17β-Estradiol decreases hypoxic induction of erythropoietin gene expression

HARSHINI MUKUNDAN, THOMAS C. RESTA, AND NANCY L. KANAGY
Vascular Physiology Group, Department of Cell Biology and Physiology, Health Sciences Center, University of New Mexico, Albuquerque, New Mexico 87131-5218

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Mukundan, Harshini, Thomas C. Resta, and Nancy L. Kanagy. 17β-Estradiol decreases hypoxic induction of erythropoietin gene expression. Am J Physiol Regulatory Integrative Comp Physiol 283: R496–R504, 2002. First published April 4, 2002; 10.1152/ajpregu.00573.2001.—Exposure to chronic hypoxia induces erythropoietin (EPO) production to facilitate oxygen delivery to hypoxic tissues. Previous studies from our laboratory found that ovariectomy (OVX) exacerbates the polycythemic response to hypoxia and treatment with 17β-estradiol (E2-β) inhibits this effect. We hypothesized that E2-β decreases EPO gene expression during hypoxia. Because E2-β can induce nitric oxide (NO) production and NO can attenuate EPO synthesis, we further hypothesized that E2-β inhibition of EPO gene expression is mediated by NO. These hypotheses were tested in OVX catheterized rats treated with E2-β (20 μg/day) or vehicle for 14 days and exposed to 8 or 12 h of hypoxia (12% O2) or normoxia. We found that E2-β treatment significantly decreased EPO synthesis and gene expression during hypoxia. E2-β treatment did not induce endothelial NO synthase (eNOS) expression in the kidney but potentiated hypoxia-induced increases in plasma nitrates. We conclude that E2-β decreases hypoxic induction of EPO. However, this effect does not appear to be related to changes in renal eNOS expression.

CHRONIC EXPOSURE TO HYPOXIA can cause a number of pathophysiological conditions including pulmonary hypertension and chronic mountain sickness. Hypoxic induction of erythropoiesis leads to polycythemia, which may contribute to the development of these diseases. Erythropoiesis is mediated by the small glycoprotein hormone erythropoietin (EPO; Refs. 3, 12). In adults, EPO is primarily synthesized in the kidney and stimulates red blood cell synthesis in bone marrow. During hypoxia, elevated red blood cell numbers help to compensate for compromised oxygen delivery to tissues.

The physiological and biochemical properties of EPO have been extensively studied, but there are few studies to investigate gender differences in regulation of the hormone. It has been demonstrated that 17β-estradiol (E2-β) can influence the expression of hypoxia-inducible genes such as vascular endothelial growth factor and endothelin-1 (ET-1) (4, 11, 58). However, estrogen regulation of EPO gene expression is not well understood. In some tissues such as the uterus and ovaries, E2-β has been shown to induce EPO production (34, 67). However, this regulation seems to be independent of pathways involved in hypoxic stimulation of EPO synthesis. In contrast, several investigators have shown that women living at high altitude have a lower hematocrit compared with men at the same altitude. Peschle et al. (40) observed that administration of estrogen benzoate (10 µg/day) to ovariectomized (OVX) rats attenuated increases in plasma iron following hypoxia. Studies from our laboratory (46) have also shown that OVX augments hypoxia-induced increases in hematocrit in rats (0.5 atm, 4 wk) and that E2-β prevents this augmentation. These findings are consistent with the hypothesis that E2-β decreases hypoxic induction of EPO gene expression.

One potential mechanism for E2-β regulation of EPO is through activation of nitric oxide (NO) synthesis. E2-β has been shown to induce NO production by both genomic and nongenomic mechanisms (1, 27). In addition, NO can reduce EPO gene induction during hypoxia (62). Therefore, we hypothesize that E2-β decreases EPO gene expression by increasing NO synthesis in OVX rats.

METHODS

All animal protocols were reviewed and approved by the University of New Mexico Institutional Animal Care and Use Committee.

