL (2005) Acute renal failure and cardiac surgery: marching in place or moving ahead? *J Am Soc Nephrol* 16: 12–14.
- Roos A, Rastaldi MP, Calvaresi N, Oortwijn BD, Schlagwein N, et al. (2006) Glomerular activation of the lectin pathway of complement in IgA nephropathy is associated with more severe renal disease. *J Am Soc Nephrol* 17: 1724–1734.
- Zhou W, Farrar CA, Abe K, Pratt JR, Marsh JE, et al. (2000) Predominant role for C5b-9 in renal ischemia/reperfusion injury. *J Clin Invest* 105: 1363–1371.
- Hart ML, Walsh MC, Stahl GL (2004) Initiation of complement activation following oxidative stress. In vitro and in vivo observations. *Mol Immunol* 41: 165–171.
- Hansen PB, Schnermann J (2003) Vasoconstrictor and vasodilator effects of adenosine in the kidney. *Am J Physiol Renal Physiol* 285: F590–F599.

- Vallon V, Muhlbauer B, Osswald H (2006) Adenosine and kidney function. *Physiol Rev* 86: 901–940.
- Morrison RR, Talukder MA, Ledent C, Mustafa SJ (2002) Cardiac effects of adenosine in A(2A) receptor knockout hearts: uncovering A(2B) receptors. *Am J Physiol Heart Circ Physiol* 282: H437–444.
- Gross ER, Gross GJ (2007) Pharmacologic therapeutics for cardiac reperfusion injury. *Expert Opin Emerg Drugs* 12: 367–388.
- Sitkovsky M, Lukashev D (2005) Regulation of immune cells by local-tissue oxygen tension: HIF1 alpha and adenosine receptors. *Nat Rev Immunol* 5: 712–721.
- Sitkovsky MV, Lukashev D, Apasov S, Kojima H, Koshiba M, et al. (2004) Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A2A receptors. *Annu Rev Immunol* 22: 657–682.
- Linden J (2005) Adenosine in tissue protection and tissue regeneration. *Mol Pharmacol* 67: 1385–1387.
- Fredholm BB (2007) Adenosine, an endogenous distress signal, modulates tissue damage and repair. *Cell Death Differ* 14: 1315–1323.
- Xu Z, Mueller RA, Park SS, Boysen PG, Cohen MV, et al. (2005) Cardioprotection with adenosine A2 receptor activation at reperfusion. *J Cardiovasc Pharmacol* 46: 794–802.
- Merighi S, Mirandola P, Varani K, Gessi S, Leung E, et al. (2003) A glance at adenosine receptors: novel target for antitumor therapy. *Pharmacol Ther* 100: 31–48.
- Grenz A, Zhang H, Hermes M, Eckle T, Klingel K, et al. (2007) Contribution of E-NTPDase1 (CD39) to renal protection from ischemia-reperfusion injury. *FASEB J* 21: 2863–2873.
- Grenz A, Zhang H, Eckle T, Mittelbronn M, Wehrmann M, et al. (2007) Protective role of ecto-5'-nucleotidase (CD73) in renal ischemia. *J Am Soc Nephrol* 18: 833–845.
- Eltzschig HK, Abdulla P, Hoffman E, Hamilton KE, Daniels D, et al. (2005) HIF-1-dependent repression of equilibrative nucleoside transporter (ENT) in hypoxia. *J Exp Med* 202: 1493–1505.
- Eltzschig HK, Thompson LF, Karhausen J, Cotta RJ, Ibla JC, et al. (2004) Endogenous adenosine produced during hypoxia attenuates neutrophil accumulation: coordination by extracellular nucleotide metabolism. *Blood* 104: 3986–3992.
