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Estrogen Modulates Xanthine Dehydrogenase/Xanthine Oxidase Activity by a Receptor-Independent Mechanism

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ABSTRACT

Hypoxia causes up-regulation and activation of xanthine dehydrogenase/xanthine oxidase (XDH/XO) *in vitro* and in the lungs *in vivo*. This up-regulation, and the likely corresponding production of reactive oxygen species, may underlie the pathogenesis of an array of disorders. Thus, compounds that prevent hypoxia-induced increase in XDH/XO activity may provide a therapeutic strategy in such disorders. The antioxidant properties of estrogens have been demonstrated in several studies. However, the effect of these compounds on XDH/XO has not been explored previously. The aim of this study was to investigate the effects of estrogen on hypoxia-induced increase in XDH/XO activity. Rat pulmonary artery microvascular endothelial cells were exposed to normoxia or hypoxia in the presence or absence of 17β- or 17α-estradiol. The XDH/XO enzyme and gene promoter activities were measured in different groups of cells. Hypoxia caused a twofold increase in XDH/XO enzymatic and promoter activity. Either of the estradiol stereoisomers prevented the hypoxia-induced increase in XDH/XO enzymatic activity, but not the promoter activity. ICI 182,780, an antagonist of the estrogen receptor, failed to block the inhibitory effect of estradiol on XDH/XO. In conclusion, 17α- and 17β-estradiol modulate the hypoxia-induced regulation of XDH/XO activity at a posttranscriptional level by a receptor-independent mechanism. *Antioxid. Redox Signal.* 5, 705–711.

INTRODUCTION

ESTROGEN, THE FEMALE SEX HORMONE, has been found to exert an atheroprotective effect through mechanisms that are not fully elucidated (46). Estrogen deficiency in rats results in an increase in vascular free radical production and enhanced angiotensin II-induced vasoconstriction, leading to endothelial dysfunction (44). Several studies have demonstrated an antioxidant effect of estrogens *in vivo* and *in vitro* (2, 37, 38, 43, 47). Other studies have suggested both a prooxidant and an antioxidant role for estrogens (23, 26, 42). An attenuation of reactive oxygen species (ROS)-induced damage may partly explain the effects of this hormone on vasculature (46). Antioxidant actions of this hormone have also been proposed to explain its protective effect on neuronal cells (4, 14). Finally, estrogen replacement has been hypothesized to confer protection against ROS-related diseases such as Alzheimer's and Parkinson's diseases (15).

Xanthine dehydrogenase/xanthine oxidase (XDH/XO) is a potent source of free radicals, and has been pathogenetically implicated in various disorders, such as ischemia–reperfusion, radiation injury, and the adult respiratorydistress syndrome (ARDS) (27). XDH/XO, a purine catabolizing enzyme system, catalyzes the oxidation of hypoxanthineto xanthine and xanthine to uric acid (17). Under certain conditions, XDH/XO can generate superoxide and hydrogen peroxide when it utilizes

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molecular oxygen (O_2, the preferred electron acceptor for the xanthine oxidase form) instead of NAD^+ (the preferred electron acceptor for the xanthine dehydrogenase form) (1). Our laboratory has demonstrated an up-regulation and activation of XDH/XO in response to hypoxia *in vitro* and *in vivo* (18, 19, 24, 49).

The effects of estradiol on the XDH/XO system have not been examined. We aimed to study the modulatory effects of 17β-estradiol and its nonreceptor binding stereoisomer, 17α-estradiol, on the hypoxia-induced up-regulation of XDH/XO in cultured rat pulmonary microvascular endothelial cells (RPMEC). Our findings indicate that estradiol can block the activation of XDH/XO by hypoxia. The fact that 17α-estradiol also blocked the hypoxia-induced activation of XDH/XO suggests that this nonestrogenic compound might have antioxidant properties.

MATERIALS AND METHODS

Materials and reagents

RPMI 1640, phosphate-free Dulbecco's modified Eagle's medium, fetal bovine serum, dialyzed fetal bovine serum, penicillin G potassium, streptomycin, fungizone, and glutamine were obtained from Invitrogen–Life Technologies (Carlsbad, CA, U.S.A.). EDTA, Tris, 17β-estradiol, 17α-estradiol, ICI 182,780, and dithiothreitol were from Sigma (St. Louis, MO, U.S.A.).