Animal preparation. Female Sprague-Dawley rats (body wt 250–300 g; Harlan Industries) were anesthetized with a mixture of ketamine (100 mg/kg im) and acepromazine (1 mg/kg im). Ovaries were removed via bilateral laparotomy. Rats were allowed 2 wk for recovery and depletion of endogenous estrogen stores. After recovery, femoral arterial and venous catheters were surgically implanted, and mini osmotic pumps (Alzet model 2002) containing lipid-soluble E2-β (20 µg/24 h) or vehicle (polypropylene glycol) were implanted subcutaneously at the base of the neck (46). We have previously shown that this dose of E2-β yields plasma...
levels of the hormone of 102 ± 11 pg/ml in the E2-β-treated animals compared with <5 pg/ml in the vehicle-treated animals (17, 18). These levels are within the physiological range of circulating estrogen in the rat (5, 7, 30). One week after catheter surgery, rats were placed in a Plexiglas chamber (25 × 15 × 10 cm) that contained fresh bedding and were allowed to adapt to their new environment for 30 min before experimentation. After this adjustment period, the chamber was flushed with room air or a hypoxic gas (12% O₂) for 8 or 12 h. The percent oxygen in the chamber was monitored continually with an oxygen analyzer (model S-3A/1, Applied Electrochemicals). Plasma samples were collected at 0, 4, 8, and 12 h of exposure. Only animals with hematocrit ≥38% postcatheterization were used, because a decrease in hematocrit could induce EPO production. To avoid decreases in hematocrit during the experimental period, red blood cells were resuspended in saline and infused back into the animal after each sample collection. Uterine weight was measured as an indicator of E2-β delivery (14, 31, 39). At the final time point, blood gases were measured to confirm exposure conditions using a blood gas analyzer (model ABL-5, Radiometer).

In the 8-h exposure studies, kidneys were harvested from anesthetized animals (pentobarbital sodium; 30 mg/kg body wt ip) and frozen in liquid nitrogen for subsequent analysis of EPO gene expression. In the 12-h exposure studies, the right kidney was fixed and prepared for immunohistochemistry studies. The left kidney was frozen in liquid nitrogen for Western analyses and RNA extraction.

Radioimmunoassay. Plasma samples were collected through the arterial catheter at 0, 4, 8, and 12 h. EPO protein was assayed using a double-antibody RIA kit (DiaSorin). This kit is a competitive-binding, diisuequilibrium RIA utilizing recombinant human EPO for both tracer and standards and a goat anti-EPO primary antibody (EPO-trac). Human and rat EPO cDNA share >80% homology including some very highly conserved regions (66). This RIA, although based on human EPO, has been used for analysis of EPO from other species (26, 33) including rat plasma (22–24), and the antibody is 100% cross-reactive with rat EPO. The manufacturer (DiaSorin) reports a percent covariance of within-assay variability across 20 replicates as 9.8 for samples ranging in concentration from 11.1 to 254.6 mU/ml. We conducted assays in triplicate to minimize within-assay variability. For the same range of sample concentrations, the between-assay variability is reported by the manufacturer as percent covariance of 11.88. The between-assay variability for four separate assays (data in Fig. 1) was 21.68%. In addition, a plasma sample from a hypoxic rat was used as an internal control for interassay variability and to normalize data by dividing all samples by this value for each assay. The differences between groups were statistically significant with or without normalization. The minimum detectable concentration of EPO was 4.4 mU/ml and the maximum detection limit was 300 mU/ml. Linearity with dilution is reported by the manufacturer for samples from 2- to 100-fold dilution. We evaluated linearity in three rat plasma samples. Linearity was maintained with 10-fold dilution in these samples.

RNA isolation. Total RNA was extracted from the kidneys of rats treated as outlined using the TRIzol extraction method described elsewhere (36). Briefly, kidneys were homogenized, total RNA was extracted and precipitated with isopropyl alcohol, washed in ethanol, and resuspended in RNase- and DNase-free water. RNA quantity and quality were determined by spectrophotometry and agarose gel electrophoresis, respectively.

