A₃ adenosine receptor knockout mice are protected against ischemia- and myoglobinuria-induced renal failure

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Submitted 24 July 2002; accepted in final form 8 October 2002

Lee, H. Thomas, Ayuko Ota-Setlik, Hua Xu, Vivette D. D'agati, Marlene A. Jacobson, and Charles W. Emala. A₃ adenosine receptor knockout mice are protected against ischemia- and myoglobinuria-induced renal failure. Am J Physiol Renal Physiol 284: F267–F273, 2003. First published October 15, 2002; 10.1152/ajprenal.00271.2002.—A₃ adenosine receptor (AR) activation and inhibition worsened and improved, respectively, renal function after ischemia-reperfusion (I/R) injury in rats. We sought to further characterize the role of A₃ ARs in modulating renal function after either I/R or myoglobinuric renal injury. A₃ knockout mice had significantly lower plasma creatinines compared with C57 controls 24 h after I/R or myoglobinuric renal injury. C57 control mice pretreated with the A₃ AR antagonist [3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-(3-iodobenzyl)-N-carbamoyladenosine (IB-MECA)] demonstrated improved or worsened renal function, respectively, after I/R or myoglobulinuric renal injury. Higher doses of IB-MECA were lethal in C57 mice subjected to renal ischemia. H₁ but not H₂ histamine receptor antagonist prevented death in mice pretreated with IB-MECA before renal ischemia. Improvement in renal function was associated with significantly improved renal histology. In conclusion, preischemic A₃ AR activation (0.125 mg/kg IB-MECA) exacerbated renal I/R injury in mice. Mice lacking A₃ ARs or blocking A₃ ARs in wild-type mice resulted in significant renal protection from ischemic or myoglobinuric renal failure.

METHODOLOGY

A₃KO mice. Homozygous A₃KO (A₃⁻/⁻) mice breeding pairs were provided by Merck Research Laboratories (West Point, PA). Generation and initial characterization of the A₃KO mice have been described in detail previously (23, 26). The A₃KO mice were obtained after they were backcrossed against C57BL/6 mice for 12 generations to obtain a congenic line. C57BL/6 mice (Harlan Laboratories, Indianapolis, IN) served as background A₃⁺/+ controls (C57).

Renal injury protocol. A₃KO and C57 mice (25–30 g body wt) were anesthetized with pentobarbital sodium (50 mg/kg or to effect ip) and placed supine on a heating pad under a warming light to maintain body temperature between 36 and 38°C. Additional pentobarbital sodium was given as needed on the basis of response to tail pinch. Bilateral flank incisions were made, and the left kidney was subjected to 30 min of ischemia with microaneurysm clips after right nephrectomy. The duration of ischemia was chosen to maximize reproducibility of renal injury and to minimize mortality in these models.

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mice. Separate groups of C57 and A3KO mice were injected with 8.5 mg/kg glycerol intramuscularly after 16 h of water deprivation to induce myoglobinuric renal injury. Some C57 mice were pretreated either with 3-ethyl-5-benzyl-2-methyl-6-(3-iodobenzyl)-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5 dicarboxylate (MRS-1191; 1 mg/kg ip), a selective A3 AR antagonist, or with 0.125 mg/kg N8-(3-iodobenzyl)-N-methyl-5’-carbamoyladenosine (IB-MECA; 0.0625, 0.125, 0.25, 0.5, 1, and 2 mg/kg ip), a selective A3 AR agonist, 15 min before renal ischemia or glycerol injection.

To determine whether histamine release by mast cell degranulation induced by IB-MECA plays any role in lethality renal ischemia or glycerol injection, some C57 mice were pretreated with 3-ethyl-5-benzyl-2-methyl-6-(3-iodobenzyl)-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5 dicarboxylate (MRS-1191; 1 mg/kg ip), a selective A3 AR antagonist, or with 0.125 mg/kg N8-(3-iodobenzyl)-N-methyl-5’-carbamoyladenosine (IB-MECA; 0.0625, 0.125, 0.25, 0.5, 1, and 2 mg/kg ip), a selective A3 AR agonist, 15 min before renal ischemia or glycerol injection.

