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Evidence against a major role of adenosine in oxygen-dependent regulation of erythropoietin in rats

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Evidence against a major role of adenosine in oxygen-dependent regulation of erythropoietin in rats. This *in vivo* study investigated whether adenosine (ADO) plays a role in oxygen-dependent production of erythropoietin (EPO). Exposure of rats to 0.075% carbon monoxide (CO) for four hours was used as a stimulus for EPO production. To inhibit potential effects of ADO, rats were treated with the non-specific ADO antagonist theophylline, the selective ADO A1 receptor blockers DPCPX and KW-3902, the selective ADO A2 receptor blocker DMPX, and AOPCP, an inhibitor of 5′-ectonucleotidase, an ADO generating enzyme that is expressed on the surface of EPO producing cells. To stimulate ADO receptor activity, animals were treated with the selective ADO A1 and A2 receptor agonists CHA and CGS 21680, the ADO reuptake inhibitors dipyridamole and soluflazine and the ADO desaminase inhibitor EHNA. At doses known to interfere with ADO signal transmission *in vivo*, none of these substances either influenced EPO serum levels in normoxic rats or affected the approximately 30-fold rise in EPO serum levels and the increase in renal EPO mRNA after exposure to carbon monoxide. Continuous administration of theophylline to normoxic rats for seven days did not alter hematocrit, hemoglobin or EPO serum levels. Taken together, these experiments do not support the hypothesis that ADO plays an important role in the regulation of EPO production.

A major physiological mechanism by which mammals respond to hypoxia is through stimulation of erythropoiesis, which results in an increased blood oxygen-carrying capacity. This response is mediated by oxygen-dependent production of the glycoprotein hormone erythropoietin (EPO) in liver and kidneys [1, 2]. The major control of EPO production occurs at the level of its mRNA and involves changes in EPO gene transcription rate [3]. An enhancer sequence has been identified that mediates hypoxia-induced activation of EPO gene activity, and nuclear proteins binding to this sequence are identified [4–7]. However, the cellular mechanisms by which hypoxia is sensed remain unknown.

Recently, peritubular renal fibroblasts and perisinusoidal Ito cells have been identified as the renal and one of the hepatic cell populations producing EPO [8–10]. A common characteristic of these cells used for their identification is the expression of 5′-nucleotidase on their surface, which splits adenosine monophosphate (AMP) into adenosine (ADO) and phosphate [11, 12]. ADO has since long been postulated to be a modulator of EPO production [13]. Indeed, renal interstitial levels of adenosine were reported to increase in response to increased tubular workload or decreased oxygen supply [14, 15], and an increase of ADO results in hemodynamic changes [16].

The effect of ADO on EPO production *in vivo* has not been directly investigated thus far, but a potentially important role of ADO has been inferred from studies in human hepatoma cells, used as an *in vitro* model of EPO production, and from studies measuring erythropoiesis in polycythemic mice [13]. Also, in patients with post-transplant erythrocytosis that may be due to inappropriate EPO production, the non-specific ADO antagonist theophylline was found to reduce EPO levels and hematocrit [17]. In contrast, adding the non-selective ADO agonist N^6-ethylcarboxamidoadenosine or forskolin, which generates the second messenger cyclic AMP, to the perfusate of isolated perfused rat kidneys did not alter the oxygen dependent production of EPO [18] and EPO mRNA [19].

In view of these contradictory findings, and that selective ADO A1 and A2 receptor agonists and antagonists as well as other compounds that potentially alter interstitial ADO availability have recently become available, we used such compounds to examine the role of ADO for EPO formation in normoxic and hypoxic rats.

METHODS

Animals

Male Wistar rats weighing 200 to 280 g were kept on a regular 12-hour dark-light cycle with free access to food and water. All animals were handled for three to five days before the studies were performed. The experiments were approved by the Animal Care and Use Committee of the University of Göttingen.

