Lack of effect of extracellular adenosine generation and signaling on renal erythropoietin secretion during hypoxia

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Submitted 24 May 2007; accepted in final form 5 September 2007

Grenz A, Zhang H, Weingart J, von Wietersheim S, Eckle T, Schnermann J, Köhle C, Kloor D, Gleiter CH, Vallon V, Eltzschig HK, Osswald H. Lack of effect of extracellular adenosine generation and signaling on renal erythropoietin secretion during hypoxia. Am J Physiol Renal Physiol 293: F1501–F1511, 2007. First published September 12, 2007; doi:10.1152/ajprenal.00243.2007.—Previous studies have yielded conflicting results as to whether extracellular adenosine generation and signaling contribute to hypoxia-induced increases in renal erythropoietin (EPO) secretion. In this study, we combined pharmacological and genetic approaches to elucidate a potential contribution of extracellular adenosine to renal EPO release in mice. To stimulate EPO secretion, we used murine carbon monoxide exposure (400 and 750 parts per million CO, 4 h), ambient hypoxia (8% oxygen, 4 h), or arterial hemodilution. Because the ecto-5’-nucleotidase (CD73, conversion of AMP to adenosine) is considered the pacemaker of extracellular adenosine generation, we first tested the effect of blocking extracellular adenosine generation with the specific CD73-inhibitor adenosine 5’-(α,β-methylene) diphosphate (APCP) or by gene-targeted deletion of cd73. These studies showed that neither APCP-treatment nor targeted deletion of cd73 resulted in changes of stimulated EPO mRNA or serum levels, although the increases of adenosine levels in the kidney following CO exposure were attenuated in mice with APCP treatment or in cd73−/− mice. Moreover, pharmacological studies using specific inhibitors of individual adenosine receptors (A1AR, DPCPX; A2AAR, DMPX; A2BAR, PSB 1115; A3AR, MRS 1191) showed no effect on stimulated increases of EPO mRNA or serum levels. Finally, stimulated EPO secretion was not attenuated in gene-targeted mice lacking A1AR−/−, A2AAR−/−, A2BAR−/−, or A3AR−/−. Together, these studies combine genetic and pharmacological in vivo evidence that increases of EPO secretion during limited oxygen availability are not affected by extracellular adenosine generation or signaling.

adenosine receptors; ecto-5’-nucleotidase; CD73; carbon monoxide

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THE GLYCOPROTEIN HORMONE ERYTHROPoIETIN (EPO) is an essential growth factor for the regulation and differentiation of erythrocytic progenitor cells in bone marrow, thus regulating the supply of red blood cells (19). EPO is predominantly synthesized in the kidneys and to a smaller extent also in the liver (~5% of total EPO production) (18, 19). EPO concentrations are decreased in patients with malignant, infectious, autoimmune, or renal diseases (26). Since the 1980s, recombinant human erythropoietin has been available and can be used clinically in the treatment of anemia from different etiologies (1). The most important transcription factor for hypoxic stimulation of EPO production is the hypoxia-inducible factor 1 (HIF-1) (43). During hypoxia, HIF-1 is stabilized and finally results in the transcriptional induction of its target genes, such as EPO, by binding to an hypoxia-response element within the EPO promoter, resulting in prompt and robust induction of EPO (46).

Several studies have implicated extracellular adenosine in the modulation of EPO secretion (3, 8, 30, 33, 51). Extracellular adenosine is derived mainly from phosphohydrolysis of adenosine 5’-monophosphate (AMP). Ecto-5’-nucleotidase (CD73), an ubiquitously expressed glycosyl phosphatidylinositol-anchored ectoenzyme, is the pacemaker of this reaction (49). Because of its transcriptional induction by hypoxia (18), CD73-dependent adenosine generation is particularly prominent during conditions of limited oxygen availability (48, 49). Furthermore, recent studies have shown that the EPO-producing peritubular renal fibroblasts express high amounts of ecto-5’-nucleotidase on their surface (2).

Extracellular adenosine produced by CD73 can signal through any of four extracellular adenosine receptors (A1AR, A2AAR, A2BAR, or A3AR). All four ARs have been associated with tissue protection in a variety of physiological settings (45). In the kidney, CD73-dependent adenosine production plays a central role in the regulation of tubuloglomerular feedback (5, 50) and during ischemic preconditioning (15).