- Thompson LF, Eltzschig HK, Ibla JC, Van De Wiele CJ, Resta R, et al. (2004) Crucial role for ecto-5'-nucleotidase (CD73) in vascular leakage during hypoxia. *J Exp Med* 200: 1395–1405.
- Eltzschig HK, Ibla JC, Furuta GT, Leonard MO, Jacobson KA, et al. (2003) Coordinated adenine nucleotide phosphohydrolysis and nucleoside signaling in posthypoxic endothelium: role of ectonucleotidases and adenosine A2B receptors. *J Exp Med* 198: 783–796.
- Eckle T, Faigle M, Grenz A, Laucher S, Thompson LF, et al. (2007) A2B adenosine receptor dampens hypoxia-induced vascular leak. *Blood* 111: 2024–2035.
- Khoury J, Ibla JC, Neish AS, Colgan SP (2007) Antiinflammatory adaptation to hypoxia through adenosine-mediated cullin-1 deneddylation. *J Clin Invest* 117: 703–711.
- Kohler D, Eckle T, Faigle M, Grenz A, Mittelbronn M, et al. (2007) CD39/ectonucleoside triphosphate diphosphohydrolase 1 provides myocardial protection during cardiac ischemia/reperfusion injury. *Circulation* 116: 1784–1794.
- Eckle T, Krahn T, Grenz A, Kohler D, Mittelbronn M, et al. (2007) Cardioprotection by ecto-5'-nucleotidase (CD73) and A2B adenosine receptors. *Circulation* 115: 1581–1590.
- Day YJ, Huang L, McDuffie MJ, Rosin DL, Ye H, et al. (2003) Renal protection from ischemia mediated by A2A adenosine receptors on bone marrow-derived cells. *J Clin Invest* 112: 883–891.
- Lee HT, Gallos G, Nasr SH, Emala CW (2004) A1 adenosine receptor activation inhibits inflammation, necrosis, and apoptosis after renal ischemia-reperfusion injury in mice. *J Am Soc Nephrol* 15: 102–111.
- Delacretaz E (2006) Clinical practice. Supraventricular tachycardia. *N Engl J Med* 354: 1039–1051.
- Bertolet BD, Anand IS, Bryg RJ, Mohanty PK, Chatterjee K, et al. (1996) Effects of A1 adenosine receptor agonism using N6-cyclohexyl-2'-O-methyladenosine in patients with left ventricular dysfunction. *Circulation* 94: 1212–1215.
- Huang DY, Vallon V, Zimmermann H, Koszalka P, Schrader J, et al. (2006) Ecto-5'-nucleotidase (cd73)-dependent and -independent generation of adenosine participates in the mediation of tubuloglomerular feedback in vivo. *Am J Physiol Renal Physiol* 291: F282–288.
- Castrop H, Huang Y, Hashimoto S, Mizel D, Hansen P, et al. (2004) Impairment of tubuloglomerular feedback regulation of GFR in ecto-5'-nucleotidase/CD73-deficient mice. *J Clin Invest* 114: 634–642.
- Krieg T, Cohen MV, Downey JM (2003) Mitochondria and their role in preconditioning's trigger phase. *Basic Res Cardiol* 98: 228–234.
- Downey JM, Davis AM, Cohen MV (2007) Signaling pathways in ischemic preconditioning. *Heart Fail Rev* 12: 181–188.
- Chatterjee PK, Patel NS, Sivarajah A, Kvale EO, Dugo L, et al. (2003) GW274150, a potent and highly selective inhibitor of iNOS, reduces experimental renal ischemia/reperfusion injury. *Kidney Int* 63: 853–865.