Compounds and solvents

The following compounds were used and dissolved in 0.85% NaCl unless otherwise indicated: theophylline (Euphyllin 200®; a gift of Byk Gulden GmbH, Konstanz, Germany); DPCPX (9-Cyclopentyl-1,3-dipropylxanthine; RBI, Natick, MA, USA) dissolved in saline, ethanol, and DMSO in a relation of 2:1:1; KW-3902 [8-(3-noradamantyl-1,3-dipropylxanthine)] (a gift of Kyowa Hakko Kogyo Co., Ltd., Düsseldorf, Germany), dissolved in saline containing 1% vol/vol DMSO and 1% vol/vol 1 N NaOH;

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DMPX (3,7-dimethyl-1-propargylxanthine; RBI), dissolved in saline containing 10% ethanol; AOPCP (α,β-methyleneadenosine 5′-diphosphate; Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany); CHA (N^6-cyclohexyladenosine; Sigma-Aldrich); CGS 21680 (2-[p-(2-carboxyethyl)phenetylamino]-5′-N-ethylcarboxamidoadenosine) (a gift of Ciba-Geigy Corporation, Summit, NJ, USA); dipyridamole (Persantin®; Dr. Karl Thomae GmbH, Biberach/Riβ, Germany); R 64719 (soluflazine; a gift of Janssen Research Foundation, Beerse, Belgium); EHNA, (erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride), RBI. When necessary, thiopental (Trapanal®, Byk Gulden GmbH) was used as an anesthetic.

Experiments

Functional anemia caused by normobaric carbon monoxide inhalation was used as a stimulus for EPO production using a technique reported earlier [20]. Together with a control group, treated animals were exposed in a plexiglass chamber for four hours to a gas mixture of 0.075% CO in synthetic air (Messer Griesheim, Duisburg, Germany). Each animal received a single dose (in mg/kg body wt) of the respective compound i.p. 15 minutes before being exposed to hypoxia in the chamber. The only exception was AOPCP, which was injected i.m. 90 minutes prior to the exposure to CO. Groups of up to six animals treated with a particular drug were exposed to hypoxia together with controls, which were treated with the same volume of the respective solvent as the actively treated animals. Within 15 minutes after the end of exposure to this gas mixture the animals were decapitated and the trunk blood collected. Immediately thereafter, the left kidney was taken and frozen in liquid nitrogen for mRNA extraction. Serum and tissue samples were kept at −80°C until analysis.

Some compounds were also given under normoxic conditions (ambient air) to study their effects on basal EPO production.

In a second experiment, a long-term treatment with theophylline and placebo (0.85% saline) was carried out. A theophylline infusion rate of 2 mg/kg/hr over seven days was achieved by implanting 2 Alzet® osmotic pumps (Model 2ML1; Charles River, Sulzfeld, Germany) subcutaneously in the back. At the end of this experiment the animals were bled for determination of EPO and theophylline concentrations as well as hemoglobin and hematocrit.

In a separate set of experiments, renal tissue ADO levels were measured under normoxic conditions and under CO exposure in untreated control animals and animals treated with AOPCP, EHNA, soluflazine and dipyridamole (5 animals each), as described [14]. Animals exposed to CO received thiopental (80 mg/kg i.p.) a few minutes before the end of the four hour exposure and were then returned on a heating table placed in the chamber, which was subsequently flushed to re-achieve the CO atmosphere. When anesthesia was effective animals were quickly removed from the chamber, laparotomized and the left kidney gently mobilized, removed and frozen within 1 to 3 seconds with a Wollenberger-clamp precooled in liquid nitrogen [14]. These kidneys were then kept in liquid nitrogen until homogenization for ADO analysis.

Assays

Erythropoietin. Serum EPO concentrations were determined by radioimmunoassay as described earlier [21] with the use of a rabbit antiserum raised against pure recombinant human EPO and iodinated human EPO (Amersham International) as a tracer. A rat serum pool enriched in EPO was prepared by exposing donor animals to hypoxia and was used as standard after calibration against the II International Reference Preparation by In Vivo Bioassay. Samples from one experiment were always analyzed in parallel in the same assay.

EPO-mRNA. In selected animals total renal RNA was prepared using an acid guanidinium thiocyanate-phenol-chloroform extraction method [22]. EPO mRNA was measured by RNAse protection as previously described [23]. An RNA probe was continuously labeled from a genomic rat EPO DNA template (*Pst*I/*Sac*I fragment containing 132 bp of exon V and approximately 300 bp of the adjoining intron (kindly provided by Dr. P.J. Ratcliffe, Oxford, UK) using SP 6 polymerase and alpha ^{32}P-GTP (410 Ci/mmol; Amersham, UK). In brief, 100,230 g of total RNA was hybridized to 0.7×10^6 cpm of radiolabeled probe in 80% formamide, 40 mM piperazine-N,N′-bis(2-ethane sulfonic acid), pH 6.4, 400 mmol/liter NaCl, 1 mM EDTA at 60°C overnight, and RNAse digestion was carried out at 20°C for 30 minutes. Protected fragments were separated by electrophoresis on a denaturing 10% polyacrylamide gel, and visualized on a phosphorimager.