Experimental studies and human trials on the effect of adenosine analogs and AR antagonists on EPO secretion have shown conflicting results (8, 12, 13, 29, 31–33, 41, 51). Nakashima et al. (28) showed an increase of EPO production in hepatocellular carcinoma (Hep3B) cells following adenosine treatment. These studies showed that neither APCP-treatment nor targeted deletion of cd73 resulted in changes of stimulated EPO mRNA or serum levels, although the increases of adenosine levels in the kidney following CO exposure were attenuated in mice with APCP treatment or in cd73−/− mice. Moreover, pharmacological studies using specific inhibitors of individual adenosine receptors (A1AR, DPCPX; A2AAR, DMPX; A2BAR, PSB 1115; A3AR, MRS 1191) showed no effect on stimulated increases of EPO mRNA or serum levels. Finally, stimulated EPO secretion was not attenuated in gene-targeted mice lacking A1AR−/−, A2AAR−/−, A2BAR−/−, or A3AR−/−. Together, these studies combine genetic and pharmacological in vivo evidence that increases of EPO secretion during limited oxygen availability are not affected by extracellular adenosine generation or signaling.
A2AAR antagonist, whereas A2BAR and A2BR agonists increased EPO mRNA levels (8). Furthermore, selective A2BAR inhibition produced a significant inhibition of EPO secretion in mice exposed to hypoxia, suggesting that EPO secretion is stimulated by activation of adenylyl cyclase by A2AAR and A2BAR (8). However, another group (12) could not reproduce the above findings using different adenosine synergistic and antagonistic compounds in models of hypoxia-stimulated EPO production in rats. A very elegant study by Ozuyaman et al. (37) has shown that cd73 mice do not respond to hypoxic stimuli with different EPO plasma levels compared with wild-type mice. However, and in contrast to other studies showing elevated adenosine levels with hypoxia or ischemia (15, 16, 21, 49), renal adenosine levels were not increased in this study (37). Moreover, in patients with erythrocytosis and high EPO serum concentrations following kidney transplantation, EPO levels were attenuated by treatment with theophylline, a non-selective AR antagonist (3). Again, other studies could not confirm these findings (10, 17).

Based on these conflicting findings, we used a combination of pharmacological and genetic approaches to define in more detail the contribution of extracellular adenosine generation and to clarify the role of the different adenosine receptor subtypes in the hypoxia-induced EPO secretion. In these studies, no influence of extracellular adenosine generation and AR signaling on stimulation of EPO secretion was found.

MATERIALS AND METHODS

Mice. All mice, weighing 22–32g, were kept on a regular 12:12-h dark-light cycle with free access to standard mice chow (Altromin 1320; Lage, Germany) and water. Experimental protocols were approved in accordance with the German Law on the Protection of Animals. C57BL/6J mice were obtained from Charles River (Sulzfeld, Germany). Mice deficient in cd73, A1AR, A2AAR, or A2BAR on the C57BL/6 strain or in A2AR on the CD1 strain were generated, validated, and characterized as described previously (7, 24, 40, 47, 49). PCR genotyping and real-time RT-PCR for determination of expression patterns of CD73 and ARs confirmed successful knockdown of renal cd73, A1AR, A2AAR, or A2BAR in the respective mice. In short, measurement of cd73 or AR mRNA expression confirmed intermediate levels of CD73 or ARs in heterozygotes and their absence in homozygous mutant mice (Supplemental Fig. 1). (Supplemental data for this article are available at the American Journal of Physiology-Renal Physiology website.)

Marine carbon monoxide or 8% O2 exposure. For carbon monoxide (CO) exposure [200 to 1,400 parts per million (ppm) CO], conscious male and female mice (paired in all groups, n = 5–7 per group) were placed into a ventilated cage as previously described (9). For carbon monoxide exposure [200 to 1,400 parts per million (ppm) CO], conscious male and female mice (paired in all groups, n = 5–7 per group) were placed into a ventilated cage as previously described (9). After group (12) could not reproduce the above findings using different adenosine synergistic and antagonistic compounds in models of hypoxia-stimulated EPO production in rats. A very elegant study by Ozuyaman et al. (37) has shown that cd73 mice do not respond to hypoxic stimuli with different EPO plasma levels compared with wild-type mice. However, and in contrast to other studies showing elevated adenosine levels with hypoxia or ischemia (15, 16, 24, 49), renal adenosine levels were not increased in this study (37). Moreover, in patients with erythrocytosis and high EPO serum concentrations following kidney transplantation, EPO levels were attenuated by treatment with theophylline, a non-selective AR antagonist (3). Again, other studies could not confirm these findings (10, 17).