38. Basile DP (2007) The endothelial cell in ischemic acute kidney injury: implications for acute and chronic function. *Kidney Int* 72: 151–156.
39. Eckle T, Grenz A, Kohler D, Redel A, Falk M, et al. (2006) Systematic evaluation of a novel model for cardiac ischemic preconditioning in mice. *Am J Physiol Heart Circ Physiol* 291: H2533–2540.
40. Grenz A, Eckle T, Zhang H, Huang DY, Wehrmann M, et al. (2007) Use of a hanging-weight system for isolated renal artery occlusion during ischemic preconditioning in mice. *Am J Physiol Renal Physiol* 292: F475–F485.
41. Salvatore CA, Tilley SL, Latour AM, Fletcher DS, Koller BH, et al. (2000) Disruption of the A(3) adenosine receptor gene in mice and its effect on stimulated inflammatory cells. *J Biol Chem* 275: 4429–4434.
42. Sun D, Samuelson LC, Yang T, Huang Y, Paliege A, et al. (2001) Mediation of tubuloglomerular feedback by adenosine: evidence from mice lacking adenosine 1 receptors. *Proc Natl Acad Sci U S A* 98: 9983–9988.
43. Ledent C, Vaugeois JM, Schiffmann SN, Pedrazzini T, El Yacoubi M, et al. (1997) Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. *Nature* 388: 674–678.
44. Yang D, Zhang Y, Nguyen HG, Koupenova M, Chauhan AK, et al. (2006) The A2B adenosine receptor protects against inflammation and excessive vascular adhesion. *J Clin Invest* 116: 1913–1923.
45. Huang DY, Boini KM, Osswald H, Friedrich B, Artunc F, et al. (2006) Resistance of mice lacking the serum- and glucocorticoid-inducible kinase SGK1 against salt-sensitive hypertension induced by a high-fat diet. *Am J Physiol Renal Physiol* 291: F1264–F1273.
46. Kloor D, Delabar U, Muhlbauer B, Luippold G, Osswald H (2002) Tissue levels of S-adenosylhomocysteine in the rat kidney: effects of ischemia and homocysteine. *Biochem Pharmacol* 63: 809–815.
47. Jablonski P, Howden BO, Rae DA, Birrell CS, Marshall VC, et al. (1983) An experimental model for assessment of renal recovery from warm ischemia. *Transplantation* 35: 198–204.
48. Horny HP, Sillaber C, Menke D, Kaiserling E, Wehrmann M, et al. (1998) Diagnostic value of immunostaining for tryptase in patients with mastocytosis. *Am J Surg Pathol* 22: 1132–1140.
49. Yang Z, Day YJ, Toufektsian MC, Ramos SI, Marshall M, et al. (2005) Infarct-sparing effect of A2A-adenosine receptor activation is due primarily to its action on lymphocytes. *Circulation* 111: 2190–2197.
50. Lee HT, Emala CW (2000) Protective effects of renal ischemic preconditioning and adenosine pretreatment: role of A(1) and A(3) receptors. *Am J Physiol Renal Physiol* 278: F380–F387.
51. Lee HT, Emala CW (2002) Preconditioning and adenosine protect human proximal tubule cells in an in vitro model of ischemic injury. *J Am Soc Nephrol* 13: 2753–2761.
52. Eckle T, Fullbier L, Wehrmann M, Khoury J, Mittelbronn M, et al. (2007) Identification of ectonucleotidases CD39 and CD73 in innate protection during acute lung injury. *J Immunol* 178: 8127–8137.
53. Colgan SP, Eltzschig HK, Eckle T, Thompson LF (2006) Physiological roles of 5'-ectonucleotidase (CD73). *Purinergic Signalling* 2: 351–360.
54. Chen ZJ (2005) Ubiquitin signalling in the NF-kappaB pathway. *Nat Cell Biol* 7: 758–765.
55. Haugen E, Nath KA (1999) The involvement of oxidative stress in the progression of renal injury. *Blood Purif* 17: 58–65.
56. Yan L, Muller CE (2004) Preparation, properties, reactions, and adenosine receptor affinities of sulfophenylxanthine nitrophenyl esters: toward the development of sulfonic acid prodrugs with peroral bioavailability. *J Med Chem* 47: 1031–1043.
57. Shiva S, Sack MN, Greer JJ, Duranski M, Ringwood LA, et al. (2007) Nitrite augments tolerance to ischemia/reperfusion injury via the modulation of mitochondrial electron transfer. *J Exp Med* 204: 2089–2102.
58. Cai Z, Manalo DJ, Wei G, Rodriguez ER, Fox-Talbot K, et al. (2003) Hearts from rodents exposed to intermittent hypoxia or erythropoietin are protected against ischemia-reperfusion injury. *Circulation* 108: 79–85.
59. Yang CW, Li C, Jung JY, Shin SJ, Choi BS, et al. (2003) Preconditioning with erythropoietin protects against subsequent ischemia-reperfusion injury in rat kidney. *FASEB J* 17: 1754–1755.
60. Ozuyaman B, Ding Z, Buchheiser A, Koszalka P, Braun N, et al. (2006) Adenosine produced via the CD73/ecto-5'-nucleotidase pathway has no impact on erythropoietin production but is associated with reduced kidney weight. *Pflugers Arch* 452: 324–331.
61. Grenz A, Zhang H, Weingart J, von Wietersheim S, Eckle T, et al. (2007) Lack of effect of extracellular adenosine generation and signalling on renal erythropoietin secretion during hypoxia. *Am J Physiol Renal Physiol* 00243.02007.
62. Liu Y, Yang XM, Iliodromitis EK, Kremastinos DT, Dost T, et al. (2008) Redox signaling at reperfusion is required for protection from ischemic preconditioning but not from a direct PKC activator. *Basic Res Cardiol* 103: 54–59.
63. Philipp S, Yang XM, Cui L, Davis AM, Downey JM, et al. (2006) Postconditioning protects rabbit hearts through a protein kinase C-adenosine A2b receptor cascade. *Cardiovasc Res* 70: 308–314.
64. Kuno A, Critz SD, Cui L, Solodushko V, Yang XM, et al. (2007) Protein kinase C protects preconditioned rabbit hearts by increasing sensitivity of adenosine A2b-dependent signaling during early reperfusion. *J Mol Cell Cardiol* 43: 262–271.
65. Okusa MD, Linden J, Huang L, Rosin DL, Smith DF, et al. (2001) Enhanced protection from renal ischemia-reperfusion [correction of ischemia-reperfusion] injury with A(2A)-adenosine receptor activation and PDE 4 inhibition. *Kidney Int* 59: 2114–2125.
66. Day YJ, Huang L, Ye H, Li L, Linden J, et al. (2006) Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: the role of CD4+ T cells and IFN-gamma. *J Immunol* 176: 3108–3114.