Real-time quantitative PCR. EPO mRNA levels were determined by one-step real-time RT-PCR. Probes and primer sequences were generated from the rat EPO sequence using Primer Express software. Total RNA was quantified in real time using a double dye-labeled fluorogenic oligonucleotide probe (53) and an automated fluorescence-based detection system. The probe was labeled 5’ with a fluorogenic reporter dye, 6-carboxy-fluoresceine (FAM), and 3’ with a quencher dye, 6-carboxy-tetramethylrhodamine (TAMRA, generated by PE Biosystems). The nucleotide sequence of the probe 5’-(FAM)-AAAGCCGCTCACCCTCGAACACTCA-(TAMRA)-3’ corresponds to segment 471–494 of rat EPO mRNA. The primer sequences specific for rat EPO cDNA are: forward primer: 5’-AAAGCCATACGTTGGGCATCTG-3’; and reverse primer: 5’-CCGGGAAGCTTGCAGAAGTA-3’. One-step RT-PCR was performed in 96-well optical plates and run on a TaqMan (ABI Prism 7700 Sequence Detection System, PE Applied Biosystems). Reverse transcription was carried out at 48°C for 30 min followed by a 40-cycle PCR. The PCR cycle was used was 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min. The reaction mixture contained magnesium chloride (5.5 mM), forward and reverse primers (10 μM each), probe (2.4 μM), dNTPs (100 μM each), RNase inhibitor (20 U/μl), AmpliTaq Gold DNA polymerase (5 U/μl), and mULV reverse transcriptase (50 U/μl). Data were analyzed using the sequence-detection system software program provided with the TaqMan. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was measured as an internal control using a rodent primer and probe kit. Although it has been previously shown that GAPDH mRNA is elevated upon exposure to hypoxia (69), we did not observe any change in expression under any of our experimental conditions. The GAPDH kit uses a different dye (Vic) as the fluorescent signal and TAMRA as the quencher. There was no difference in the amplification of GAPDH mRNA between experimental groups. All reagents were from PE Applied Biosystems unless otherwise stated.

Determination of plasma levels of NO by chemiluminescence detection. Plasma nitrites and nitrates (NOx) were measured in samples collected at 0, 4, 8, and 12 h using a Sievers 270B NO chemiluminescence analyzer (65). Plasma was diluted in a 1:10 ratio in PBS containing 5% dextran. Dilute sample (100 μl) was injected into the reaction chamber containing a solution of vanadium chloride in concentrated HCl at 90°C. The chamber also contained an antifoaming agent to minimize protein-induced frothing. NOx in the sample was reduced under these conditions and the NO generated was translated to voltage by a photomultiplier tube. Plasma NOx concentrations were calculated from a sodium nitrate standard curve. Final data are expressed as micromoles NOx.

Determination of renal NO synthase expression by Western blotting. Expression of all three NO synthase (NOS) isoforms has been previously shown that GAPDH mRNA is elevated upon exposure to hypoxia (69), we did not observe any change in expression under any of our experimental conditions. The Bradford method (Bio-Rad protein assay) and then separated in 15% polyacrylamide gels. Separated proteins were blocked and probed with a monoclonal antibody specific for one of the three NOS isoforms (1:1,000 dilution, Transduction Laboratories). In a parallel set of experiments, the proteins separated were blotted with a monoclonal antibody for α-actin (1:5,000 dilution, Transduction Laboratories). Enhanced chemiluminescence was used to visualize labeled protein and the relative quantity of protein was determined with Sigma-
Gel software. Densitometric units were quantitated by normalization to the standard run on the same gel and to the density of α-actin bands. Molecular weight markers and respective protein standards were used to confirm specific NOS isoform detection and to normalize between gels. In a separate experiment, 5, 10, 20, 30, 40, and 50 μg of protein/lane were analyzed to ensure linearity.