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Pharmacological studies. In subsets of experiments, CD73 or A1AR, A2AAR, A2BR, or A3AR were blocked. The doses of all substances that we have tested were based on earlier reports in which clear antagonistic effects were demonstrated on different organ function in experimental animals. We used 8-phenyltheophylline (8-PT) as a nonspecific AR antagonist (10 mg/kg ip) (14) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 1 mg/kg ip) (20, 44), 3,7-dimethyl-1-propargylxanthine (DMPX; 1 mg/kg ip) (42), PSB 1115 (10 mg/kg ip) (7), and RSR1119 (1 mg/kg ip) (25) as specific AR subtype inhibitors. CD73 was inhibited using adenosine 5’-(α,β-methylene) diprophosphate (DAPP; 2 mg ip) (7, 15). All drugs were given 30 min before the experimental procedure. All reagents were purchased from Sigma-Aldrich.

Assays. Serum EPO concentrations were determined via quantitative enzyme-linked immunosorbent assay (EPO ELISA; Medac, Wedel, Germany). Hematocrit was measured by routine Coulter counter technique.

Transcriptional analysis. To assess the CD73, A1AR, A2AAR, A2BR, and A3AR transcript levels in the different mouse strains, kidneys were excised under condition controls in wild-type, heterozygote, and gene-targeted mice, and transcript levels were determined. Total RNA was isolated from kidney tissue using the total RNA isolation NucleoSpin RNA II kit according to the manufacturer’s instructions (Macherey & Nagel, Düren, Germany). For this purpose tissue frozen in liquid nitrogen was homogenized in the presence of RA1 lysis buffer (Micro D8 homogenizer; ART-Labotechnik, Mulheim, Germany), and after filtration, lysates were loaded on NucleoSpin RNA II columns, followed by desalting and DNasel 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). Primer sets (sense sequence, antisense sequence, and transcript size, respectively) for the following genes were cd73 (5’-CA AACCAC TG-3’; 5’-TGGC TCA CT TGT GAC AGG AC-3’, 123 bp); A1AR (5’-AGG GAG GGG TCA AGA ACT GT-3’, 5’-TCC CAG TCT CGT CCT CGT TT-3’, 109 bp); A2AAR (5’-GAA GAC CAT GAG GAT GCT TT-3’, 5’-GAG TAT GGG CCA ATG GGA GT-3’, 253 bp); A2BR (5’-GGA AGG ACT TGC TCT CCA-3’, 5’-GGG CAG CAA CTC AGA AAA CT-3’, 322 bp); and A3AR (5’-CCA TTC GCT CCT TGT TT-3’, 5’-TCC CTG ATC ATT ACC ACG GCAC TC-3’, 334 bp). Each target sequence was amplified using increasing numbers of cycles of 94°C for 1 min, 58°C for 0.5 min, and 72°C for 1 min. Murine β-actin mRNA (sense primer: 5’-ACA TTG GCA TGG CTT TGT TT-3’, antisense primer: 5’-GTT TGC CCC AAC CAA CGT CT-3’) was amplified in identical reactions to control for the amount of starting template.

For measurement of renal EPO transcript, total RNA from mice kidney was isolated using PeqGold RNAPure (PeqLab, Erlangen, Germany). Synthesis of cDNA was performed using oligo(dT)18 and random hexamers as primers and avian myeloblastosis virus reverse transcriptase (PeqLab, Erlangen, Germany). PCR was carried out on the LightCycler instrument with the FastStart DNA Master SYBR green I kit (Roche, Mannheim, Germany). Primer sequences used for amplification were as follows: Epo (RefSeq accession no. NM_007942): sense, 5’-AGG GAG GGG TCA AGA ACT GT-3’, antisense, 5’-GGA AGG ACT TGC TCT CCA-3’. Relative expression ratio of the target gene EPO was calculated according to Pfaffl et al. (38).

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N ice-cold perchloric acid. Tissue adenosine levels were determined as described previously (21).

Statistical methods. Data were compared using two-factor ANOVA or Student’s t-test where appropriate. All values are means ± SE. P values <0.05 were considered to be statistically significant.