## Editors' Summary

**Background.** Throughout life, the kidneys perform the essential task of filtering waste products and excess water from the blood to make urine. Each kidney contains about a million small structures called nephrons, each of which contains a filtration unit consisting of a glomerulus (a small blood vessel) intertwined with a urine-collecting tube called a tubule. If the nephrons stop working for any reason, the rate at which the blood is filtered (the glomerular filtration rate or GFR) decreases and dangerous amounts of waste products such as creatinine build up in the blood. Most kidney diseases destroy the nephrons slowly over years, producing an irreversible condition called chronic renal failure. But the kidneys can also stop working suddenly because of injury or poisoning. One common cause of “acute” renal failure in hospital patients is ischemia—an inadequate blood supply to an organ that results in the death of part of that organ. Heart surgery and other types of surgery in which the blood supply to the kidneys is temporarily disrupted are associated with high rates of acute renal failure.

**Why Was This Study Done?** Although the kidneys usually recover from acute failure within a few weeks if the appropriate intensive treatment (for example, dialysis) is provided, acute renal failure after surgery can be fatal. Thus, new strategies to protect the kidneys from ischemia are badly needed. Like other organs, the kidneys can be protected from lethal ischemia by pre-exposure to several short, nonlethal episodes of ischemia. It is not clear how this “ischemic preconditioning” increases renal resistance to ischemia but some data suggest that the protection of tissues from ischemia might involve a signaling molecule called extracellular adenosine. This molecule binds to proteins called receptors on the surface of cells and sends signals into them that change their behavior. There are four different adenosine receptor—A1AR, A2AAR, A2BAR, and A3AR—and in this study, the researchers use ischemic preconditioning as an experimental strategy to investigate which of these receptors protects the kidneys from ischemia in mice, information that might provide clues about how to protect the kidneys from ischemia.

**What Did the Researchers Do and Find?** The researchers first asked whether ischemic preconditioning protects the kidneys of mice strains that lack the genes for individual adenosine receptors (*A1AR*<sup>-/-</sup>, *A2AAR*<sup>-/-</sup>, *A2BAR*<sup>-/-</sup>, and *A3AR*<sup>-/-</sup> mice) from subsequent ischemia. Using a hanging-weight system, they intermittently blocked the renal artery of these mice before exposing them to a longer period of renal ischemia. Twenty-four hours later, they assessed the renal function of the mice by

measuring their blood creatinine levels, GFRs, and urine production. Ischemic preconditioning protected all the mice from ischemia-induced loss of kidney function except the *A2BAR*<sup>-/-</sup> mice. It also prevented ischemia-induced structural damage and inflammation in the kidneys of wild-type but not *A2BAR*<sup>-/-</sup> mice. These results suggest that A2BAR may help to protect the kidneys from ischemia. Consistent with this idea, ischemic preconditioning did not prevent ischemia-induced renal damage in wild-type mice treated with a compound that specifically blocks the activity of A2BAR. However, wild-type mice (but not *A2BAR*<sup>-/-</sup> mice) treated with an A2BAR agonist (which activates the receptor) retained their kidney function after renal ischemia without ischemic preconditioning. Finally, the researchers report that A2BAR has to be present on the blood vessels in the kidney to prevent ischemia-induced acute renal failure.

**What Do These Findings Mean?** These findings suggest that the protection of the kidneys from ischemia and the renal resistance to ischemia that is provided by ischemic preconditioning involve adenosine signaling through A2BAR. They also suggest that adenosine might provide protection against ischemia-induced damage by blocking inflammation in the kidney although other possible mechanisms of action need to be investigated. Importantly, these findings suggest that A2BAR might be a therapeutic target for the prevention of renal ischemia. However, results obtained in animals do not always reflect the situation in people, so before A2BAR agonists can be used to reduce the chances of patients developing acute renal failure after surgery, these results need confirming in people and the safety of A2BAR agonists need to be thoroughly investigated.

**Additional Information.** Please access these Web sites via the online version of this summary at <http://dx.doi.org/10.1371/journal.pmed.0050137>.

- The US National Institute of Diabetes and Digestive and Kidney Diseases provides information on how the kidneys work and what can go wrong with them, including a list of links to further information about kidney disease
- The MedlinePlus encyclopedia has a page on acute kidney failure (in English and Spanish)
- Wikipedia has pages on acute renal failure, ischemia, ischemic preconditioning, and adenosine (note that Wikipedia is a free online encyclopedia that anyone can edit; available in several languages)

