Adenosine A<sub>2A</sub> and A<sub>2B</sub> receptor activation of erythropoietin production

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Received 15 March 2001; accepted in final form 27 June 2001

Fisher, James W., and Jesse Brookins. Adenosine A<sub>2A</sub> and A<sub>2B</sub> receptor activation of erythropoietin production. Am J Physiol Renal Physiol 281: F826–F832, 2001. First published July 12, 2001; 10.1152/ajprenal.0083.2001.—We have examined the effects of adenosine receptors and protein kinases A and C in the regulation of erythropoietin (Epo) production using hepatocellular carcinoma (Hep3B) cells in culture and in vivo in normal mice under normoxic and hypoxic conditions. CGS-21680, a selective adenosine A<sub>2A</sub> agonist, significantly increased levels of Epo in normoxic Hep3B cell cultures and in serum of normal mice under both normoxic and hypoxic conditions. CGS-21680 also produced a significant increase in Epo mRNA levels in Hep3B cell cultures. SCH-58261, a selective adenosine A<sub>2A</sub> receptor antagonist, significantly inhibited the increase in medium levels of Epo in Hep3B cell cultures exposed to hypoxia (1% O<sub>2</sub>). Enprofylline, a selective adenosine A<sub>2B</sub> receptor antagonist, significantly inhibited the increase in plasma levels of Epo in normal mice exposed to hypoxia. Chelerythrine chloride, an antagonist of protein kinase C activation, significantly inhibited hypoxia-induced increases in serum levels of Epo in normal mice. A model is presented for adenosine in hypoxic regulation of Epo production that involves kinases A and C and phospholipase A<sub>2</sub> pathways.

MATERIALS AND METHODS

Chemicals

Enprofylline and CGS-21680 were obtained from Sigma Chemical (St. Louis, MO). Penicillin G, streptomycin, L-glutamine, sodium pyruvate, nonessential amino acids, trypsin, Eagle’s minimum essential medium (EMEM), and Dulbecco’s phosphate-buffered saline were obtained from GIBCO-BRL (Life Technologies). Chelerythrine chloride was obtained from Alexis (San Diego, CA). SCH-58261 was provided by Schering-Plough Research Institute (Milan, Italy). All other chemicals were purchased from Sigma Chemical, with the exception of those specifically described.

In Vivo Epo Studies in Mice

Briefly, CD1 strain female mice were used in these studies. Enprofylline, a selective adenosine A<sub>2B</sub> receptor antagonist in saline, was administered intravenously in a single dose in a volume of 0.2 ml. Chelerythrine and 2-p-[2-carboxyethyl]phenethylamino-5'-N-ethylcarboxamidoadenosine (CGS-21680) were administered subcutaneously in 0.2 ml of saline. After the injection of one of the above compounds, or saline as the control, the mice were exposed to 2 or 4 h of hypoxia (0.42 atm) in a hypobaric chamber. After hypobaric stimulation, the mice were immediately anesthetized with ether and exsanguinated via cardiac puncture. Blood samples were collected via cardiac puncture in heparinized tubes, and the

F826
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plasma was separated by centrifugation. Epo levels in the plasma were determined by a sensitive Epo RIA. The details of the RIA used in our laboratory have been published previously (14). The mean basal level of Epo in our Hep3B cell culture medium in cells incubated for 24 h under normoxic control conditions for the five CGS-21680 experiments was 18.53 ± 5.0 mU/ml. The minimal detectable level of Epo in this assay was 1.56 mU/ml. Epo levels are expressed as milliunits per milliliter.

Hep3B Cell Cultures

Human Hep3B cells, obtained from the American Type Culture Collection (ATCC), were transferred to 75-cm² canted neck tissue culture flasks (Corning, Corning, NY). The cells were placed in a water-jacketed incubator (model no. 31580; Forma Scientific) and incubated in monolayer cell cultures in EMEM supplemented with 10% fetal bovine serum, 0.1 mmol/l nonessential amino acids, 1 mmol/l sodium pyruvate, penicillin G (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO₂-95% air at 37°C. For the CGS-21680 experiments, 7.6 × 10⁶ cells were transferred to each 75-cm² tissue flask, and, for the mRNA experiments, 15.0 × 10⁶ cells were transferred to each 75-cm² flask (Sarstedt). The cells were then incubated for 24 h in a normoxic (20% O₂-5% CO₂-75% N₂) atmosphere, after which several experiments were performed. For the SCH-58261 experiments, cells were transferred to each 75-cm² flask. Cytotoxicity testing of each of the Hep3B cell culture experiments was carried out utilizing the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) technique (4).