Fixation of kidneys for immunohistochemical analysis. Rat kidneys were fixed for immunohistochemistry by the paraformaldehyde perfusion technique described elsewhere (45). Briefly, rats were anesthetized with pentobarbital sodium (15 mg/kg iv), the chest was opened, and 0.1 ml of heparin was injected into the left ventricle. The ascending aorta was cannulated and the systemic circulation was flushed for 10 min at a flow rate of 48 ml/min with perfusion buffer [physiological saline solution containing bovine serum albumin (4%), papaverine (10⁻⁴ M), and heparin (0.3 ml/kg body wt)] and then for 10–25 min with a fixative solution [PBS containing paraformaldehyde (4%), glutaraldehyde (0.1%), and papaverine (10⁻⁴ M)]. Cross sections of kidneys (4–5 mm) were immersed in fixative solution for 4 h at room temperature. After fixation, slices were cryoprotected overnight in PBS containing 20% sucrose before immunostaining.

Immunostaining for renal endothelial NOS and EPO. Renal EPO and endothelial NOS (eNOS) protein expression were further analyzed by immunohistochemistry (18, 21). Rat kidney slices were placed in specimen molds containing embedding medium (Tissue-Tek OCT compound) and frozen in isobutane cooled with liquid nitrogen. Transverse sections (10 μm) were cut and thaw-mounted on glass (Superfrost Plus) slides. Sections were treated with 0.33% hydrogen peroxide to inhibit endogenous peroxidases, blocked with buffer containing both goat and horse serum (4% each), and incubated with a combination of a mouse monoclonal antibody for eNOS (1:1,000 dilution, Transduction Laboratories) and a rabbit polyclonal antibody for EPO (1:500 dilution, Santa Cruz Biotechnologies) for 1 h at room temperature followed by overnight incubation at 4°C. Sections were subsequently probed with a mixture of a rat-adsorbed, biotinylated horse anti-mouse IgG (1:400 dilution, Vector Laboratories) and human-adsorbed alkaline phosphatase-labeled goat anti-rabbit IgG (1:400 dilution, Stressgen) for 1 h at room temperature. Antibody-labeled proteins were stained using a Vector Blue staining kit (Vector Laboratories) for EPO followed by incubation with the peroxidase substrate 3,3′-diaminobenzidine tetrahydrochloride dihydrate (0.07%) in hydrogen peroxide (0.002%) for eNOS. Sections were dehydrated, cleared with a xylene-free clearing medium (Fisher Laboratories), and permanently mounted using Vectamount xylene-free mounting medium (Vector Laboratories). Nonspecific binding was evaluated by substituting total mouse IgG and rabbit serum for the primary antibodies. Densitometric analysis of immunocytochemical labeling was performed as described previously (44). Staining intensity is expressed in optical density (OD) units according to the calculation

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\text{OD} = -\log_{10}(\text{GL}_{\text{specimen}}/\text{GL}_{\text{white}})
\]

where GL_{specimen} is the gray level of the stained image and GL_{white} is the gray level of the image obtained from an area of the microscope slide absent of tissue. Dividing the numerator by GL_{white} compensates for uneven field illumination and for the OD contributed by the glass slide, Vectamount, and coverslip. Specific staining was defined as the difference in staining intensity of sections incubated with primary antibodies and staining intensity of mouse IgG and rabbit serum-exposed sections. NOS staining intensity was calculated as the average specific staining OD. Fifteen to twenty arteries were analyzed for each section and averaged to obtain a single n value. OD was calibrated using a stepped OD filter (Edmund Scientific). All measurements were made using green-wavelength illumination (×40 objective). Vessel images were generated with a Dage-MTI charge-couple device 72 video camera from a Nikon Optiphot microscope and processed with a MetaMorph Imaging System (Universal Imaging).