Assessment of renal injury. Renal function was assessed by measuring plasma creatinine 24 h after renal ischemia or glycerol injection by using a commercially available colorimetric method (Sigma). For histological preparations, explanted kidneys were bisected along the long axis and were fixed in 10% formalin solution overnight. After automated dehydration through a graded alcohol series, transverse kidney slices were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin-eosin. Morphological assessment was performed by an experienced renal pathologist (V. D. D’Agati) who was blinded to the treatment for each animal. A grading scale of 0–4, as outlined by Jablonski et al. (10), was used for the histopathological assessment of I/R- or myoglobinuric damage of the proximal tubules.

Measurement of plasma histamine levels. Plasma histamine levels were measured in A3KO and C57 mice 20 min after IB-MECA or compound 48/80 treatment (5 min after initiation of renal ischemia) by using an ELISA specific for histamine (Bio-Rad, Hercules, CA) using a primer annealing temperature of 60°C. The products were resolved in a 6% polyacrylamide gel and stained with Syber green (Roche, Indianapolis, IN) for analysis with a FluorS Multi Imager (Bio-Rad, Hercules, CA).

Chemicals. Unless otherwise specified, all reagents were purchased from Sigma (St. Louis, MO).

Statistics and data analysis. The data were analyzed with Student’s t-test when comparing means between two groups or with one-way analysis of variance plus Dunnett post hoc multiple comparison test to compare mean values across multiple treatment groups.

RESULTS

Genotyping of wild-type and A3KO C57 mice. To confirm the genotypes of mice used in these studies, genomic DNA extracted from the tail was analyzed by PCR for the expression of the full-length A3 AR (wild-type or C57 mice) or the plasmid insert (PGKneo) originally used to disrupt the A3 AR gene (A3KO mice) (23). The full-length A3 AR (~220 bp) was only detected in C57 wild-type mice, whereas the PGKneo insert (~290 bp) was only detected in the A3KO mice (Fig. 1).

Renal function assessment. C57 control and A3KO mice that underwent sham operations had similar baseline hemodynamic values (heart rate and blood pressure) and renal function [creatinine (Cr) = 0.3 ± 0.1 mg/dl, n = 3, for C57, and 0.3 ± 0.1 mg/dl, n = 3, for A3KO]. However, 24 h after renal I/R injury, A3KO...
mice had significantly lower plasma creatinine (Cr = 0.7 ± 0.1 mg/dl, n = 8) compared with C57 controls (Cr = 3.0 ± 0.3 mg/dl, n = 8, P < 0.05, Fig. 2). A3KO mice were also protected against myoglobinuric renal injury compared with control mice as demonstrated by reduced creatinines at 24 h (Cr = 1.8 ± 0.3 mg/dl, n = 8 for A3KO, vs. 4.1 ± 0.2 mg/dl, n = 4, for C57, P < 0.05, Fig. 2).

To further illustrate the role of the A3 AR in modulating renal function after injury, control C57 mice were pretreated with an A3 AR antagonist or agonist before I/R or glycerol-induced myoglobinuric injury. C57 mice pretreated with MRS-1191 (a selective A3 AR antagonist) demonstrated improved renal function after either I/R-induced or glycerol-induced renal injury (Cr = 1.8 ± 0.2 mg/dl, n = 9, for MRS-1191+I/R, or 3.1 ± 0.2 mg/dl, n = 6, for MRS-1191+glycerol, respectively, P < 0.05; Fig. 2) compared with I/R or glycerol injury alone. In contrast, C57 mice pretreated with IB-MECA, a selective A3 AR agonist, failed to survive the ischemic period at doses of 2 (n = 6), 1 (n = 6), 0.5 (n = 6), or 0.25 mg/kg (n = 6). At 0.125 mg/kg IB-MECA, C57 mice survived renal I/R injury and demonstrated significantly worsened renal function (Cr = 4.5 ± 0.2 mg/dl, n = 6, P < 0.05, compared with I/R injury alone; Fig. 2). C57 mice pretreated with 0.0625 mg/kg IB-MECA before renal I/R did not exhibit significantly worsened renal function (Cr = 3.4 ± 0.2 mg/dl, n = 4). Selective A3 AR agonist (Cr = 0.6 ± 0.1 mg/dl, n = 5, after 0.125 mg/kg IB-MECA) or antagonist (Cr = 0.6 ± 0.1 mg/dl, n = 4, after 1 mg/kg MRS-1191) has no impact on renal protection observed in A3KO mice subjected to I/R injury.