Cell culture and exposure to hypoxia

RPMEC were a gift from Dr. Una Ryan (Avant Immunotherapeutics, Needham, MA, U.S.A.) and were cultured as previously described (12). For estradiol exposure, 1 mM stock solutions of 17α- and 17β-estradiol were made in ethanol. Required dilutions were performed in ethanol. Corresponding concentrations of ethanol were used in control cells. For hypoxic exposure, cells were placed in humidified airtight incubation chambers (Billups-Rothenberg, Del Mar, CA, U.S.A.) and gassed with 3% O_2, 5% CO_2, and balance N_2. The hypoxic chambers were kept in a 37°C incubator for the duration of the experiment. Normoxic cells were kept in a tissue culture incubator maintained at 5% CO_2 and 37°C.

Xanthine oxidase activity measurements

The activities of xanthine dehydrogenase and xanthine oxidase in response to different treatments were assayed using a slight modification of a fluorimetric assay that measures both xanthine oxidase and xanthine dehydrogenase activities (3). The principle of the assay involves the conversion of pterin into the fluorescent product isoxanthopterin. The rate of product formation with oxygen as the electron acceptor represents the activity of xanthine oxidase, and the combined activities of xanthine oxidase and xanthine dehydrogenase are measured with methylene blue as the electron acceptor. In brief, cells were washed once in phosphate-buffered saline, then scraped off the plate in 50 m*M* sodium phosphate (pH 7.4), 1.5 mg/ml dithiothreitol, and 1× Protease Inhibitor Cocktail 3 (Calbiochem, San Diego, CA, U.S.A.). The cells were sonicated for 5 s, and centrifuged at 10,000 *g* for 5 min. The supernatant was collected and assayed immediately, or stored at −80°C overnight.

XDH/XO promoter activity in endothelial cells

A DNA construct containing a 6,000-bp fragment of the rat XDH/XO promoter driving the expression of firefly luciferase was introduced into RPMEC by electroporation. As transient transfections of this construct were highly variable, and precluded consistent quantitative assessment of the effect of different treatments on promoter activity, we generated stably transfected cells. The stable transfectants were produced by cotransfection of the promoter–reporter construct with pcDNA3.1 (1:10 DNA ratio), which conveys to cells resistance to Geneticin. One day after cotransfection, the cells were treated with Geneticin. A week later, colonies formed by Geneticin-resistant cells were individually trypsinized, passed into 96-well dishes, and maintained in Geneticin-containing media. After two more passages, 35-mm dishes derived from each colony were assayed for luciferase activity. Out of several clones screened, two clones that express a significant baseline luciferase activity were obtained. These clones were further expanded, and used to study the effect of hypoxia and 17α- or 17β-estradiol treatment on the XDH/XO promoter activity. The firefly luciferase activity was assayed using a kit from Promega (Madison, WI) according to the manufacturer's instructions. In brief, cells were lysed and the substrate (beetle luciferin) was added to the lysate. Next, chemiluminescence was measured using a luminometer.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), western blotting, and immunoprecipitation

Aliquots from the cell lysates prepared as described above were assayed for protein using the Bradford protein assay (6) and then diluted with 2× Laemmli loading buffer for SDS-PAGE (25). Equal amounts of protein were then loaded in each well of 4–20%Tris/glycine gels. After electrophoresis for 90 min at 125 V constantvoltage, the gel was blotted onto an Immobilon-P membrane by electrophoretictransfer at 25 V constant voltage overnight. The membrane wasthen washed, blocked with 5% milk, and probed with antibodiesagainst xanthine oxidase (LabVision). The immunoreactive bands were visualized using a secondary antibody conjugated to horseradishperoxidase and a chemiluminescent substrate according to the manufacturer'sinstructions (SuperSignal, Pierce-Rockford, IL). The intensity of the bandswas quantified using a Molecular Dynamics densitometer and ImageQuant software.

Statistical analysis

The values plotted in all figures are means, and the error bars reflect the standard deviation from the mean. Statistical analysis was carried out using SPSS (SPSS, Inc, Chicago, IL, U.S.A.). Student's *t* test was used to determine if differences between control and estradiol-treated groups were statistically significant. Analysis of variance was used when more than two groups were compared. Statistical significance was considered at $p < 0.05$.