RESULTS

EPO secretion is induced by CO exposure, ambient hypoxia, or arterial hemodilution. To study the effect of extracellular adenosine production and signaling on hypoxia-associated EPO secretion, we first tested different murine models of EPO stimulation by limited O₂ availability. As a first experimental strategy, we exposed mice to CO (200 to 1,400 ppm). As shown in Fig. 1, A and B, increasing concentrations of CO, applied over 4 h, were associated with elevated hemoglobin-CO (HbCO) levels (to 48% with 1,400 ppm) and increasing EPO serum levels, with the latter peaking at ~1,000 ppm CO. Furthermore, we performed CO exposure over 3, 4, or 5 h at 400 or 750 ppm CO (Fig. 1, C and D). Based on these initial experiments, all subsequent experiments were performed using 400 or 750 ppm CO for 4 h, resulting in HbCO concentrations of 25 or 35%, respectively. The second experimental strategy exposed the mice to ambient hypoxia. Exposure to 8% (instead of 21%) O₂ over 4 h was associated with an 11-fold increase in EPO serum concentrations (Fig. 1E) P < 0.05. For the third model of stimulating EPO secretion, we used arterial hemodilution. In fact, the applied maneuver reduced the hematocrit by 23, 32, and 41% after 3, 8, and 24 h, which was accompanied by EPO increases to 60 ± 9, 176 ± 34, and 396 ± 63 mU/ml, respectively (Fig. 1, F and G) P < 0.05. Together, these studies indicate the feasibility of investigating EPO secretion in the above models of limited O₂ availability (CO exposure, ambient hypoxia, or arterial hemodilution).

Pharmacological inhibition or genetic deletion of CD73 does not alter hypoxia-induced EPO secretion. After having shown dramatic increases in EPO stimulation with CO exposure, ambient hypoxia, or arterial hemodilution, we next pursued the consequences of blocking extracellular adenosine generation by pharmacological inhibition of CD73. Mice received APCP (2 mg ip), a dose previously shown by our group (15) to inhibit adenosine generation under hypoxic conditions, 30 min before the experimental procedure and were then exposed for 4 h to 400 or 750 ppm CO or to ambient hypoxia (8% O₂). Vehicle-treated controls showed significant increases of renal EPO mRNA (Fig. 2, A and B) or EPO serum levels (Fig. 2, C and D), but these responses were not affected by APCP treatment (Fig. 2, A–D). Similarly, EPO stimulation by hemodilution was not affected by APCP treatment (Fig. 2E). These studies provide pharmacological evidence that inhibition of extracellular adenosine generation by APCP does not affect hypoxia-induced EPO secretion.

After having shown in pharmacological studies that EPO secretion is not affected by inhibition of CD73, we next pursued the same lines of studies in a genetic model. For this purpose, we used previously characterized cd73<sup>−/−</sup> mice (49). Exposure of cd73<sup>−/−</sup> mice to 400 or 750 ppm CO or to 8% O₂ resulted in a stimulation of EPO mRNA (Fig. 3, A and B) or EPO serum concentrations (Fig. 3, C and D) that was not different from that of littermate wild-type controls. Similarly, arterial hemodilution did not alter EPO serum concentration differently in cd73<sup>−/−</sup> mice compared with their littermate controls (Fig. 3E). Together, these studies provide strong pharmacological and genetic evidence that renal EPO stimulation by hypoxia does not depend on CD73-dependent adenosine generation.

Adenosine renal tissue content is reduced in CD73<sup>−/−</sup> mice following hypoxic stimuli. To assess to which extent CD73 may contribute to renal tissue adenosine content, we determined adenosine in kidneys of cd73<sup>−/−</sup> mice and their littermate controls under normoxic and three hypoxic conditions. Under normoxic conditions, adenosine tissue contents were similar in wild-type mice of all three groups. However, cd73<sup>−/−</sup> mice exhibited significantly lower adenosine tissue levels under normoxic and hypoxic conditions (750 ppm CO, 8% O₂, or hemodilution) compared with wild-type mice (Fig. 4, A–C).