Data analysis and statistics. Data are reported as means ± SE. Two- or three-way ANOVA with Tukey’s post hoc test were used where applicable to compare between groups and treatments. Values for n represent numbers of animals. A P value of ≤0.05 was considered statistically significant.

RESULTS

Arterial blood gases, hematocrit, and uterine weight. Table 1 illustrates arterial blood gases after 8 h of hypoxic or normoxic exposure. Exposure to hypoxia caused a smaller decrease in arterial PO₂ in both vehicle- and E2-β-treated groups. In addition, arterial PCO₂ decreased and pH increased as anticipated with hypoxic stimulation of ventilation. E2-β has been shown to be a ventilatory stimulant (2). However, there were no significant differences in blood gas values between the two groups under either normoxic or hypoxic conditions, which suggests that ventilatory stimulation by E2-β did not mediate the observed effect on EPO synthesis.

Hematocrit was maintained during the exposure period by reinfluising red blood cells to prevent anemia-mediated induction of EPO. There was no significant decrease in hematocrit between the initial and final time points (Table 1).

Uterine weights were determined as an index of E2-β or vehicle treatment. Average uterine weight was significantly greater in E2-β-treated animals than in control animals (E2-β, 0.562 ± 0.011 vs. controls, 0.097 ± 0.005 g; n = 12 animals). Increased uterine weight has been previously used as an indicator of E2-β treatment (14, 31, 39).

Plasma EPO. Exposure to hypoxia caused a significant increase in plasma EPO in both the E2-β-treated and vehicle-treated groups (Fig. 1). A significant increase was seen after 4 h of hypoxia with a further increase after 8 h of hypoxia in the vehicle-treated group. However, there was no further increase in the E2-β-treated group at 8 h compared with 4 h, and

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Values are means ± SE; n = 6 animals/group. *P < 0.05 vs. normoxic counterparts.
plasma levels of EPO were significantly lower in the E2-β-treated compared with vehicle-treated rats at this time point (Fig. 1).

To evaluate whether E2-β persistently decreases or only delays the onset of EPO synthesis, animals were exposed to 12 h of hypoxia and plasma EPO levels measured by RIA. We found that exposure to 12 h of hypoxia did not further increase plasma EPO in either group. Rather, the difference in plasma levels between the E2-β-treated and vehicle-treated groups was maintained (Fig. 1). There were no changes in plasma EPO levels during normoxia.

Renal EPO mRNA. To determine whether E2-β attenuation of EPO synthesis was mediated at the level of gene expression, EPO mRNA levels were determined in kidneys from animals treated with E2-β or vehicle and exposed to hypoxia or normoxia. GAPDH mRNA was used as an internal control. It has been previously reported that GAPDH mRNA is induced upon hypoxic exposure and estrogen treatment (47, 48). However, we did not observe any difference in gene expression under any of our experimental conditions. Real-time PCR analysis revealed that exposure to 8 h of hypoxia significantly increased EPO mRNA in both the E2-β- and vehicle-treated groups (Fig. 2). However, the increase was significantly greater in vehicle-treated animals compared with those treated with E2-β. Renal EPO mRNA levels were lower after 12 h of hypoxia compared with 8 h of hypoxia, although they were still significantly elevated compared with normoxic groups. These results are consistent with plasma EPO data and previous observations in the literature (29, 54).

Plasma NOx. Treatment with E2-β can induce NOS gene expression in a number of organs including the kidney (1, 18). Also, E2-β can directly stimulate NOS activity, thereby increasing NO availability (27). To examine whether E2-β treatment increases NO synthesis, plasma levels of NOx were determined by chemiluminescence detection. Interestingly, we found that hypoxia elevated plasma NOx in both E2-β- and vehicle-treated groups exposed to hypoxia (Fig. 3). This hypoxic increase was further augmented by E2-β treatment, although no significant effect of E2-β was observed in normoxic rats. Exposure of animals to 12 h of hypoxia did not cause any further increase in plasma NOx in either group. However, plasma NOx remained elevated in both groups compared with the normoxic counterparts. These data suggest that E2-β treatment increases NO release but do not evaluate the source of the elevated NO production. Therefore, Western blotting was employed to determine whether E2-β upregulates expression of renal NOS isoforms.