RESULTS

Hypoxia increases XDH/XO enzymatic activity and XDH/XO promoter activity in RPMEC

A significant (twofold, $p < 0.0001$) increase in XDH/XO enzymatic activity occurred in response to a 24-h exposure to hypoxia (Fig. 1A). This is consistent with our previous studies, which demonstrated an increase in XDH/XO activity after exposure to hypoxia in vitro and in vivo (18, 19, 24, 49). To determine if transcription contributed to the increase in enzyme activity, we tested the effect of hypoxia on the XDH/XO gene promoter. RPMEC were stably transfected with DNA constructs containing 6,000 bp of the XDH/XO gene promoter driving the expression of firefly luciferase, and then exposed to hypoxia for 24 h. The exposure of RPMEC to hypoxia caused a significant (2.4-fold, $p < 0.0001$) increase in XDH/XO promoter activity (Fig. 1B).

Hypoxia increases the expression of estrogen receptor (ER) β, but not ERα in RPMEC

Many of the effects of estradiol are mediated via the two known ERs, ERα and ERβ. We used immunoblotting techniques to detect the presence of either of these receptors in RPMEC, and to study possible alterations after exposure to hypoxia. Immunoblotting with specific antibodies against the α or β forms of the protein failed to detect any ERα receptor isoform in RPMEC. The ERβ isoform protein was present in normoxic cells and increased significantly in response to a 24-h exposure to hypoxia (Fig. 2).

17α- and 17β-estradiol attenuate hypoxia-induced XDH/XO activity in RPMEC

To study the effect of estradiol on hypoxia-induced increase in XDH/XO activity, RPMEC were exposed to different concentrations of 17α- and 17β-estradiol prior to a 24-h exposure to hypoxia. Both 17α-estradiol (Fig. 3A) and 17β-estradiol (Fig. 3B) caused attenuation in hypoxia-induced increase in XDH/XO activity. 17β-estradiol had no effect on baseline XDH/XO activity. The attenuation was seen at all doses tested (10^{-10}–10^{-7} *M*), although the effect was not dose-dependent ($p < 0.05$ for all tested doses of either estradiol).

17α- and 17β-estradiol do not inhibit XDH/XO gene promoter

To determine if the attenuation of hypoxia-induced increase in XDH/XO transcription was one of the underlying mechanisms for the decrease in XDH/XO activity, we treated XDH/XO promoter constructs with 17α- or 17β-estradiol before exposure to hypoxia for 24 h. Hypoxia caused an increase in XDH/XO promoter activity. However, the presence of estradiol, 17α- or 17β-, did not attenuate this increase, suggesting that the effect of estradiol is occurring at a post-transcriptional level (Fig. 4).

17β-Estradiol-induced modulation of XDH/XO activity is not receptor mediated

To determine if the inhibitory effect of 17β-estradiol on hypoxia was mediated through ERs, we repeated the experiments in the presence of ICI 182,780 (10 μ*M*), an ER antagonist. There was no change in the effect of 17β-estradiol on XDH/XO activity with the addition of ICI 182,780 for the duration of the experiment (Fig. 5). These results are consistent with the fact that 17β-estradiol, which binds the ER, and 17α-estradiol, which does not bind the ER, both resulted in a similar blockade of the activation of XDH/XO by hypoxia.

DISCUSSION

In this study, we have demonstrated that both 17α- and 17β-estradiol cause an attenuation of the expected increase in XDH/XO activity in response to hypoxia. This effect does not appear to be mediated by the traditional ERs. Our results also demonstrate that hypoxia can induce the expression of ERβ.

