

# Differential regulation of the inducible nitric oxide synthase gene by estrogen receptors 1 and 2

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## Abstract

Estrogen has both rapid and longer term direct effects on cardiovascular tissues mediated by the two estrogen receptors, ESR1 and ESR2. Previous work identified that estrogen regulates the expression of inducible nitric oxide synthase (NOS2A) in vascular smooth muscle cells (VSMC). ESR2 knockout mice have vascular dysfunction due to dysregulation of NOS2A expression and these mice are hypertensive (Zhu *et al. Science* 2002 **295** 505–508). Here, we report studies to examine the differential regulation of *NOS2A* gene expression by ESR1 and 2. Immunoblotting and RT-PCR studies revealed that different VSMC lines expressed different levels of ESR1 and ESR2 protein and mRNA. VSMC from different vascular beds were studied, including aortic VSMC expressing ESR1 and radial (Rad) VSMC expressing ESR2. E<sub>2</sub> inhibited NO production and NOS2A protein expression in aortic VSMC. Human NOS2A promoter–reporter studies revealed suppression of NOS2A reporter activity by E<sub>2</sub> in

aortic VSMC, and stimulation of NOS2A reporter activity by E<sub>2</sub> in Rad arterial VSMC. In heterologous expression studies of COS-7 cells lacking endogenous ER, E<sub>2</sub> treatment of COS-7 cells did not alter NOS2A reporter activity in the presence of ESR1, while reporter activity increased 2.3-fold in the presence of ESR2. Similar experiments in COS-7 cells using the selective estrogen receptor modulator raloxifene showed that raloxifene caused a reduction in NOS2A reporter activity with ESR1 coexpression and an increase with ESR2 coexpression. Rat VSMC expressing ESR2 but not ESR1 also showed increased NOS2A reporter activity with E<sub>2</sub> treatment, an effect lost when ESR1 was introduced into the cells. Taken together, these data support that hNOS2A transcription is regulated positively by ESR2 and negatively by ESR1 in VSMC, supporting differential actions of these two estrogen receptors on a physiologically relevant gene in VSMC.

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## Introduction

Physiological effects of estrogen on vascular smooth muscle cells (VSMC) are mediated by two intracellular estrogen receptors (ESR1 and 2), which regulate transcription of target genes following ligand activation through binding to specific DNA target sequences (Kannel *et al.* 1976, Marcus *et al.* 1994, Gardin *et al.* 1995, Mendelsohn & Karas 1999). Regulation of specific genes in VSMC by estrogen is still not well understood. Recent studies have suggested that nitric oxide (NO) plays an important role in estrogen-mediated effects on VSMC. NO is generated by a family of NO synthases (NOS2A) that catalyze the conversion of the amino acid L-arginine to citrulline (Moncada & Higgs 1993). *In vivo*, estrogen reduces vasoconstriction in vessels from which the endothelium has been removed from both humans and wild-type animals (Mügge *et al.* 1997, Binko & Majewski 1998), an effect that is blocked by pharmacological inhibition of NOS2A (Binko & Majewski 1998). Estrogen has been shown to increase the expression and activity of NOS2A in VSMC

(Zhu *et al.* 2002), and this effect is responsible for vascular dysfunction in mouse vessels from ESR2 knockout mice.

Estrogen receptors bind to a specific DNA sequence, the estrogen response element (ERE), in the promoters of many estrogen-responsive genes. However, there are no full palindromic ERE sequence elements for the consensus vitellogenin ERE identified thus far in any of those genes identified to be regulated by estrogen in vascular tissue, including the human *NOS2A* gene. Several studies have shown that selective ER modulators can modulate gene expression by mechanisms that are independent of binding to a classical ERE (Paech *et al.* 1997, Ray *et al.* 1997, Srivastava *et al.* 1999). There is also considerable evidence indicating that ERs may also function by interacting with other transcription factors, including JUND, specificity protein-1, and nuclear factor-κB, bound to their specific response elements in target genes that do not contain EREs (Ray *et al.* 1997, Webb *et al.* 1999, Sanches *et al.* 2002). Moreover, it is evident that estrogen effects are not only dependent on the target gene but also reliant on the cell context. In a similar fashion, the cAMP

regulatory element (CRE; Johansson *et al.* 1999) motif of corticotropin-releasing hormone promoter region can be affected by the action of estrogen receptor in human placental syncytiotrophoblast cells (Ni *et al.* 2004). Nitric oxide production from VSMC can be pathophysiological or physiological, depending in part on the degree of NOS2A activation in a given setting. For example, the general hypotension occurring in septic shock is a result of overwhelming vasodilatory actions of NO released by NOS2A in the context of septicemia. By contrast, in atherosclerosis and balloon-induced injury, increased production of NO by cGMP may provide beneficial effects by inhibiting platelet and leukocyte adhesion as well as the proliferation of VSMC (Galea & Feinstein 1999). In this study, we examined the molecular mechanisms for estrogen-stimulated changes in NOS2A gene expression and the roles of ESR1 and ESR2. We found that transcriptional induction of NOS2A by estradiol was promoted by ESR2 but attenuated by ESR1 in several VSMC and in heterologous expression studies.