Isolation of RNA

Hep3B cells were incubated for 6 h under normoxic conditions in the presence or absence of the test substance. Total cellular RNA was isolated by use of the RNazol B method (TEL-TEST, Friendswood, TX). The isolated RNA was further purified by use of RQ1 RNase-free DNase (Promega, Madison, WI). The integrity of each RNA sample was verified by agarose-formaldehyde gel electrophoresis and quantified spectrophotometrically.

Quantitation of RNA

Quantitative RT-PCR was performed using the technique of Fandrey and Bunn (6), as previously described, but with some modifications. Total RNA (250 ng) in each sample was reverse transcribed by incubation with 1 unit of rTth DNA polymerase (PerkinElmer) per 10 μl of the reaction mixture, containing 100 μM deoxynucleotide triphosphate (dNTP; PerkinElmer), 25 pmol of Epo or β-actin downstream primer, 5 mM manganese acetate [Mn(OAc)₂], and 1 × EZ buffer (PerkinElmer) for 30 min at 60°C. The reverse transcription was terminated by placing the tube on ice. The resultant cDNA was used for PCR. Each sample was measured for Epo cDNA and β-actin cDNA in separate tubes with the use of specific primers. The sequence of Epo primers was kindly provided by Dr. Joaquin Fandrey (Univ. of Bonn, Bonn, Germany). These primers yielded a 253-base pair fragment (6). The upstream and downstream primers for β-actin were ATCTGGCACCACACCTCTACAATG and GGGGTGTTC-AAGGTCTCAAAAC, respectively, which yielded a 137-base pair cDNA fragment. PCR was performed by incubation of 250-ng/μl samples of cDNA with 5 mM Mn(OAc)₂, 200 μM dNTP, 1 unit/10 μl of reaction mixture rTth DNA polymerase, and 1 × EZ buffer (PerkinElmer) for 30 min at 60°C.
quantity of 30 b-actin primers were never combined in the same tube. A
ried out for 40 cycles under identical conditions. Epo and
Robocycler-40 system. PCR using the Epo primers was car-

ase, 1× EZ buffer, 1 μCi [32P]dCTP (ICN), and 25 pM of
b-actin primers and carried out for 35 cycles (30 s at 95°C, 30 s at 60°C, and 40 s at 72°C) with the use of the Strategene
Robocycler-40 system. PCR using the Epo primers was car-
p-actin primers were never combined in the same tube. A
quantity of 30 μl of the final PCR reaction was electrophoresed using 2% agarose (Promega) in 1× Tris-boric acid-
EDTA (TBE) buffer containing 0.36 μg/ml of ethidium bromide. The bands corresponding to the cDNA product were
excised and mixed with scintillation cocktail, and counts per
minute were determined on a Beckman beta counter. Epo
and b-actin cDNA obtained from PCR of the reverse transcribed RNA was used to generate standard curves. The
cDNA was amplified by PCR, and the resultant amplified product was divided into small fractions that were reamplified.
The purity of the final product was confirmed by elec-
trophoresis. If a single band of the appropriate size was
obtained, the final product was cleaned using a Wizard PCR
purification system (Promega) to remove primers. The
cleaned product was again electrophoresed to confirm that it
contained only the desired DNA. The cDNA was quantified
spectrophotometrically after purification. Standard curves
for Epo mRNA or b-actin mRNA (10⁻¹ to 10⁻⁷ μg/tube) were
prepared by simultaneously amplifying the appropriate samples of cDNA in separate tubes. Every PCR amplified in-
cluded a standard curve. All results are expressed as nano-
grams Epo cDNA per nanograms