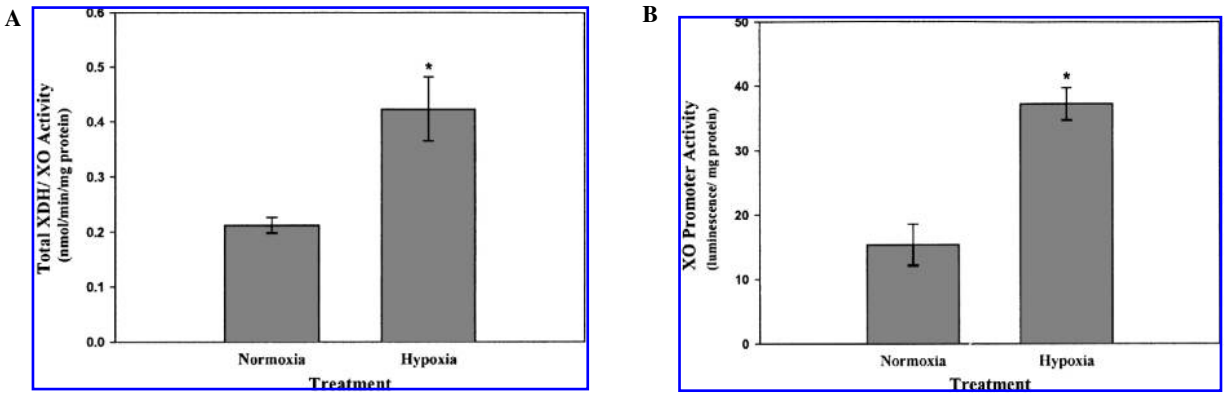

FIG. 1. Hypoxia causes up-regulation of XDH/XO in RPMEC. (**A**) Exposing RPMEC to hypoxia for 24 h causes a twofold increase in total (XDH/XO) activity. *$p < 0.05$ versus normoxia. (**B**) Hypoxia causes an increase in XDH/XO promoter activity. To study the changes in XDH/XO promoter activity, RPMEC were transfected with 6,000-bp DNA construct of rat XDH/XO promoter driving the expression of firefly luciferase. The resulting luminescence was measured using luciferase assay and used as an indicator of XDH/XO promoter activity. Exposure of these cells to hypoxia for 24 h causes a 2.4-fold increase in XDH/XO promoter activity. *$p < 0.05$ versus normoxia.

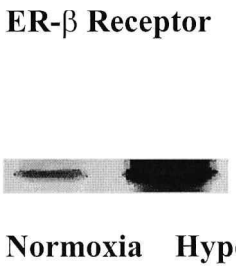

FIG. 2. Effect of hypoxia on the ER. RPMEC were exposed to hypoxia for 24 h, and the ER protein level was determined by immunoblotting with specific antibodies against the α or β forms of the protein. Whereas the ERα receptor isoform was not detectable in RPMEC, the ERβ isoform protein was present in normoxic cells and significantly increased in response to hypoxic exposure.

Estrogens have been used as therapeutic agents for a variety of medical disorders. Postmenopausal estrogen replacement is associated with a decrease in the risk of neurological disorders such as Alzheimer's and Parkinson's diseases (15, 48). Experimental evidence suggests a possible role for estrogens in acute ischemic central nervous system injury (35). Recent studies support a role for these compounds in attenuation of experimentally induced pulmonary hypertension in animal models, as well as treatment of perinatal pulmonary hypertension (13, 31, 36). Diverse mechanisms have been proposed to explain the salutary effects of estrogen. Based on our observations, we believe that modulation of the XDH/XO enzyme system may play a role in the therapeutic properties of estrogen.

XDH/XO is a complex molybdoflavoenzyme that is the terminal enzyme of human purine catabolism (17). XDH/XO is believed to be involved in the pathogenesis of diseases such as systemic hypertension (29), pulmonary hypertension (20, 41), ischemia–reperfusion injury (16, 33), and ARDS (34). Therefore, factors that modulate the metabolism and activity of XDH/XO may play a role in the pathogenesis or therapy of these disorders. Work from our laboratory and others have shown that hypoxia up-regulates XDH/XO at transcriptional, translational, as well as posttranslational levels (17). This enzyme, and particularly the oxidase form, can generate ROS such as superoxide and hydrogen peroxide, which can cause direct cell injury (17). Alternatively, xanthine oxidase-derived superoxide can react with nitric oxide to form peroxynitrite, a potent oxidant.

Hypoxia is a common physiological response to various cardiac, pulmonary, and vascular disorders, and an increase in XDH/XO may be one of the plausible mechanisms through which it exerts its deleterious effects. This hypothesis is supported by the experimental evidence that hypoxia–induced increase in XDH/XO can contribute to ischemia–reperfusion injury (21). Increased plasma levels of XDH/XO have been reported in patients subjected to ischemia–reperfusion induced by aortic cross-clamp procedures (40) or liver transplantation (32). Further experimental support for the pathogenetic role of XDH/XO in hypoxic conditions comes from the observation that perfusion of isolated rat lung with the effluent of ischemic–reperfused liver causes pulmonary microvascular permeability, a phenomenon that is inhibited by the XDH/XO inhibitor allopurinol (45). In addition, antioxidants and free radical scavengers alleviate XDH/XO-induced acute lung injury (9). We speculate that alleviation of oxidative stress through inhibition of hypoxia-induced activation of XDH/XO may be partly responsible for the suggested protective, as well as a therapeutic, role of estrogens in these and other disorders characterized by hypoxia. Furthermore, inhibition of XDH/XO improves endothelial dysfunction in hypercholesterolemic rabbits (11), as well as in human subjects with type 2 diabetes and mild hypertension (8).