## Materials and Methods

### Materials

17 $\beta$ -estradiol (E<sub>2</sub>) was purchased from Sigma Chemical Co. ICI 182 780 was obtained from Tocris (Ballwin, MO, USA). COS-7 cell line, which is an African green monkey fibroblast-like cell line, and A10 cell line, which was derived from the thoracic aorta of embryonic rats, were obtained from American Type Culture Collection (Rockville, MD, USA). Rabbit polyclonal anti-ESR2 antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

### Cell culture

Human aortic (Ao), coronary (Cor), and radial (Rad) arterial VSMC were cultured in our laboratory as previously described. Ao and Cor arterial smooth muscle cells were immortalized, and Rad arterial smooth muscle cells were primary culture. These arterial smooth muscle cells were harvested from human surgical and autopsy specimens as described (Karas *et al.* 1994). These VSMC were developed in our laboratory by the explants method and characterized by both morphology and immunohistochemical studies of expression of smooth muscle cell-specific  $\alpha$ -actin. Ao and Cor arterial smooth muscle cells were immortalized by retroviral constructs containing the E<sub>6</sub> and E<sub>7</sub> human papillomavirus proteins as we have reported previously (Pace *et al.* 1999). Ao and Rad arterial smooth muscle cells were derived from male donors, and Cor arterial smooth muscle cells were derived from 26-year-old female. COS-7 cells were used to selectively express transfected ERs as this cell line expresses neither ESR1 nor ESR2. A10 rat VSMC were used as this cell line expresses only ESR2 (Takahashi *et al.* 2003). These cells were maintained in phenol-red free Dulbecco's modified Eagle's medium (Gibco BRL, Grand

Island, NJ, USA) supplemented with 10% charcoal-stripped, estrogen-free fetal calf serum, and 0.5% penicillin–streptomycin (Gibco BRL) at 37 °C, in humidified incubator at 5% CO<sub>2</sub> which changed every other day.

### Nitrite determination

NO in the culture medium was measured using the Griess diazotization reaction (Griess Reagent Kit for Nitrite Determination, Molecular Probes, Eugene, OR, USA) to detect nitrite formed by the spontaneous oxidation of NO.

### Western blot analysis

To prepare whole-cell lysates, cells were lysed in cell lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ M/ml leupeptin, 10  $\mu$ g/ml aprotinin, and 1 mM sodium orthovanadate). The insoluble material was excluded by centrifugation. The protein concentration in the supernatant of tissue homogenate was determined using DC protein assay reagent (Bio-Rad), and adjusted the same concentration with cell lysis buffer. The resulting supernatant was mixed with SDS-PAGE loading buffer and was subjected to SDS-PAGE. Cellular lysates were fractionated by 10% SDS-PAGE, electrophoretically transferred to nitrocellulose membrane, and then analyzed for immunoreactivity with a rabbit anti-NOS2A antibody (BD Transduction Laboratories, San Jose, CA, USA). Following incubation with the primary antibody, the membrane was exposed to a horseradish peroxidase-conjugated secondary anti-rabbit antibody (GE Healthcare, Buckinghamshire, UK), subjected to Electrochemi-luminescence (ECL) plus (GE Healthcare), and exposed to film. Equal loading was verified by stripping and reprobing the blots with mouse anti  $\beta$ -actin (GE Healthcare).

### Reverse transcriptase-PCR (RT-PCR)

Cells were starved for 24 h and then stimulated in the absence or presence of 10 nM 17 $\beta$ -estradiol (E<sub>2</sub>) or 100 mg/ml lipopolysaccharide (LPS; Sigma) for 24 h before harvest. Total RNA was isolated from VSMC with RNeasy (Qiagen). Total RNA of 1.0  $\mu$ g was used as a template for first-strand cDNA synthesis using random hexamer as primer in the presence of Superscript reverse transcriptase (Invitrogen). One milliliter of cDNA was subsequently used as a template for PCR amplification using *Taq* polymerase (New England Biolabs, Ipswich, MA, USA) using specific primers of NOS2A,  $\beta$ -actin, ESR1, and ESR2 respectively.