Western blotting for renal NOS isoforms. To further investigate the hypothesis that E2-β-induced NO production mediates the attenuation of EPO induction, expression levels of all three NOS isoforms in the kidney were analyzed by Western blotting. However, we did not detect either the inducible or neuronal isoforms of NOS (iNOS and nNOS, respectively), although we did detect the respective positive controls (rat brain homogenates for nNOS and macrophage lysate for iNOS, Transduction Laboratories; lung homogenate from a lipopolysaccharide-treated rat was used as a second positive control for iNOS). In all kidney homogenates, eNOS was detected. However, we did not observe any difference in protein expression between groups (Fig. 4A). This was further evaluated by immunohistochemistry.

Immunohistochemistry for renal eNOS and EPO. E2-β regulation of eNOS expression in the kidney was further evaluated by immunohistochemistry. The double-staining technique also evaluated the relative proximity of EPO- and eNOS-expressing cells in the kidney.

Fig. 1. Normalized plasma erythropoietin (EPO) concentrations from vehicle- and 17β-estradiol (E2-β)-treated animals exposed to hypoxia (12% O2) or normoxia for 0, 4, 8, or 12 h as measured by RIA. E2-β treatment decreased hypoxia-induced increases in plasma EPO. *P ≤ 0.05, significantly different from 0-h values; #P ≤ 0.05, significantly different from normoxic group at that time point; †P ≤ 0.05, significantly different from vehicle; n = 8 animals/group.

Fig. 2. EPO mRNA levels in kidneys from vehicle- and E2-β-treated animals exposed to hypoxia (12% O2) or normoxia for 8 or 12 h. EPO mRNA was quantitated by real-time PCR using glyceraldehyde-3-phosphate dehydrogenase mRNA as internal control. E2-β treatment decreased induction of EPO gene expression during hypoxia. *P ≤ 0.05, significantly different from normoxic groups; #P ≤ 0.05, significantly different from vehicle-treated groups; †P ≤ 0.05, significantly different from the 8-h time point; n = 6 animals/group.
Kidney eNOS was detected by staining with 3,3′-diaminobenzidine tetrahydrochloride dihydrate with peroxide, whereas EPO was detected with Vector-blue alkaline phosphatase staining (Fig. 5). Specific staining for eNOS was observed primarily in endothelial cells of renal arteries. However, consistent with the Western analyses, there was no induction of eNOS expression with E2-β treatment (Fig. 4B). On the contrary, eNOS expression tended to be lower in both normoxic and hypoxic E2-β-treated groups compared with the respective vehicle controls, although this difference was only significant between normoxic groups.

EPO staining was present in proximal tubule cells in the cortico-medullary border (Fig. 5A). Minimal EPO staining was observed in sections from animals exposed to normoxia, but staining intensity was markedly increased in sections from 12-h hypoxic rats. Consistent with plasma EPO and renal EPO mRNA data, staining appeared to be less in sections from E2-β-treated animals compared with vehicle-treated rats after hypoxia (Fig. 5B).

**DISCUSSION**

Previous studies from our laboratory and others led us to hypothesize that E2-β decreases hypoxic induction of EPO in rats by increasing NO production. The major findings from this study are 1) E2-β decreased hypoxic induction of plasma EPO and renal EPO mRNA expression in OVX rats (see Figs. 1 and 2); 2) exposure to hypoxia increased plasma NOx, and this induction was further augmented by E2-β treatment (see Fig. 3); and 3) E2-β did not increase immunoreactive renal eNOS (see Fig. 4A).