Adenosine. As described earlier [14], the frozen kidneys were powdered under liquid nitrogen and the sample weighed. The homogenate was then treated with 0.6 N perchloric acid at 0°C. After adding 50 nmol methyl-adenosine (m-ADO) as internal standard the supernatant was recovered after centrifugation and adjusted to pH 7.2 to 7.4 with 2 M potassium carbonate. $KClO_4$ originating during this procedure was removed by centrifugation and the supernatatnt filtrated. The quantitaive analysis of ADO was performed with HPLC as described earlier [24]. In brief, 2 ml of tissue extract were applied onto phenylboronate columns (Frankfurt am Main, Germany), washed twice with citrate buffer solution and eluted with 0.5 ml HCl (0.1 mol/liter). A total of 50 μl of the eluate were applied onto the HPLC system with a reversed phase column (C 18, 3 μm). The UV absorbance detector (Sykam, Germany) was set at 254 nm. The mobile phase was an aquaeous solution of 0.01 mmol/liter ammonium phosphate and 6% methanol; increasing percentages of acetonitril were added by a gradient system. ADO concentration was calculated by peak area corrected for internal standard peak area. Absolute recovery of ^{3}H-labeled ADO was 87 to 89%. Precision of the analytical procedure (recovery of ADO added to the tissue and calculated for internal standard) was ± 3% in the concentration range from 5×10^{-7} to 10^{-4} mol/liter.

Dipyridamole. Serum samples were analyzed using an HPLC with UV detection (280 nm) using propranolol as an internal standard [25]. In brief, a Li Chrospher 5 μm reversed-phase C18 column was used. Dipyridamole and propranolol were eluted with 37% acetonitrile in 0.02 M phosphate buffer containing 0.01 M tetramethylethylenediamine at pH 2.9. The calibration curve was linear in the range of 20 to 1000 ng/ml ($r = 0.9895$).

Theophylline. Theophylline was measured using a fluorescence polarization immunoassay (Tdx® Theophyllin 2; Abbott Diagnostics).

Hemoglobin and hematocrit. These variables were measured using a routine Coulter counter technique.

Statistics

Each study group and their respective controls were compared using the Mann-Whitney test. A P value < 0.05 was considered significant.

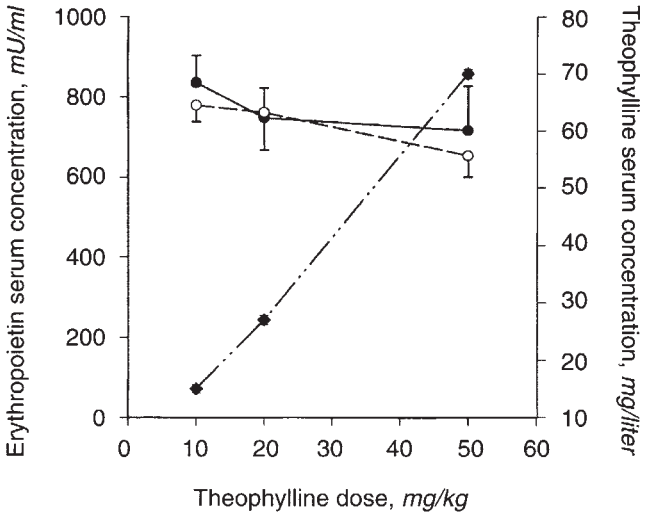

Fig. 1. Effect of different doses of theophylline on serum EPO concentrations in animals exposed for four days to 0.075% carbon monoxide (controls, ○; theophylline treated animals, ●). The theophylline concentrations at the end of the exposure to hypoxia are shown by filled lozenges. Data represent means ± SEM; $N = 6$ to 8 animals/group.