### Plasmid constructs

The plasmids pGL3-8296 contained the full-length hNOS2A promoter was kind gift from Dr Joel Moss (The Pulmonary-Critical Care Medical Branch, NHLBI, National Institutes of Health, Bethesda, MD, USA; Arnold *et al.* 2001). The human estrogen receptor 1 (ESR1) expression vector was a kind gift

from Dr Pierre Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Collège de France, Illkirch Cedex, France).

### Transient transfection

Transient transfection was performed with 1.0 µg reporter plasmid DNA for wild-type hNOS2A promoter-directed luciferase expression per well using the Lipofectamine Plus transfection reagent (Invitrogen) according to the manufacturer's protocol. In some experiments, ESR1 or ESR2 expression plasmid was cotransfected to detect alteration of hNOS2A promoter activity.

### Pharmacological treatment and luciferase assay

At 24 h after transfection, cells were treated with E<sub>2</sub> dissolved in ethanol; the latter did not exceed 0.01% final concentration. Controls received the same amount of ethanol. The cells were then further incubated for 24 h under the same conditions.

After incubation, the cells were harvested and luciferase assays were performed with the PicaGene dual seapansy system (Toyo Inc., Tokyo, Japan) following the manufacturer's protocol. Firefly-luciferase activity and seapansy-luciferase activity were measured as relative light units with a luminometer (Lumat LB9507, EG&G, Berthold, Bad Wildbad, Germany). The firefly-luciferase activity was then normalized relative to the seapansy-luciferase activity to determine the transfection efficiency.

### Statistical analysis

Statistical significance of the results was determined by Student's *t*-test for experiments with two groups or by performing an ANOVA followed by Fisher's least significance difference test for experiments with more than two groups; *P* < 0.05 was accepted as statistically significant.

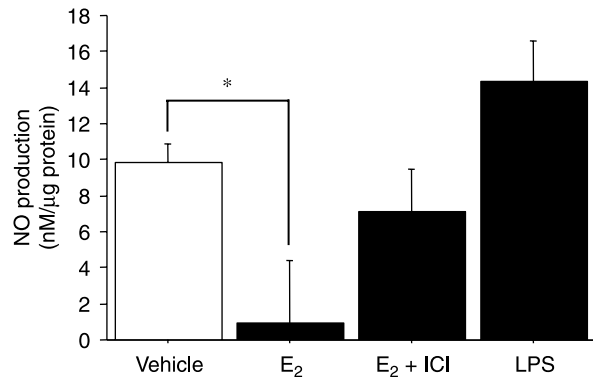
## Results

### Effect of estradiol E<sub>2</sub>, ICI 182 780, and LPS on NO production in aortic VSMC

E<sub>2</sub> stimulation of NO production in VSMC was first examined in cultured aortic VSMC (Ao184 cells) in the absence or presence of the estrogen receptor antagonist ICI 182 780, with LPS used as a positive control (Fig. 1). NO synthesis was determined by assaying the culture supernatants for nitrite, using Griess reagent. NO production was suppressed by E<sub>2</sub> (10 nM), and this effect was significantly reversed by ICI 182 780 (1 µM).

### Western blot analysis of NOS2A protein in VSMC

To explore whether the effects of NO production by E<sub>2</sub> were different between aortic VSMC (Ao184) and Cor arterial

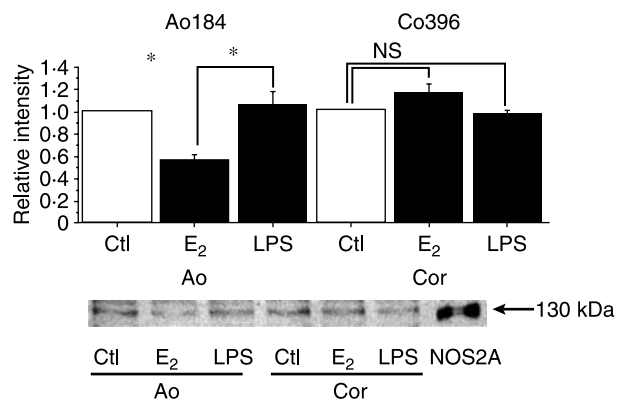


**Figure 1** The effects of E<sub>2</sub>, ICI, and LPS on NO production in aortic vascular smooth muscle cells. Aortic VSMC was treated with E<sub>2</sub> (10 nM), ICI 182 780 (1 µM), and LPS (100 µg/ml). NO synthesis was determined by assaying the culture supernatants for nitrite, using Griess reagent. Values are expressed as means ± s.e.m. of three individual experiments, performed in triplicate. \**P* < 0.05, significantly different from the vehicle.