Immunohistochemistry studies confirm that hypoxic induction of EPO was diminished in tissues from rats treated with E2-β and that there was no increase in renal eNOS protein expression with either E2-β treatment or hypoxia (Figs. 4B and 5).

Reports on estrogen regulation of EPO synthesis during hypoxia are controversial. As early as 1941, Vollmer and Gorden (referenced in 20) observed that OVX increases the rate of erythropoiesis in rats. More recently, it was demonstrated that hypoxia-induced increases in hematocrit are greater in male Hilltop Sprague-Dawley rats compared with their female counterparts (38). Moore et al. (35) have shown a decreased polycythemic response to hypoxia in female rats compared with males. We have also observed that OVX exacerbates hypoxia-induced increases in hematocrit and that E2-β treatment inhibits this effect of OVX (46). In contrast, others have demonstrated (41) that estrogen does not affect or actually enhances the polycythemic response to hypoxia. The purpose of this study was to determine whether estrogen regulates hypoxic induction of red blood cell synthesis as evidenced by changes in EPO synthesis. These data indicate that E2-β inhibits EPO induction during hypoxia by decreasing gene expression.

There are several possible mechanisms by which E2-β may inhibit EPO gene expression during hypoxia. One is through increased NO synthesis. It has been previously shown that all three NOS isoforms are expressed in the kidney (63) and that E2-β can induce NOS gene expression in the kidney (42, 43) and other organ systems (32, 56). Furthermore, E2-β has been shown to acutely increase eNOS activity that results in
elevated production of NO (1, 27). However, others have shown (49, 64) that E2-/H9252 treatment does not affect expression of renal NOS isoforms. NO regulation of EPO synthesis is also much debated. NO has been reported to attenuate EPO gene expression under multiple conditions (20, 57), although some investigators have demonstrated that NO can induce EPO synthesis during hypoxia in Hep3B cells (37, 68). We therefore hypothesized that E2-/H9252 increases NO production to attenuate EPO gene expression. However, Western analyses and immunohistochemistry studies demonstrate a tendency for renal eNOS protein expression to be decreased, not increased, with E2-/H9252 treatment. Given that NO induces EPO production during hypoxia (37), it is possible, although speculative, that decreased eNOS expression during E2-/H9252 treatment contributes to decreased EPO synthesis during hypoxia. In any case, these results clearly indicate that E2-/H9252-mediated attenuation of EPO synthesis does not require increased renal eNOS protein expression.

Hypoxic induction of EPO gene expression is mediated by the transcription factor hypoxia inducible factor-1 (HIF-1). Although several investigators have shown that NO attenuates HIF-1 activity/stability during hypoxia thereby decreasing expression of hypoxia-induced genes (57), other studies suggest that HIF-1α is stabilized in the presence of NO (28, 50, 51). Therefore, it is possible that the apparent decrease in renal eNOS protein expression contributed to downregulation of HIF-1-dependent EPO synthesis in the estrogen-treated rats.

Estrogen has also been shown to stimulate eNOS activity by nongenomic mechanisms (21). Indeed, in the current study, plasma NOx was increased after hypoxia, and this effect was augmented by E2-/H9252 treatment. Therefore, it appears that E2-/H9252 augments hypoxia-induced NO production and this augmentation correlates with the decrement in EPO synthesis. However, it is not clear whether E2-/H9252-induced NOx is responsible for attenuation of EPO gene expression during hypoxia.

The present study examined E2-/H9252-induced NO as one potential mechanism by which E2-/H9252 may decrease EPO gene expression during hypoxia. However, there are other mechanisms by which this might occur. A recent study from our laboratory demonstrates that E2-/H9252 reduces HIF-1-mediated ET-1 gene expression (11). Because hypoxic regulation of both ET-1 and EPO is mediated by HIF-1, it is possible that E2-/H9252 regulates both genes directly through interaction with the common factor, HIF-1. Alternatively, E2-/H9252 may interfere with EPO gene expression by directly binding to the steroid hormone response element present in the EPO promoter (25).