IB-MECA (0.5 mg/kg) also exacerbated renal function in C57 mice after myoglobinuric renal injury (Cr = 4.8 ± 0.1 mg/dl, n = 4, P < 0.05, compared with myoglobinuric injury alone). C57 mice injected with IB-MECA (2 mg/kg) alone without I/R or myoglobinuric injury survived and showed normal renal function. When C57 (n = 4) and A3KO (n = 4) mice were pretreated with compound 48/80 (5 mg/kg) and subjected to renal I/R injury, all the mice died during renal ischemia.

Increase in plasma histamine by IB-MECA or compound 48/80 is lethal when associated with renal ischemia. A3 AR activation causes mast cell degranulation and histamine release in rodents (6, 22, 26). We questioned whether the systemic elevations in histamine played a role in the lethality and impaired renal function of A3 AR activation coupled with renal ischemia and reperfusion. We measured plasma histamine levels in A3KO and C57 mice after IB-MECA or compound 48/80 (induces mast cell degranulation) pretreatment by using a mouse-specific ELISA. A3KO and control C57 mice that underwent sham operations had similar baseline plasma histamine levels (4.6 ± 1.0 and 5.0 ± 1.6 ng/ml, respectively, n = 3). IB-MECA (1 mg/kg) increased plasma histamine concentration to 226 ± 24.8 ng/ml in C57 mice (n = 6) and to 31.0 ± 4.3 ng/ml in A3KO mice (n = 4, P < 0.01 vs. C57) 20 min after injection (5 min after initiation of renal ischemia). Plasma histamine concentrations increased in C57 and A3KO mice pretreated with compound 48/80 (320 ± 8.8 ng/ml, n = 4, and 249 ± 29.3 ng/ml, n = 5, respectively).

Because all control C57 mice pretreated with compound 48/80 or 0.25–2.0 mg/kg IB-MECA and all A3KO mice pretreated with compound 48/80 died during ischemia, we questioned whether inhibition of histamine receptor subtypes would alter lethality or renal function. Thirty minutes of pretreatment with H1 (diphenhydramine, 10 mg/kg ip) but not with H2 (ranitidine, 10 mg/kg ip) histamine receptor antagonist prevented death in C57 mice treated with IB-MECA.
and in C57 or A3KO mice treated with compound 48/80. However, pretreatment with diphenhydramine did not protect renal function after I/R injury in C57 mice treated with IB-MECA (Cr = 4.0 ± 0.2 mg/dl, n = 4). Similarly, when C57 mice were pretreated with diphenhydramine before compound 48/80 and subjected to renal I/R, they survived renal ischemia but failed to show improved renal function compared with the I/R injury alone group (Cr = 2.9 ± 0.2 mg/dl, n = 3). Therefore, although elevated plasma histamine and activation of the H1 histamine receptor coupled with renal ischemia are lethal in mice, H1 receptor activation alone cannot account for A3 AR activation-mediated exacerbation of renal function.

*Injured A3KO mice maintained improved renal histology.* In Fig. 3, enhanced renal protection of A3KO mice against I/R- and glycerol-mediated injury are further supported by representative histological slides. C57 control mice subjected to 30 min of renal ischemia (Fig. 3B) or glycerol injection (Fig. 3C) followed by 24 h of reperfusion resulted in significant renal injury, as evidenced by severe tubular necrosis, medullary congestion and hemorrhage, and the development of proteinaceous casts. A3KO mice (Fig. 3, D and E) or C57 mice (Fig. 3, F and G) pretreated with the A3 AR antagonist MRS-1191 before I/R- or glycerol-induced injury showed improved renal morphology. The Jabolonski (10) scale histology grading scores are shown in Fig. 4. Twenty-four hours after 30 min of renal ischemia or glycerol treatment in C57 control mice resulted in severe acute tubular necrosis (grade: 3.0 ± 0.3, n = 6, and 3.7 ± 0.4, n = 4, respectively). A3KO mice subjected to I/R or glycerol injury showed significant improvements in histological scoring compared with C57 mice subjected to I/R (grade: 0.7 ± 0.5, n = 4, and 1.7 ± 0.3, n = 4, respectively). Additionally, C57 mice pretreated with MRS-1191 before I/R or glycerol injury showed significant improvements in histological scoring compared with C57 mice subjected to I/R injury (grade: 2.1 ± 0.2, n = 5, and 2.3 ± 0.3, n = 4, respectively). IB-MECA-pretreated C57 mice subjected to I/R or glycerol injury failed to show statistically significant worsening histological injury compared with C57 mice (grade: 3.7 ± 0.4, n = 4, and 3.8 ± 0.4, n = 3, respectively).