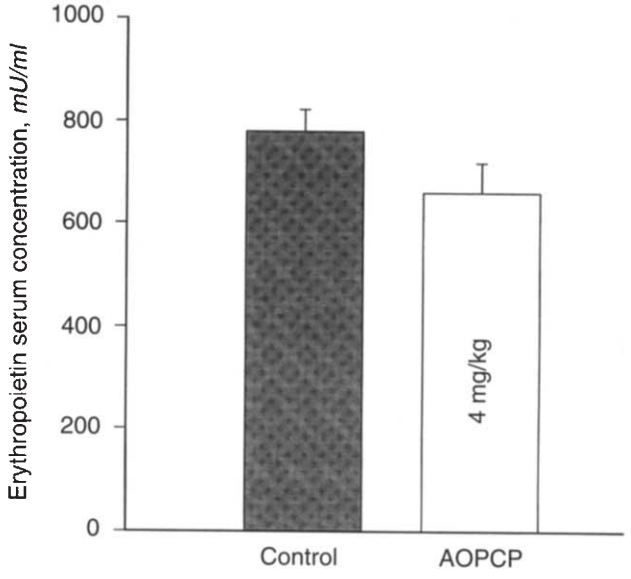

Fig. 2. Effect of the nucleotidase inhibitor (AOPCP) on serum EPO concentration in animals exposed for four hours to 0.075% carbon monoxide. Data represent means ± SEM; $N = 8$ to 10 animals/group.

RESULTS

Inhibiting ADO. To inhibit ADO availability and receptor-activity, animals were treated with the non-selective ADO receptor antagonist theophylline, with selective antagonists for ADO A 1 receptors (DPCPX and KW 3902) and ADO A 2 receptors (DMPX) and an inhibitor of 5′-ectonucleotidase (AOPCP).

Increasing ADO. To increase ADO availability and receptor-activity, animals were treated with ADO uptake inhibitors (dipyridamole, soluflazine), an ADO deaminase inhibitor (EHNA), and selective agonists for ADO A 1 (CHA) and ADO A 2 receptors (CGS 21680).

Effect of ADO antagonistic compounds on hypoxia-induced EPO levels

Exposure of animals to 0.075% CO for four hours resulted in an approximately 30-fold increase of serum EPO levels, which is in accordance with previous studies [26]. As shown in Figure 1, treatment of rats with theophylline (10 to 50 mg/kg body wt) did not have a significant effect on EPO serum levels, although, as expected, theophylline serum levels increased in a dose dependent fashion.

Figure 2 illustrates that the application of AOPCP, at a dose previously demonstrated to maximally inhibit renal activity of 5′-ectonucleotidase [27], results in a slight, but statistically not significant reduction of mean serum EPO levels.

To selectively block ADO receptors, the specific ADO A 1 receptor antagonists KW 3902 and DPCPX and the ADO A 2 receptor antagonist DMPX were used. DPCPX resulted in a slight increase of the EPO response (not statistically significant). EPO levels were not consistently different from controls with two doses of KW 3902 (1 and 3 mg/kg) as well as DMPX (Figure 3).

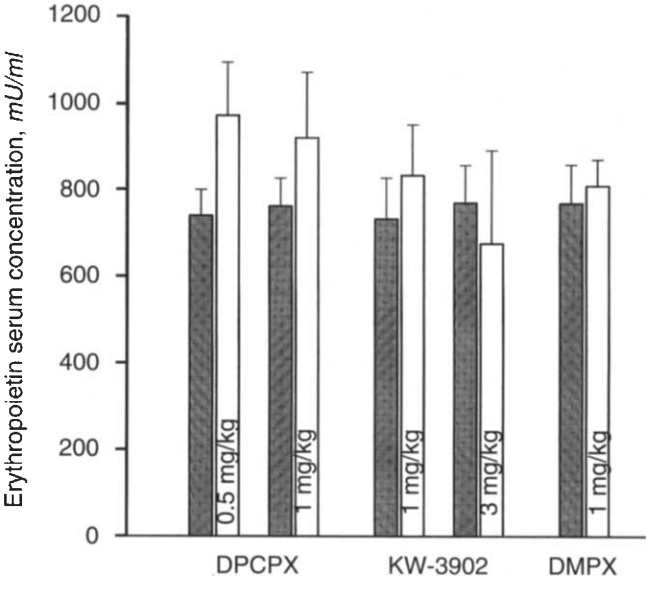

Fig. 3. Effect of different selective adenosine receptor antagonists on serum EPO concentrations in rats exposed for four hours to 0.075% carbon monoxide (adenosine A1 receptor antagonists, DPCPX, KW-3902; adenosine A2 receptor antagonist, DMPX). Data represent means ± SEM; $N = 6$ to 8 animals/group. Symbols are: (▨) control; (□) ADO antagonist.

Effect of ADO synergistic compounds on hypoxia-induced EPO levels

Figure 4 shows that inhibition of ADO uptake with either dipyridamole or soluflazine, or inhibition of ADO deamination by EHNA had no effect on EPO serum levels. In dipyridamole-treated animals the mean dipyridamole concentration four hours after i.p. injection was 0.25 ± 0.3 μg/ml.