Previous studies have shown that E2-/H9252 and other ovarian hormones function as ventilatory stimulants. It has been observed that alveolar ventilation and hypoxic ventilatory responses are greater in females than in males and such differences have been attributed to ovarian hormones (2). It has also been speculated that physiological levels of female hormones act peripherally to raise carotid body chemosensitivity...
(60), although the combined administration of estrogen and progesterin is a more effective stimulus for increased ventilation (15, 59). To evaluate whether E2-β administration stimulated ventilatory responses in our experiments, arterial PO2, PCO2, and pH were measured in vehicle- and estrogen-treated animals at the end of each experiment (see Table 1). We did not observe any apparent ventilatory stimulation by estrogen, and thus a decrease in the degree of hypoxemia could not account for the reduced EPO synthesis in the E2-β-treated group.

Recent studies by Masuda and others (10, 34) have shown that EPO is synthesized in a number of organs outside the liver and kidney. Furthermore, EPO synthesis is under tonic regulation by E2-β in some of these organs (34, 52, 53). In the uterus and ovaries, E2-β is required for hypoxic induction of EPO, which promotes angiogenesis (8). However, in the same study, E2-β did not affect hypoxic induction of EPO in the murine kidney. Although the results from this study are in contrast to ours, it must be noted that the experimental conditions in the two studies were very different. The mouse study examined the effect of acute estrogen treatment on renal EPO synthesis during hypoxia. The mice were given a bolus of estrogen 30 min before the hypoxic exposure, and the experiment was designed to compare estrogen induction of EPO in the kidney with that in the ovaries. Therefore, it is difficult to tell whether chronic estrogen treatment affects EPO mRNA synthesis in mice because that was not reported (53). These studies do suggest that more than one pathway regulates EPO production and that EPO, in turn, may regulate multiple physiological conditions. Furthermore, E2-β may regulate EPO synthesis in an organ-dependent and/or a species-dependent manner.

In the present study, a whole-animal model was used to examine the effect of E2-β on EPO gene expression. The results were verified by several techniques, one of which was immunohistochemical analysis. EPO protein was immunolabeled in proximal tubule cells of the rat kidney. However, the site of EPO synthesis within the kidney is much debated. Earlier studies have suggested multiple sites of EPO synthesis including the glomerulus and distal tubules. More recently, several investigators demonstrated EPO mRNA localized only in peritubular capillary beds of hypoxic kidneys (9, 13). Still others have demonstrated EPO synthesis in interstitial cells of peritubular capillaries (16). In our study, staining clearly demonstrated specific immunoreactivity only in proximal tubule cells of rat kidneys as confirmed by hematoxylin-eosin staining in parallel sections. There was no EPO staining in serum controls and little staining in normoxic sections, which suggests that the staining is indeed EPO specific. However, it is possible that the protein staining is an artifact of filtration rather than EPO synthesis, and several investigators have considered this point in evaluating EPO synthesis within the kidney. However, similar to our studies, both EPO protein and mRNA have been localized in renal proximal tubule cells (19, 55). Therefore, the probability that this is the site of EPO synthesis cannot be excluded either. In conclusion, the exact site(s) of EPO synthesis in the kidney is not clear, although it is interesting to note that several other secretory products including angiotensinogen and vitamin D3 are synthesized in proximal tubule cells (6), which indicates that these cells can act as sites of hormone synthesis (61).

In conclusion, in vivo hypoxic induction of EPO is decreased by E2-β. This finding is of significance because EPO induction and consequent polycythemia may contribute to the pathology of diseases like chronic mountain sickness and pulmonary hypertension. Therefore, estrogen attenuation of EPO induction may underlie some gender differences in the development of pulmonary diseases, and estrogen status might be important in the systemic response to hypoxia.

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