**DISCUSSION**

The major findings of our study are the following: 1) mice deletionally lacking A3 ARs are protected against both ischemic and myoglobinuric forms of renal injury, 2) blocking A3 ARs protected and activating A3 ARs worsened renal failure after I/R- and myoglobinuric-induced injury in C57 control mice, 3) high doses of the A3 AR agonist (IB-MECA) coupled with renal ischemia are lethal in C57 mice presumably by degranulating resident mast cells and releasing histamine, and 4) blocking H1 histamine receptors prevented A3 AR agonist- or mast cell degranulation-induced mortality during renal ischemia in C57 control or A3KO mice.

In previous studies by our laboratory, we showed that preischemic A1 or postischemic A2a AR activation protects against renal I/R injury in rats (14, 16, 17). In addition, we demonstrated in rats that A3 AR activation with IB-MECA and inhibition with MRS-1191 worsened and protected, respectively, against I/R-induced renal failure (14). In the present study, we utilized mice that lack A3 ARs to further probe the role of A3 ARs in renal I/R and myoglobinuric injury.

The A3 AR subtype is the most recently characterized member of the AR family (5, 11). Although the expression of the A3 AR subtype in the kidney has been demonstrated (18, 28), its function in the kidney is unknown. The present studies in mice agree with our previous studies in rats in that pretreatment with the highly selective A3 AR agonist IB-MECA worsened renal I/R injury, whereas pretreatment with the highly selective A3 adenosine agonist MRS-1191 protected renal function after I/R injury (14). Although MRS-1191 is a potent and selective antagonist of human A3 receptors, it has only limited potency and selectivity at the mouse A3 AR receptor (K_i ~ 1.42 μM) (12, 13). For this reason, we cannot rule out the possibility that MRS-1191 might act in part by blocking other AR subtypes. For this reason, the data derived from the use of A3KO mice are important to bolster the conclusion on the basis of the use of MRS-1191.

IB-MECA also exacerbated glycerol-induced myoglobinuric renal failure. Furthermore, mice lacking the A3 AR exhibited endogenous protection against both I/R-induced and myoglobinuric-induced renal injury. Because the A3 AR agonist alone had no effect on renal function in both rats (14) and mice, it can be concluded that A3 AR activation must be coupled with an I/R or myoglobinuric renal insult for the receptor to have a detrimental effect on renal function. The detrimental effects of preischemic A3 AR agonist treatment on the kidney differs from the heart, where significant cardiac protection with A3 AR agonist pretreatment has been reported (19).

The mechanism(s) by which A3 AR activation or inhibition exacerbates or protects against, respectively, renal injury remains to be determined. A3 adenosine agonists cause apoptosis in multiple cell lines, including cardiomyocytes, human leukemia cell lines, and human proximal tubule (HK-2) cells, via incompletely characterized mechanisms (11, 15, 24). Chronic A3 AR activation or overexpression is detrimental to cell survival (11). Moreover, overexpression of A3 AR is...
increased histamine levels in both C57 and A3KO mice. A3KO mice subjected to ischemia and reperfusion (n = 4), C57 mice pretreated with IB-MECA before ischemia and reperfusion (n = 4), C57 mice pretreated with MRS-1191 before ischemia and reperfusion (n = 3), C57 mice subjected to glycerol injury (n = 4), A3KO mice subjected to glycerol injury (n = 4), C57 mice pretreated with IB-MECA before glycerol injury (n = 3), and C57 mice pretreated with MRS-1191 before glycerol injury (n = 4). *P < 0.05 vs. sham-operated C57 mice. #P < 0.05 vs. C57 mice subjected to ischemia and reperfusion. %P < 0.05 vs. C57 mice subjected to glycerol injury. Error bars, 1 SE.