To test whether selective stimulation of ADO receptors alters the CO-induced increase in EPO levels, CHA, a selective ADO A1 receptor agonist, and CGS 21680, a selective ADO A2 receptor agonist were used. Figure 5 illustrates that neither substance affected the EPO response.

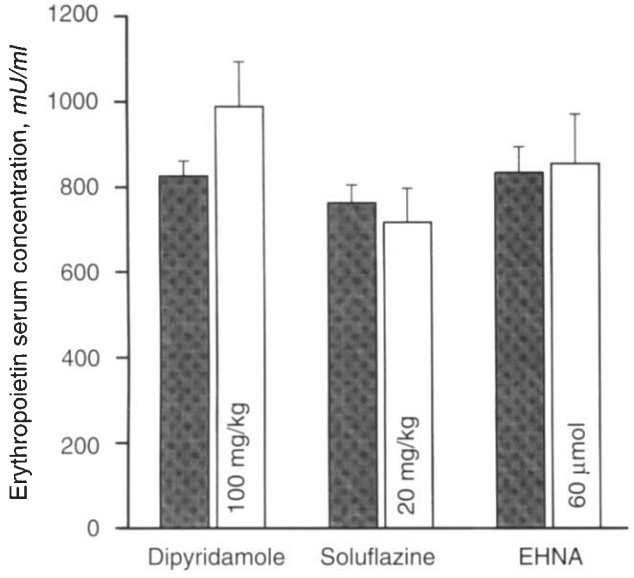

Fig. 4. Effect of different adenosine reuptake inhibitors (dipyridamole, soluflazine), and the nucleosidase inhibitor (EHNA) on serum EPO concentrations in animals exposed for four hours to 0.075% carbon monoxide. Data represent means ± SEM; N = 8 to 10 animals/group. Symbols are: (▨) control; (□) compound.

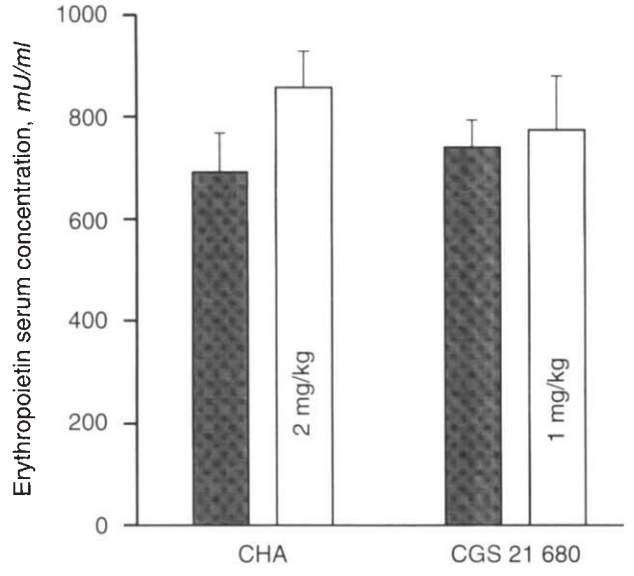

Fig. 5. Effect of selective adenosine receptor agonists on serum EPO concentrations in animals exposed for four hours to 0.075% carbon monoxide (adenosine A1 receptor agonist, CHA; adenosine A2 receptor agonist, CGS 21 680). Data represent means ± SEM; N = 6 to 8 animals/group. Symbols are: (▨) control; (□) agonist.

Effect of ADO synergistic and antagonistic compounds on EPO levels in normoxic animals

To investigate whether inhibition or stimulation of ADO A1 or A2 receptors mimick part of the hypoxic response, theophylline, the ADO A1 and A2 antagonists KW-3902 and DMPX, and the ADO A1 and A2 agonists CHA and CGS 21680 were given to normoxic animals and their serum EPO levels were determined four hours later. As shown in Table 1, none of the five substances had a significant effect.

Table 1. Erythropoietin serum concentrations under normoxic conditions (ambient air) following the i.p. application of the indicated compounds

Compound	Dose *mg/kg*	EPO concentration *mU/ml*
Controls	—	20 ± 1.8
Theophylline	10	20 ± 4.5
KW-3902	3	24 ± 1.4
DMPX	1	23 ± 6.0
CHA	2	21 ± 1.5
CGS 21 680	5	23 ± 2.1

Data are means ± SEM; N = 6 to 8 animals/group.