Experimental Groups Studied

Experiment 1. Normal female CD1 mice were injected
subcutaneously with 0.25 ml saline, 10 mg/kg chelerythrine,
250 μM/kg enprofylline, or 0.1 μM/kg CGS-21680 (iv). One
hour after injection, the mice were placed in a hypobaric (0.42
atm) chamber for 2 and 4 h, respectively. Immediately after
being removed from the hypobaric chamber, the mice were
exsanguinated and the plasma was separated from the hep-
arinized blood and stored at −70°C before Epo RIA. The
effects of CGS-21680 (0.1 μM/kg iv) on plasma levels of Epo in
normal mice under normoxic conditions and after exposure
to 4-h hypoxia are shown (see Fig. 5). The effects of 250
μM/kg enprofylline on plasma levels of Epo in normal mice
after 2- and 4-h exposure to hypoxia are also shown (see
Fig. 6). The effects of chelerythrine at 10 mg/kg (ip) on plasma
levels of Epo in normal mice after exposure to 2- and 4-h
hypoxia are shown (see Fig. 7).

Experiment 2. The effects of CGS-21680 on Epo and Epo
mRNA production by Hep3B cells were studied. Hep3B cells
were incubated in 75-cm² flasks under normoxic conditions
until 100% confluency of the cells was reached. Five flasks
were used as controls (only EMEM added) and five flasks for
each of five concentrations (1×10⁻⁹ to 1×10⁻⁵ M) of
CGS-21680 under normoxic (10% O₂) conditions. Fifteen
flasks were set up for the SCH-58261 experiments under
hypoxic (1% O₂ for 24 h) conditions (5 flasks for controls, 5
flasks with 1×10⁻⁶ M SCH-58261, and 5 flasks with 1×10⁻⁵ M SCH-58261). Thereafter, the medium and cells were
harvested from the 20 flasks and used for quantitative de-
termination of Epo (RIA) and of Epo mRNA by RT-PCR.
Figure 1 shows the effects of SCH-58261 (1×10⁻⁶ and 1×10⁻⁵ M) on Hep3B cell medium levels of Epo after exposure
to hypoxia (1% O₂).

Figure 2 shows the effects of several concentrations of
CGS-21680 on Hep3B cell culture medium levels of Epo
under normoxic conditions. Figure 3 shows the effects of
CGS-21680 on Epo mRNA in Hep3B cell cultures under
normoxic conditions.

Figure 4 shows the electrophoretic gels of Epo mRNA from
Hep3B cell cultures after hypoxia (1% O₂) and CGS-21680.

RESULTS

As seen in Fig. 1, SCH-58261 (1×10⁻⁶ and 1×10⁻⁵
M), a selective adenosine A₂A receptor antagonist, sig-
ificantly (P < 0.05) inhibited the increase in medium
levels of Epo in Hep3B cell cultures exposed to hypoxia
(1% O₂) for 24 h.

As noted in Fig. 2, when Hep3B cells were incubated
with CGS-21680 (1×10⁻⁹ to 1×10⁻⁵ M) for 24 h
under normoxic conditions, a significant (P < 0.05)
increase in Epo levels was seen in the culture medium. CGS-21680 produced a significant \( P < 0.05 \) increase in Epo mRNA levels in Hep3B cell cultures under normoxic conditions (Fig. 3).

The electrophoretic gels illustrating the effects of CGS-21680 and hypoxia on Epo mRNA are shown in Fig. 4. As noted in Fig. 4, exposure of Hep3B cells to CGS-21680 for 6 h under normoxic conditions resulted in a significant increase in Epo mRNA levels in Hep3B cells compared with normoxic controls. Also note the marked increase in Epo mRNA seen after exposure to hypoxia.

As noted in Fig. 5, CGS-21680 at 0.1 \( \mu \text{mol/kg} \) (when injected intravenously 4 h before) and at 0.1 \( \mu \text{mol/kg} \) (at the time the mice were exposed to hypoxia) significantly \( P < 0.01 \) enhanced the effects of hypoxia on plasma levels of Epo compared with hypoxia controls. In addition, CGS-21680 significantly increased plasma levels of Epo in normoxic mice compared with normoxic controls (Fig. 5). These data indicate that adenosine \( A_{2A} \) receptor activation plays a significant role in Epo regulation.