Pharmacological inhibition or genetic deletion of adenosine receptors does not alter hypoxia-induced EPO secretion. After having shown that CD73-dependent adenosine production does not affect renal EPO secretion, we next performed studies using AR antagonists. As the first step, we used the nonselective antagonist 8-PT. Intraportal treatment with 8-PT 30 min before the experimental maneuver did not influence hypoxia-induced increases in EPO serum concentrations (Table 1). Similarly, selective blockade of the A<sub>1</sub>AR (DPCPX), A<sub>2A</sub>AR and A<sub>2B</sub>AR (DMPX), A<sub>2B</sub>AR (PSB 1115), or A<sub>3</sub>AR (MRS 1119) had no impact on stimulated EPO serum concentrations (Table 1). Since compensatory upregulation or down-regulation of the receptor density might occur in the different AR gene-targeted mice, we administered a cocktail containing DPCPX, DMPX, PSB 1115, and MRS 1191 as well in the doses described above before exposure to 600 ppm CO. However, as shown in Table 1, we could not detect any effect of this cocktail of the different AR antagonists on EPO secretion.

After having demonstrated that pharmacological inhibition of ARs does not affect the EPO secretory response to different hypoxic stimuli, we next investigated the EPO response in previously characterized A<sub>1</sub>AR<sup>−/−</sup> (47), A<sub>2A</sub>AR<sup>−/−</sup> (24), A<sub>2B</sub>AR<sup>−/−</sup> (7), or A<sub>3</sub>AR<sup>−/−</sup> mice (40) and their respective littermate controls. As shown in Table 2, A<sub>1</sub>AR<sup>−/−</sup>, A<sub>2A</sub>AR<sup>−/−</sup>, A<sub>2B</sub>AR<sup>−/−</sup>, or A<sub>3</sub>AR<sup>−/−</sup> mice and their respective littersmates had approximately the same basal EPO mRNA level under normoxia, indicating that AR deletion does not interfere with basal EPO secretion. Exposure to 400 and 750 ppm CO for 4 h induced a similar, dose-dependent increase in EPO mRNA levels in all four groups of gene-targeted mice and their respective littermate controls (Table 2). EPO serum concentrations increased in parallel to EPO mRNA levels after exposure to 400 and 750 ppm CO in A<sub>1</sub>AR<sup>−/−</sup>, A<sub>2A</sub>AR<sup>−/−</sup>, A<sub>2B</sub>AR<sup>−/−</sup>, or A<sub>3</sub>AR<sup>−/−</sup> mice and their respective littermate controls (Table 2).

After having demonstrated a similar EPO secretory response in gene-targeted mice for individual ARs following CO exposure, we next exposed these mice to normobaric hypoxia. Exposure to 8% O₂ over 4 h revealed a similar increase in renal EPO mRNA levels in mice with deletion of A<sub>1</sub>AR, A<sub>2A</sub>AR, A<sub>2B</sub>AR, and A<sub>3</sub>AR compared with their respective littermate controls (Table 2). Likewise, the increase of EPO serum concentrations was similar in gene-targeted mice and their corresponding littermate controls (Table 2).

Finally, we tested the effect of arterial hemodilution on EPO stimulation and observed that EPO serum levels increased...
Fig. 1. Erythropoietin (EPO) secretion during carbon monoxide (CO) exposure, ambient hypoxia, or arterial hemodilution. A: in vivo kinetics of carboxy-hemoglobin (HbCO) expressed as a percentage of total Hb in response to increasing CO concentrations in inspired air for 4 h in C57BL/6 conscious mice. B: EPO/CO dose response curve. Serum EPO in C57BL/6 mice after 4 h of exposure to increasing CO concentrations [0–1,400 parts per million (ppm)] in inspired air. *P < 0.05 vs. mice under normoxic conditions (0 ppm CO). C: time-dependent increase in EPO concentrations after exposure to 400 ppm CO for 3, 4, and 5 h. *P < 0.05 vs. 3 h. D: time-dependent increase in EPO concentrations after exposure to 750 ppm CO for 3, 4, and 5 h. *P < 0.05 vs. 3 h. E: EPO serum concentrations in mice following exposure to 8% O₂. *P < 0.05 vs. 21% O₂. F: erythropoiesis was induced in mice by arterial hemodilution (initial bleeding of 1.25% of body weight followed by a second bleeding of 1% of body weight after 3 h by retroorbital puncture). Eight and 24 h after the initial bleeding, additional blood samples were taken (75 μl). The volume was substituted by intraperitoneal application of isotonic Ringer solution. Hematocrit before blood withdrawal (0 h) and 3, 8, and 24 h after initial hemodilution in C57BL/6 mice. *P < 0.05 vs. 0 h. G: EPO serum concentrations before blood withdrawal (time 0) and 3, 8, and 24 h after hemodilution in C57BL/6 mice. *P < 0.05 vs. 0 h. Data are derived from 5–8 mice per condition. Results are means ± SE.
similarly 3, 8, and 24 h after hemodilution in $A_1AR^{-/-}$, $A_2AAR^{-/-}$, $A_2BAR^{-/-}$, or $A_3AR^{-/-}$ mice compared with littermate controls (Table 2). Moreover, we did not detect any difference in the EPO secretory response following CO exposure (Fig. 5A), 8% O$_2$ exposure (Fig. 5B) and at 24 h following hemodilution (Fig. 5C) among the different mouse strains. Furthermore, there were no differences in hematocrit and hemoglobin levels among the different mouse populations (data not shown). Together, these studies combine pharmacological and genetic evidence that extracellular adenosine
signaling through the four known ARs does not alter basal or stimulated EPO levels.