The modulation of XDH/XO by both 17β- and 17α-estradiol is consistent with earlier studies that demonstrated antioxidant effects of both these stereoisomers (5, 7, 10). The antioxidative effect is independent of the estrogenic properties of the compound (28). Many actions of estrogen are prob-

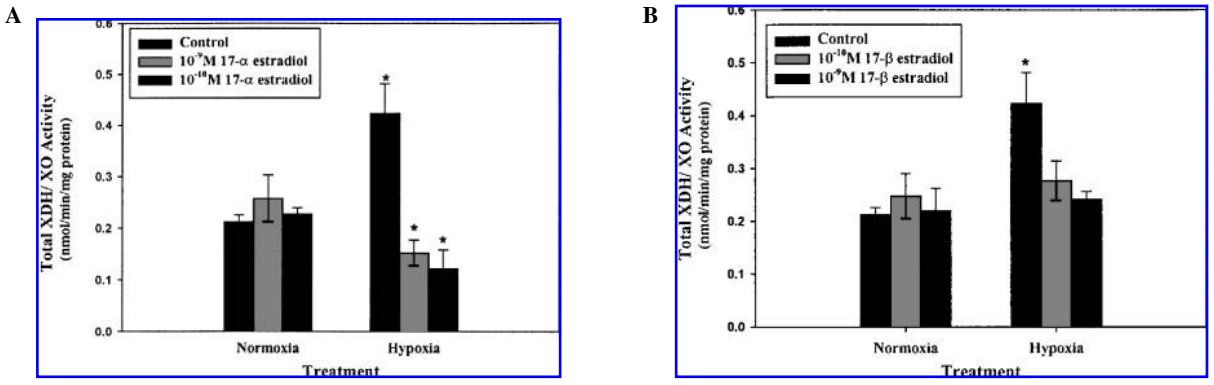

FIG. 3. 17α- and 17β-estradiol attenuate hypoxia-induced increase in XDH/XO activity. RPMEC were treated with different concentrations of 17α- or 17β-estradiol prior to exposure to hypoxia or normoxia. All tested concentrations of either estradiol (10^{-10}–10^{-7} *M*) prevented the expected increase in XDH/XO activity after 24-h exposure to hypoxia. For clarity, results with the two lowest concentrations are shown. There was no dose-response relationship between the concentration of estradiol and decrease in XDH/XO activity. *$p < 0.05$ versus normoxia. (**A**) Effect of 17α-estradiol on hypoxia-induced modulation of XDH/XO activity in RPMEC. (**B**) Effect of 17β-estradiol on hypoxia-induced modulation of XDH/XO activity in RPMEC.

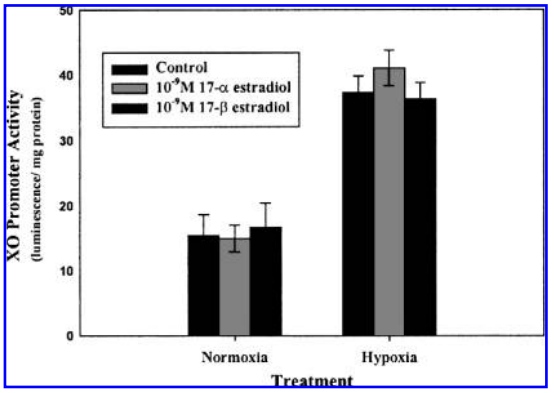

FIG. 4. Effect of 17α-estradiol and 17β-estradiol on XDH/XO gene promoter. RPMEC transfected with 6,000-bp construct of rat XDH/XO promoter were treated with either 17α-estradiol or 17β-estradiol prior to a 24-h exposure to hypoxia. In contrast to their effect on XDH/XO enzymatic activity, neither 17α-estradiol nor 17β-estradiol altered the hypoxia-induced increase in XDH/XO promoter activity.