As seen in Fig. 6, enprofylline, a selective adenosine \( A_{2B} \) receptor antagonist, at a dosage of 250 \( \mu \text{mol/kg} \), significantly \( P < 0.01 \) inhibited the rise in plasma levels of Epo in normal mice exposed to either 2 or 4 h of hypoxia. These data indicate that adenosine \( A_{2B} \) receptor activation after exposure to hypoxia is an important mechanism in the regulation of Epo production.

As noted in Fig. 7, chelerythrine (10 mg/kg), a selective PKC inhibitor, significantly \( P < 0.01 \) inhibited the increase in plasma levels of Epo in normal mice exposed to either 2 or 4 h of hypoxia.

**DISCUSSION**

Hypoxia is known to enhance the production of adenosine in endothelial cells (18). This increase in adenosine activates adenosine \( A_{2A} \) (10, 27) and \( A_{2B} \) (8, 23) receptors to initiate a cascade of events leading to several physiological responses. We have demonstrated previously that adenosine receptor activation enhances the effects of hypoxia-induced Epo production (19, 21, 27). In the present studies, we report that adenosine regulation of Epo occurs through the activation of both adenosine \( A_{2A} \) and \( A_{2B} \) receptors. We have
also found that SCH-58261, a selective adenosine A\textsubscript{2A} receptor antagonist, produces a significant inhibition of the rise in Epo levels in culture medium in Hep3B cells after exposure to hypoxia. CGS-21680, a selective adenosine A\textsubscript{2A} receptor agonist, was also found to produce a significant increase in culture medium levels of Epo and Epo mRNA in Hep3B cells under normoxic conditions and a significant increase in plasma levels of Epo in normal and hypoxic mice. Adenosine A\textsubscript{2A} receptor activation leads to the stimulation of adenylate cyclase, a rise in cAMP levels in cells, and an increase in A\textsubscript{2A} receptor mRNA (10). It is well known that A\textsubscript{2A} and A\textsubscript{2B} adenosine receptors are coupled to increasing G stimulatory (G\textsubscript{s}) proteins, and both have been demonstrated to activate adenylate cyclase in almost every cell that has been studied (8, 10, 22). We know that the activation of adenylate cyclase is an important signaling mechanism for A\textsubscript{2A} receptors, but adenylate cyclase may not be completely responsible for the important signaling mechanisms after A\textsubscript{2A} receptor activation (8).