Fig. 4. Jablonski grading scale scores for histological appearance of acute tubular necrosis from sham-operated C57 mice (n = 3), sham-operated A3KO mice (n = 3), C57 mice subjected to ischemia and reperfusion (n = 4), A3KO mice subjected to ischemia and reperfusion (n = 4), C57 mice pretreated with IB-MECA before ischemia and reperfusion (n = 4), C57 mice pretreated with MRS-1191 before ischemia and reperfusion (n = 5), C57 mice subjected to glycerol injury (n = 4), A3KO mice subjected to glycerol injury (n = 4), C57 mice pretreated with IB-MECA before glycerol injury (n = 3), and C57 mice pretreated with MRS-1191 before glycerol injury (n = 3), and C57 mice pretreated with IB-MECA (diphenylhydramine) prevented death during renal ischemia in C57 mice treated with 1 mg/kg IB-MECA, whereas pretreatment with an H2 histamine receptor blocker (ranitidine) failed to rescue C57 mice from death. Although C57 mice survived renal ischemia after H1 histamine receptor antagonist and IB-MECA, their renal function was not improved. This indicates that an increase in plasma histamine concurrent with renal ischemia is lethal in mice because of H1 histamine receptor activation. However, H1 histamine receptor activation cannot account for exacerbation of renal function after ischemia- or glycerol-induced renal injury, because pretreatment with H1 histamine blocker failed to improve plasma creatinine or histology. It remains to be determined whether other histamine receptor subtype blockades (H2, H3, or H4) with or without concomitant H1 histamine receptor blockade are required to protect against renal I/R injury.

We recognize that the background strain of mice is an important determinant of renal injury (2). In this study, we utilized commercially available C57BL/6 mice as controls because the homozygous A3KO mice were backcrossed against C57BL/6 mice for 12 generations to obtain a congenic line. Backcrossing to this

embryologically lethal in mice with prominent fragmentation of DNA (27). Additionally, mice deletionally lacking A3 ARs are protected against cardiac I/R injury by unclear mechanisms (3, 8, 9).

The A3 AR activation degranulates resident mast cells, which results in the release of stored inflammatory mediators, including histamine and proteolytic enzymes, as well as other proinflammatory mediators (e.g., IL-1β and TNF-α) (6, 22). We examined the role of mast cell degranulation and histamine release after A3 AR activation utilizing histamine receptor blockers and by measuring plasma histamine concentrations. We demonstrate that the A3 AR agonist IB-MECA profoundly increases plasma histamine levels in C57 mice (~45-fold increase). In contrast, compound 48/80 increased histamine levels in both C57 and A3KO mice. It is interesting to note that IB-MECA increased plasma histamine levels even in A3KO mice by approximately sevenfold, although this increase is significantly less than that observed in C57 mice. It may be that IB-MECA has some additional mechanism of action to increase plasma histamine levels independently of A3 AR activation.

We show that 0.125 mg/kg IB-MECA, a selective A3 AR agonist, worsened renal function after I/R renal injury. Moreover 0.5 mg/kg IB-MECA exacerbated renal injury after glycerol-mediated myoglobinuric renal injury. Surprisingly, C57 mice were very sensitive to A3 AR agonist (IB-MECA) injection when subjected to renal ischemia, because doses ≥0.25 mg/kg were lethal. Moreover, degranulation of mast cells with compound 48/80 coupled with renal ischemia was also lethal in both C57 and A3KO mice. IB-MECA and compound 48/80 injections without renal ischemia were well tolerated in both A3KO and C57 mice. The mechanism of mortality after higher doses of IB-MECA (0.25–2 mg/kg) coupled with renal ischemia is likely related to H1 histamine receptor activation. Systemic effects of histamine release on the cardiac and vascular systems likely account for mortality after high doses of IB-MECA. Death associated with elevated systemic histamine complicates the interpretation of A3 AR-mediated exacerbation of renal function after renal ischemia and is the major limitation of the present, in vivo approach.
extent makes a congenic line that is 99.99% genetically C57BL/6.

In conclusion, we demonstrate that mice lacking A₃ AR expression are protected against both I/R-induced and myoglobinuria-induced renal failure. Moreover, A₃ AR agonist and antagonist worsened and protected, respectively, I/R- and myoglobinuric-mediated renal injury in C57 control mice. The mechanism(s) of A₃ AR-mediated exacerbation of renal function after I/R or myoglobinuric injury and the signaling pathway(s) responsible for potent renal protection in A₃KO mice remain to be determined. These findings further support the potential role of a selective A₃ AR antagonist for protection against perioperative renal failure.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant RO1-DK-58547.

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