ably mediated through at least two distinct ERs, ERα and ERβ (30). An increased expression of ERs has been demonstrated with oxidative stress induced by hydrogen peroxide, Fe^{2+}, 2,2′-azobis(2-amidinopropane) dihydrochloride, and activated macrophages (39). Similar to our study, the increase was predominantly seen in ERβ receptors, the major receptor type in lung tissue, with a minimal increase in ERα (39). The physiological significance of this up-regulation is not clear. The presence of ERβ receptors in lungs and on blood vessels (30), and an up-regulation in response to hypoxia as shown in the present study, suggest a possible role of this receptor in the pathophysiology of disorders characterized by hypoxia.

Although it is possible that activation of the estradiol receptor by hypoxia may further stimulate certain effector pathways, a modulatory role for this receptor on the XDH/XO enzyme system is not borne out from our study. Our study suggests that XDH/XO modulation by estradiol is either receptor-independent or regulated by a yet unidentified ER. This is consistent with prior studies documenting the protective role of estradiol independent of the receptors. For example, Behl *et al.* demonstrated that the antioxidative protection conferred by 17β-estradiol in neuronal cells was receptor-independent (4). Similarly, Karas *et al.* showed that 17β-estradiol markedly and equally inhibited vascular injury in both wild-type and ERβ-deficient mice (22), supporting a non–receptor-dependent mechanism for this inhibition.

The mechanism(s) by which estrogens might affect post-transcriptional activation of XDH/XO need further investigation and are beyond the scopeof the current study. However, one can speculate one of several mechanisms. Posttranslational modification of the protein, such as phosphorylation (24) and sulfuration–desulfuration (17), are important in activation of the XDH/XO enzyme system. Estrogens may possibly cause posttranslational modificationof XDH/XO by preventing its phosphorylation or converting the protein to a desulfo-form, thus inhibiting its activation. Recent work has also suggested the importance of two kinases, p38 kinase and casein kinase II (CK2) in mediating the phosphorylation of XDH/XO in hypoxia (24). p38 is a stress-activated mitogen-activated protein kinase that is activated in response to a variety of stimuli, including hypoxia. CK2, a ubiquitous protein, may be important in the regulation of DNA synthesis and replication, as well as cell growth. Further studies are required to elucidate any possible involvement of kinases p38 and CK2 in the estrogen regulation of XDH/XO activation by hypoxia.

In conclusion, this study suggests that the protective properties of estrogen may be, at least in part, non–receptor-mediated and involve the regulation of XDH/XO. As such, these results may offer a new potential role for 17α-estradiol in cardiovascular and neuronal protection, while avoiding the hormonal effects seen with 17β-estradiol.

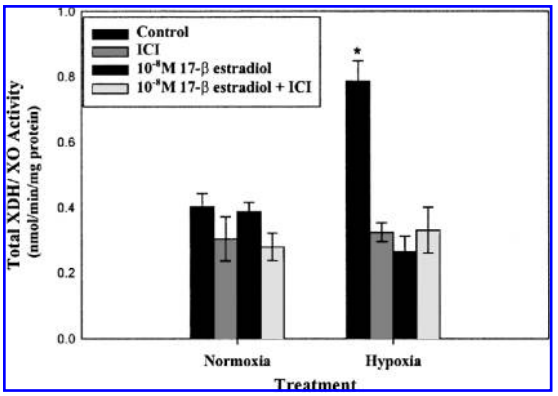

FIG. 5. ER antagonist, ICI 182,780, does not block the effects of 17β-estradiol on hypoxia-inducedincrease in XDH/XO activity. RPMEC were treated with ICI 182,780 starting 30 minutes prior to exposure to 17β-estradiol. This inhibitor failed to block the modulatory effects of 17β-estradiol on XDH/XO activity, suggesting that the process may be receptor-independent.

ACKNOWLEDGMENTS

We thank Dr. Una Ryan for providing the RPMEC.

ABBREVIATIONS

ARDS, adult respiratory distress syndrome; CK2, casein kinase II; ER, estrogen receptor; ROS, reactive oxygen species; RPMEC, rat pulmonary microvascular endothelial cells; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; XDH/XO, xanthine dehydrogenase/xanthine oxidase.

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