DISCUSSION

Contradictory results have been reported in previous pharmacological studies with regard to the role of adenosine in the regulation of EPO secretion by the kidneys. In the present study we used a combination of pharmacological and gene deletion approaches to examine the contribution of extracellular adenosine production and signaling to renal EPO secretion. Transcriptional profiling of renal tissue following exposure to three different hypoxic stimuli (CO, hypoxia, or hemodilution)
revealed a prominent induction of EPO mRNA. Pharmacological inhibition or targeted gene deletion of cd73 had no impact on hypoxia-induced increases in EPO mRNA or EPO serum concentrations. Moreover, pharmacological studies using a nonselective AR inhibitor or specific inhibitors of individual ARs or experiments in gene-targeted mice for these receptors (A1AR, A2AR, A2BR, or A3AR) showed no differences in hypoxia-induced increases in EPO mRNA or serum levels. Together, these studies provide genetic and pharmacological in vivo evidence that neither the ecto-5′-nucleotidase nor signal transduction through individual ARs is required for the stimulation of EPO by limited O2 availability.

EPO expression is known to be controlled by several transcription factors and thus possesses several regulatory DNA elements. Of primary importance are hypoxic response elements in the 3′-EPO enhancer to which specific heterodimeric HIFs (HIF-α/β) can bind (53). Besides HIFs, the EPO promoter has binding sites for GATA factors (46). GATA-2 and GATA-3 are thought to suppress EPO secretion, whereas GATA-4 induces EPO secretion by binding at the 5′-promoter (4). Moreover, the EPO promoter and the 5′-flanking region contain binding sites for NF-κB, inhibiting EPO gene expression in inflammatory diseases (23). Proinflammatory cytokines, such as TNF-α and IL-1, also have been shown to inhibit EPO expression (46). Besides these modulators of EPO secretion, other factors such as adenosine have been proposed to regulate EPO secretion.

The hypothesis that adenosine modulates EPO secretion is based on several observations. Adenosine is known to accumulate in the kidney in response to ischemia or increased tubular workload (27, 35, 36) and can change intrarenal hemodynamics by induction of vasoconstriction in the kidney and thus change local O2 concentrations (52). The enzyme ecto-5′-nucleotidase, converting extracellular AMP into adenosine, is present on the cell surface of interstitial cells, which also positively stained for EPO mRNA (2, 9, 22). Furthermore, ARs...
Erythropoietin (EPO) secretion have yielded conflicting results. In vitro investigations using the human hepatoma cell lines Hep3B and HepG2, which produce EPO in an oxygen-dependent fashion (51), showed an increase in the EPO secretory response following application of the selective A1AR agonist 6-cyclohexyladenosine (CHA) at concentrations of 10⁻⁵ and 5 × 10⁻⁵ M (28). However, cyclophosphamide, another selective A1AR agonist, inhibited (32), 5'-N-ethylcarboxamideadenosine (NECA), a nonselective AR agonist, stimulated (30), and CGS-21680 [2-p-(2-carboxyethyl) phenethyly ammo-5'-N-ethylcarboxamideadenosine hydrochloride], a selective A2AR agonist, had no effect on EPO secretion (17). Apart from inconsistencies in these findings, the extent to which EPO production in liver cells reflects regulation of the hormone in the kidney remains questionable.