We have also found in these studies that enprofylline, a selective adenosine A\textsubscript{2B} receptor antagonist,
produced a significant inhibition of the rise in plasma levels of Epo in mice exposed to hypoxia. It has been proposed that after A<sub>2</sub>B receptor activation, a significant stimulation of phospholipase C occurred in bone marrow-derived mast cells (13). In addition, the G<sub>p</sub> family of regulatory proteins (G proteins that activate phospholipase A<sub>2</sub>) has been postulated to play a significant role in the activation of A<sub>2</sub>B receptors, which are coupled to β-phospholipase C in human mast cells (7). In contrast, A<sub>2A</sub> receptors have not been found to stimulate phospholipase C (7). Adenosine A<sub>2B</sub> Receptor activation has also been found to activate adenylate cyclase to increase cAMP. We have found in the present studies that chelerythrine, a selective PKC inhibitor, produced a significant inhibition of the increase in Epo levels in culture medium of Hep3B cells and plasma levels of Epo in normal mice after exposure to hypoxia. Inhibition of Epo production by phorbol esters has been reported to be associated with down-regulation of PKCa isoenzyme in hepatoma cell cultures (9). It is possible that adenosine activation of A<sub>2A</sub> and A<sub>2B</sub> receptors is additive, in that it would appear from our previous studies (19, 21) that 5′-(N-ethylcarboxamidoadenosine) (NECA), a nonselective A<sub>2A</sub> and A<sub>2B</sub> receptor agonist, was much more potent in stimulating Epo production than CGS-21680. However, until a more selective A<sub>3</sub>B agonist is available, it will not be possible to test this hypothesis. It has been reported that A<sub>2A</sub> receptor-stimulated gene expression is regulated by the activation of adenosine A<sub>2A</sub> receptors through the stimulation of adenylate cyclase and an increase in the second messenger cAMP (25). It is also of interest that hypoxia has been reported to stimulate the expression of the adenosine A<sub>2A</sub> receptor gene in PC12 cells (10). In addition, studies in HeLa cell cultures treated with either a cAMP analog or a phorbol ester suggest that protein kinase A, but not PKC, is involved in oxygen sensing through the transcriptional factor hypoxia-inducible factor (HIF)-1 (11). Thus we postulate that both A<sub>2A</sub> and A<sub>2B</sub> adenosine receptors increase Epo mRNA through an increase in cAMP and HIF-1α. A receptor-mediated activation of phospholipase A<sub>2</sub> has been proposed (3). Agonists that provoke hydrolysis of inositol phospholipids liberate free fatty acids and lysophospholipids as well as arachidonic acid (5). Adenosine has been demonstrated in previous studies to be involved in phospholipase A<sub>2</sub> activation in biological responses (2, 17). We have reported previously that a cis-unsaturated free fatty acid, oleic acid, significantly enhanced 1-oleoyl-2-acetyl-rac-glycerol (OAG)-induced increases in medium levels of Epo in normoxic Hep3B cells, whereas a phospholipase A<sub>2</sub> inhibitor, mepacrine, significantly decreased hypoxia-induced increases in Epo production in Hep3B cells (29). We reported several years ago that eicosanoids significantly increased Epo production in vivo in mice (20). Cis-unsaturated fatty acids, including oleic, linoleic, and arachidonic acids, all of which are produced from phospholipids by the action of phospholipase A<sub>2</sub>, are known to enhance the effects of diacylglycerol (DAG) in the presence of calcium on PKC (26). We propose that an additional transcriptional factor(s), heretofore unidentified, may be important in the phospholipase C, phospholipase A<sub>2</sub>, and kinase C signal transduction pathways leading to an increase in Epo mRNA.

Our model for hypoxic regulation of Epo production is shown in Fig. 8. Hypoxia results in the depletion of ATP in cells. Hypoxia also increases ectonucleotidase activity in extracellular fluid (18), which breaks down ATP to adenosine. We propose that adenosine activates A<sub>2A</sub> and A<sub>2B</sub> receptors to stimulate adenylyl cyclase, increased cAMP, kinase A activation, increased phosphorylation of HIF-1α, and increased Epo mRNA. It has been reported that activating transcription factor-1 and cAMP-responsive element binding (CREB)-1 are the major constitutive nuclear factors binding to the HIF-1 DNA recognition site (11). A<sub>2B</sub> receptor activation also results in the stimulation of phospholipase C, which increases levels of inositol trisphosphate (IP<sub>3</sub>) and DAG (8). IP<sub>3</sub> increases intracellular calcium, which acts in concert with DAG to stimulate PKC. Kinase C activation causes phosphorylation of another transcriptional protein, which binds to a DNA domain on the Epo gene to increase Epo mRNA, and increased Epo production. We also propose that adenosine activation of a receptor linked to phospholipase A<sub>2</sub> leads to the production of cis-unsaturated fatty acids, which act in concert with DAG and calcium to stimulate PKC, leading to an increase in transcriptional proteins involved with Epo mRNA production. Our previous studies have indicated that adenosine activation of Epo production through the A<sub>2A</sub> receptor is probably through the cAMP pathway (19), which results in an increase in the effects of the transcriptional protein HIF-1α and perhaps an Epo mRNA binding protein (ERBP) (15). Further work is necessary to clarify the role of cAMP in this posttranscriptional regulation of Epo mRNA stability by ERBP. We have reported previously that with antisense oligonucleotide experiments, PKCa is involved in Epo production (15). The mechanism of Epo production is multifactorial and involves several signal transduction pathways.

This work was supported by funds from the Regents Professor in Pharmacology Fund.

REFERENCES


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ADENOSINE RECEPTORS ANDERYTHROPOIETIN