Effect of ADO synergistic and antagonistic compounds on hypoxia-induced EPO mRNA levels

To test whether ADO synergistic or antagonistic compounds might have opposite effects on EPO gene expression and EPO mRNA translation under our experimental conditions, which could result in unchanged serum EPO concentrations, renal EPO mRNA from three animals of each group with similar serum EPO concentrations was determined by RNAse protection. EPO mRNA levels in kidney tissue were similar in treatment and control groups (Fig. 6).

Effect of hypoxia and treatment with AOPCP, EHNA, soluflazine and dipyridamole on renal tissue ADO concentrations

As shown in Table 2, renal tissue ADO concentrations under basal conditions were 5.95 ± 1.03 nmol/g wet wt. This value is very similar to the concentration reported by other investigators [14, 15]. Carbon monoxide exposure alone and treatment with AOPCP had no demonstrable effect on tissue ADO concentrations. ADO levels were increased, however, in animals treated with the uptake inhibitors dipyridamole and soluflazine and the deaminase inhibitor EHNA.

Effect of long-term treatment with theophylline on EPO serum levels in normoxic rats

In view of the evidence that long-term treatment with theophylline slightly reduces EPO serum levels in healthy normoxic humans [17], we tested the effects of continuous application of the drug to normoxic rats for seven days by recording the serum EPO levels, hematocrit and hemoglobin concentration. For unknown reasons the EPO levels were slightly higher than in animals studied under basal conditions, but there was no difference between animals treated with theophylline and untreated controls studied in parallel (Table 3).

DISCUSSION

Using a variety of different ADO synergistic and antagonistic compounds *in vivo* we failed to demonstrate a significant effect of ADO on EPO production under normoxic and hypoxic conditions. Several lines of evidence suggest that this lack of an effect on EPO production is not due to inappropriate dosing or the experimental setting in which these substances were investigated.