One study (8) proposed that adenosine regulated EPO secretion through the activation of the A2AR and A2BR. The investigators postulated that A2AR and A2BR increase EPO mRNA through an increase in cAMP and HIF-1α. However, these findings of selective inhibition of EPO secretion by A2AR and A2BR antagonism could not be reproduced by other groups (11–13). These conflicting results could be due to weak selectivity of the compounds and different experimental settings. Another study (51) reported that, in vitro, the A1AR agonist CHA inhibited albuterol-stimulated ²⁰Fe incorporation. This effect was blocked by high doses of theophylline (up to 80 mg/kg). However, this theophylline dose cannot be considered to block ARs only (39). In another study (6), it was shown recently that theophylline in therapeutic concentrations (3 μM) could stimulate a histone deacetylase independently of adenosine receptors or phosphodiesterase inhibition, rendering theophylline as a substance too nonspecific to interfere with adenosine receptors.

Our current results in the mouse model confirm and extend the results of a previous, very thoroughly performed in vivo study in rats. That study, using different AR antagonists such as theophylline, 9-cyclopropyl-1,3-dipropylxanthine, 8-noradamantryl-1,3-dipropylxanthine (KW-3902), and DMPX as well as the AR agonists CHA and CGS-21680 failed to show any

Table 2. EPO mRNA and serum levels in mice following CO exposure, ambient hypoxia, or arterial hemodilution

<table>
<thead>
<tr>
<th>AR Gene-Targeted Mice</th>
<th>A1AR+/+</th>
<th>A1AR+/−</th>
<th>A2AR+/+</th>
<th>A2AR+/−</th>
<th>A2BR+/+</th>
<th>A2BR+/−</th>
<th>A3AR+/+</th>
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<tr>
<td>EPO transcript</td>
<td>1±0.1</td>
<td>0.9±0.3</td>
<td>1.3±0.2</td>
<td>1.1±0.1</td>
<td>1.0±0.3</td>
<td>1.4±0.1</td>
<td>1.2±0.2</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>EPO serum, mU/ml</td>
<td>8.2±1.7</td>
<td>9.5±1.1</td>
<td>11.8±2.4</td>
<td>8.0±2.5</td>
<td>9.9±1.5</td>
<td>9.0±2.3</td>
<td>6.8±1.3</td>
<td>6.9±1.5</td>
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<td>CO (400 ppm)</td>
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<tr>
<td>EPO transcript</td>
<td>15.0±2.6*</td>
<td>16.9±3.2*</td>
<td>12.9±3.5*</td>
<td>11.2±3.1*</td>
<td>16.4±4.2*</td>
<td>15.4±5*</td>
<td>13.0±1.2*</td>
<td>14.8±3.1*</td>
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<tr>
<td>EPO serum, mU/ml</td>
<td>150.2±20*</td>
<td>127.4±14.1*</td>
<td>128.2±19.0*</td>
<td>117.3±15.3*</td>
<td>126.7±16.0*</td>
<td>122.7±11.8*</td>
<td>105.0±11.9*</td>
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<tr>
<td>EPO transcript</td>
<td>40.0±6.2*</td>
<td>41.4±5.1*</td>
<td>36.0±6.4*</td>
<td>34.6±4.0*</td>
<td>37.8±9.8*</td>
<td>37.0±3.2*</td>
<td>39.4±5.9*</td>
<td>37.6±8.4*</td>
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<tr>
<td>EPO serum, mU/ml</td>
<td>375.1±70.1*</td>
<td>357.2±71.8*</td>
<td>412.7±40.4*</td>
<td>392.28.3*</td>
<td>366.7±76.2*</td>
<td>385.8±26.3*</td>
<td>343.5±62.8*</td>
<td>356.0±36.6*</td>
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<td>8% O₂</td>
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<tr>
<td>EPO transcript</td>
<td>18.0±3.1*</td>
<td>17.8±2.5*</td>
<td>13.3±1.7*</td>
<td>13.8±2.4*</td>
<td>15.8±1.9*</td>
<td>15.3±3.4*</td>
<td>18.0±2.9*</td>
<td>17.5±4.9*</td>
</tr>
<tr>
<td>EPO serum, mU/ml</td>
<td>117.1±18.7*</td>
<td>120.3±16.8*</td>
<td>114.5±14.6*</td>
<td>118.3±20.9*</td>
<td>122.0±10.9*</td>
<td>127.0±9.5*</td>
<td>135.0±7.6*</td>
<td>133.2±15.8*</td>
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</table>