First, the doses of all substances tested were based on earlier

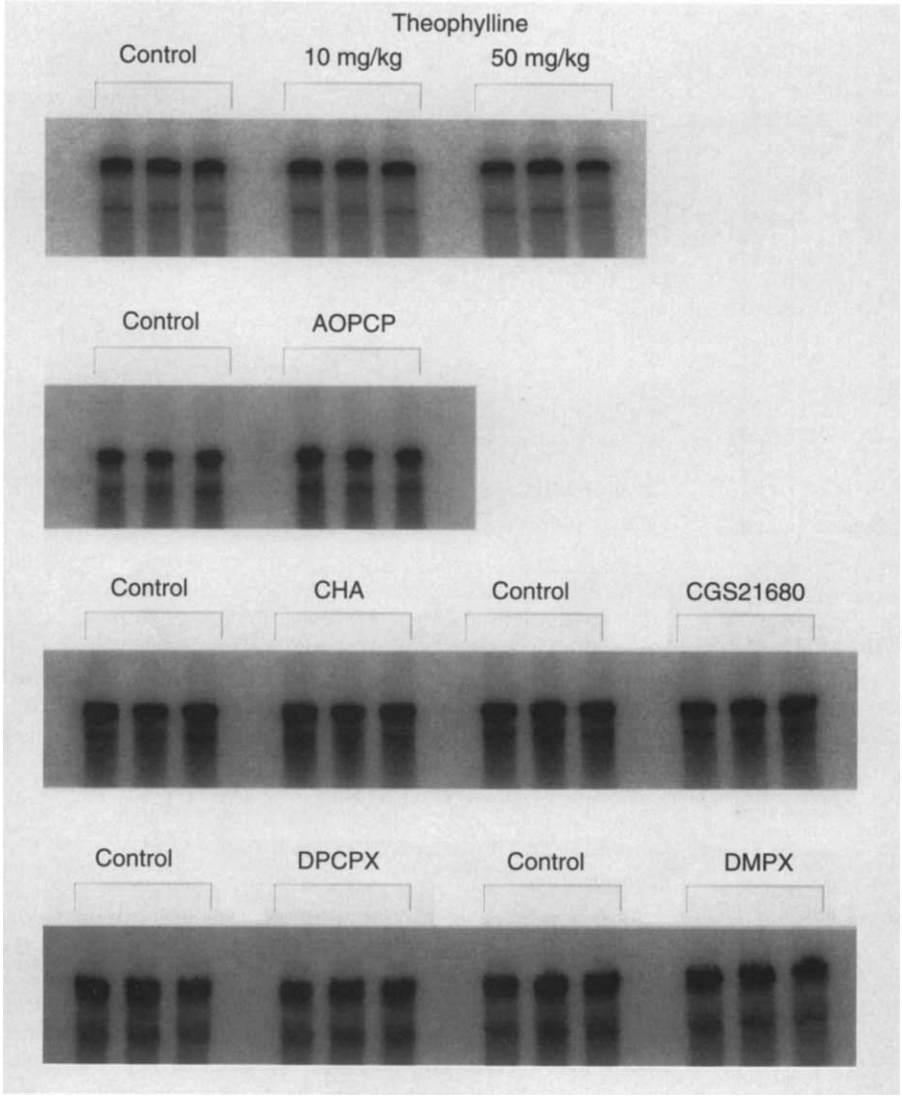

Fig. 6. Autoradiographs of RNase protection assays for EPO mRNA in the kidneys of rats exposed to carbon monoxide (0.075%) for four hours and treated with the indicated compounds rats (N = 3 animals/treatment). There was no difference in the EPO mRNA signal in any group of treated animals compared with their respective controls (doses: non-specific ADO antagonist theophylline as indicated, 5′-ectonucleotidase inhibitor AOPCP 4 mg/kg, ADO A1 receptor agonist CHA 2 mg/kg, ADO A2 receptor agonist CGS 21680 1 mg/kg, ADO A1 receptor antagonist DPCPX 1 mg/kg, ADO A2 antagonist DMPX 1 mg/kg).

Table 2. Renal tissue concentrations of adenosine

Condition	Compound	Dose	ADO concentration *nmol/g wet wt*
Normoxia	—		5.9 ± 1.0
Carbon monoxide	—		5.7 ± 0.6
Carbon monoxide	AOPCP	4 mg/kg	5.8 ± 0.9
Carbon monoxide	EHNA	60 umol	20.8 ± 5.8[a]
Carbon monoxide	soluflazine	20 mg/kg	21.4 ± 6.5[a]
Carbon monoxide	dipyridamole	100 mg/kg	10.9 ± 2.9[a]

Data are means ± SEM; $N = 5$ animals/group
[a] Significant difference from untreated controls exposed to CO ($P < 0.05$)

Table 3. EPO concentrations, theophylline concentrations, hematocrit and hemoglobin at the end of a 7 day treatment with theophylline (2 mg/kg/h) or placebo (0.85% NaCl) using osmotic minipumps (normoxic conditions)

Treatment	Drug concentration *mg/liter*	Hematocrit *%*	Hemoglobin *g%*	EPO concentration *mU/ml*
Placebo	—	38.4 ± 0.8	13.0 ± 0.3	39 ± 3.1
Theophylline	10.2 ± 1.2	39.6 ± 0.5	13.3 ± 0.2	40 ± 1.1

Data are means ± SEM; $N = 8$ animals/group.

reports in which clear adenosine-synergistic and antagonistic effects were demonstrated on different organ functions in experimental animals. Thus, theophylline concentrations following single injections at the lowest dose used in our animals were in the range of the accepted lower effective threshold of 10 mg/ml [28]. The doses of DPCPX used in our study had a protective effect in glycerol-induced acute renal failure, a model disease considered to be caused by increased ADO levels in the kidney [29, 30]. KW-3902 had a clear beneficial effect on cephaloridine-induced acute renal failure (as measured by creatinine clearance, kidney histology, and proteinuria) at the lower dose used in our experiments [31]. DMPX has been shown to have a maximal effect at the dose used on NECA-induced hypothermia and locomotor activity depression [32]. CHA and CGS 21 680 have been shown to be active at the dose chosen [33–35]. Dipyridamole plasma concentrations were in the order of magnitude where an inhibition of

ADO reuptake was demonstrated [36]. Soluflazine, which was selected because its use is not confounded by inhibition of phosphodiesterases as may be the case with dipyridamole, was administered at a higher dose compared to a study by Phillips, O'Reagan and Walter, who failed to demonstrate an effect on interstitial ADO levels following infusion of 0.5 mg/kg [37]. AOPCP has been shown to have its maximum inhibitory effect on 5'-ectonucleotidase at a dose of 4 mg/kg about 1.5 hours after i.m. administration [27]. EHNA was used in the same dose as by Paul, Rothmann and Meagher, who reported an increase in erythropoietic activity in mice [38].