EPO mRNA and serum levels were measured in AR-deficient mice and their respective littermate controls after 4 h of exposure to CO (400 or 750 ppm), after 4 h of exposure to 8% O₂, or after arterial hemodilution (see text for details). Data are derived from 5–8 mice per condition. Results are means ± SE. *P < 0.05 vs. normoxic control mice.
effects of these compounds on EPO secretory responses under normoxic or hypoxic conditions (12). Moreover, the same group administered APCP or dipyridamole to increase extracellular adenosine concentrations, but these maneuvers were also without effect on renal EPO mRNA or EPO serum concentrations, suggesting that adenosine is not critical for normal regulation of EPO secretion. These observations were confirmed in two human trials in which the induction of EPO

Fig. 5. EPO serum levels in the different mouse populations following exposure to carbon monoxide (CO), ambient hypoxia, or arterial hemodilution. A: EPO serum concentrations after 4 h of exposure to 750 ppm CO. B: EPO serum concentrations after 4 h of exposure to 8% O2. C: EPO serum concentrations 24 h after hemodilution in CD73 or A1 adenosine receptor (A1AR), A2AAR, A2BAR, or A3AR gene-targeted mice and their respective controls. Data are derived from 5–8 mice per condition. Results are means ± SE.
secretion by phlebotomy (13) or exposure to hypobaric hypoxia in healthy volunteers was not modified by theophylline, a nonselective AR antagonist, or dipyridamole, an adenosine reuptake inhibitor (11). Notably, and in accordance with our current findings, Ozuyaman et al. (37) very recently demonstrated that the EPO secretory response to hypoxia is unaffected in cd73−/− mice compared with their controls, despite the fact that these mice have another genetic background compared with the mice used in this study. The finding in our three models of systemic hypoxia that adenosine tissue content did not change compared with normoxic conditions emphasizes the difference between systemic hypoxia and complete renal ischemia by clamping the renal artery in which adenosine tissue levels increases at least fivefold (36). Interestingly, mice lacking CD73 activity exhibit significantly lower adenosine levels in the kidney even under systemic hypoxia than under normoxia (Fig. 4), indicating the importance of extracellular adenosine generation. Ozuyaman et al. (37) also reported unchanged adenosine tissue content (20 nmol/g) under hypoxic conditions (1,000 ppm CO, 8% O2). However, the renal adenosine tissue content in our study was between 3 and 4 nmol/g wet wt, which agrees well with data reported in the literature for rats (21, 36) and mice (15, 16). The differences between absolute adenosine tissue content reported by Ozuyaman et al. and those of the literature are unexplained at present. In addition, concentrations of renal extracellular adenosine (50–200 nmol/l) do not correspond to the whole tissue content of adenosine (2–5 μmol/kg wet wt) (52).

The doses of all substances that we have tested were based on earlier reports in which clear antagonistic effects were demonstrated on different organ functions in experimental animals. Thus the dose of DPCPX showed a protective effect in glycerol-induced acute renal failure (20, 44), the dose of DMPS had a maximal effect on NECA-induced hypothermia and locomotor activity depression (42), and the dose of MRS DMPX had a maximal effect on NECA-induced hypothermia in glycerol-induced acute renal failure (20, 44), the dose of MRS 1119 was protective in ischemia-reperfusion and myoglobinuria-mediated renal injury (25). Furthermore, we could show that the dose of theophylline was protective following renal transplantation (14) and that the dose of APCCP or PSB 1115 abolished the renal or cardioprotective effects of ischemic preconditioning, respectively (7, 15). To provide confidence that the results of this study do not reflect in our hands a lack of sensitivity and/or specificity for the detection of EPO, we refer to our previous investigations showing an increase or decrease of the EPO secretory response in rats following renal denervation or in rats on a high-salt diet, respectively, following 4 h of exposure to 600 ppm CO compared with controls.

In conclusion, considering the entirety of our findings obtained with knockout mice and adenosine receptor-inhibiting substances, it appears unlikely that an increase of adenosine concentration in the peritubular renal interstitium modulates a hypoxia-induced increase in the production or release of EPO. Together, the present experiments demonstrate that neither adenosine generation by ecto-5′-nucleotidase nor signaling through individual ARs is required for stimulation of EPO secretion in response to hypoxic stimuli.

ACKNOWLEDGMENTS

We acknowledge Rosemarie Maier and Renate Riehle for technical assistance and Cathérine Ledent and Marlene Jacobson for kindly providing gene-targeted mice for A2aAR and A3AR, respectively.

GRANTS

This investigation was supported by the Federal Ministry of Education, Science, Research and Technology (BMBF 01EC0001).

REFERENCES


ROLE OF ADENOSINE IN EPO SECRETION