Second, following the application of EHNA, soluflazine and dipyridamole we found an increase in ADO levels in whole kidney homogenates (Table 2). Although this finding cannot prove the local activation of ADO receptors on EPO producing cells, it indicates that the three substances were effective in enhancing ADO availability in the kidney. Interestingly, the measurement of renal ADO concentrations also showed that there was no difference between ADO levels in normoxic animals and animals exposed to carbon monoxide, indicating that the effect of this type of hypoxia on ADO accumulation is different from renal ischemia, which was reported to result in a two- to sixfold increase of renal ADO levels [14, 15]. Since carbon monoxide is a very potent stimulus for EPO production, leading to an approximately 30-fold increase in serum hormone levels within the brief four hour period, the conclusion that this increase in EPO production is not mediated by a rise in ADO levels is supported further. As in a previous study [27] AOPCP, the inhibitor of 5'-ectonucleotidase, did not lead to a detectable reduction of basal tissue ADO levels, although when applied under exactly the same the conditions, AOPCP was found to inhibit the ischemia-induced rise in ADO concentrations [27]. The contribution of 5'-ectonucleotidase to ADO production may thus be much more significant under conditions of increased ADO production than under basal conditions.

Third, theophylline and ADO A1 and A2 receptor agonists and antagonists had no effect on hypoxia-induced EPO production, and were also ineffective in normoxic animals. In the short-term experiments we cannot exclude that any of the substances investigated inhibited the basal EPO production; taking into account the stability of EPO mRNA and EPO protein, a moderate inhibition may not become apparent within four hours. However, the unchanged EPO levels do indicate that the modulation of ADO does not mimick the hypoxic response.

Finally, the failure to observe an increase of hypoxia-induced EPO serum levels with any of the ADO synergistic substances was not due to the fact that stimulation by 0.075% CO was already maximal, because we found a further 2.5-fold increase of EPO levels under the same experimental conditions when the animals were treated, such as with the beta 2 adrenoceptor agonist salbutamol (data not shown).

In conclusion, considering the entirety of our findings obtained with direct and indirect adenosinergic stimulatory and inhibitory substances, it appears unlikely that an increase in ADO concentrations in the peritubular renal interstitium, where the EPO producing fibroblasts are located, mediates the large hypoxia-induced increase in production of the hormone or modulates it in an important fashion.

These findings are in contrast to previous work, which was interpreted to indicate an important role of ADO for EPO production [13]. In previous *in vivo* experiments, however, EPO was not directly measured, but ^{59}Fe incorporation was used as indicator of erythropoietic activity. In these experiments ADO increased ^{59}Fe incorporation and the ADO A1 receptor agonist CHA inhibited albuterol-stimulated ^{59}Fe incorporation [39]. This effect was blocked by high doses of theophylline (up to 80 mg/kg), which cannot be considered to block ADO receptors only [28].

In *in vitro* studies using the human hepatoma cell lines Hep G2 and Hep 3B, which produce EPO in an oxygen dependent fashion [40], the selective ADO A 1-receptor agonist CHA increased hypoxia-induced EPO secretion at concentrations of 10^{-5} and 5×10^{-5} M [41], but another selective ADO A-1 receptor agonist, cyclopentyladenosine inhibited [42], NECA (a non-specific ADO agonist [43]) stimulated [44] and CGS 21 680, a selective ADO A2 receptor agonist, had no effect on EPO secretion [45]. Apart from inconsistencies in these findings, the extent to which EPO production in these liver cell lines reflects regulation of the hormone in the kidney, which contributes the majority of EPO *in vivo*, remains unclear.

Bakris et al first reported [17] that in humans increased erythropoiesis after renal transplantation may be corrected by treatment with theophylline, and they observed a decrease in EPO levels in patients treated with theophylline. However, this has not been confirmed unequivocally by other investigators, who have found that in many patients theophylline is not effective [45, 46]. Noteworthy, our experiments found that theophylline not only had no acute effect on EPO levels (Fig. 1), but also had no effect on EPO and hemoglobin levels as well as hematocrit after seven days of treatment (Table 2). It is important to recognize that the mechanisms controlling EPO secretion in post-transplant erythrocytosis are unknown and may well be different from those operating in intact kidneys. In fact, in contrast to the findings by Bakris et al, others observed no effect of theophylline on EPO levels in normoxic healthy adults [47]. We also have recently found no effect of theophylline and dipyridamole on EPO production that was stimulated in healthy volunteers by phlebotomy [24] or exposure to hypobaric hypoxia [48].

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