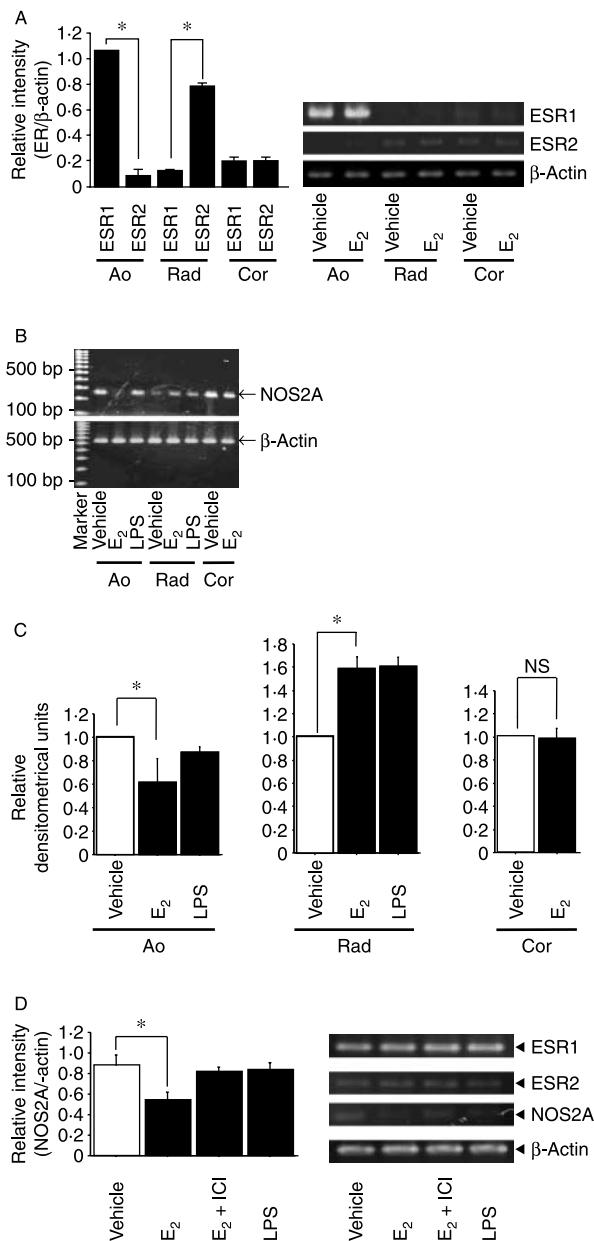
VSMC (Co396), western blot analysis of NOS2A protein following estrogen treatment was studied (Fig. 2). Protein was extracted from VSMC treated with vehicle (0.1% ethanol), E<sub>2</sub> (10 nM), or LPS (100 µg/ml) for 24 h. Mouse macrophage cell lysate stimulated with IFNG (10 ng/ml) and LPS (100 µg/ml) for 12 h, served as a positive control. Although E<sub>2</sub> attenuated NOS2A protein expression in Ao184 cells (cf. Fig. 1), E<sub>2</sub> did not affect NOS2A protein expression in Co396 cells.

### The effects of E<sub>2</sub> on ERs and NOS2A mRNA expression

To examine the potential mechanisms for the difference in NOS2A induction in different VSMC, the distribution of



**Figure 2** Western blot analysis of NOS2A protein in VSMC. Protein was extracted from VSMC (Ao, Cor) treated with vehicle, E<sub>2</sub> (10 nM), or LPS (100 µg/ml) for 24 h. Mouse macrophage cell lysate, which was stimulated with IFNG (10 ng/ml) and LPS (1 µg/ml) for 12 h, was served as a positive control. Values are expressed as means ± s.e.m. of three individual experiments, performed in triplicate. \**P* < 0.05, significantly different from the vehicle.

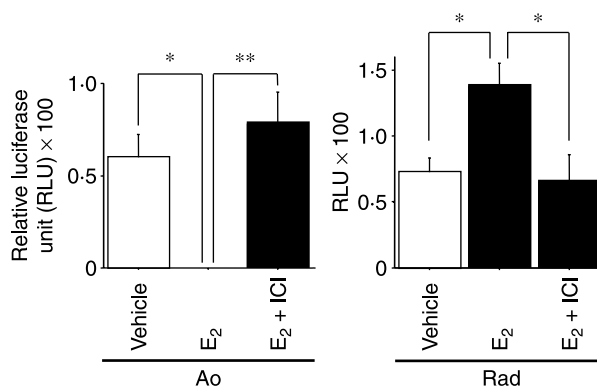


**Figure 3** The effects of E<sub>2</sub> on ERs and NOS2A mRNA expression. (A) mRNA expressions of estrogen receptors 1 (ESR1) and 2 (ESR2) were measured by RT-PCR in various vascular smooth muscle cells. Ao, aortic vascular smooth muscle cells; Rad, radial arterial vascular smooth muscle cells; Cor, coronary arterial vascular smooth muscle cells. (B and C) NOS2A mRNA expression was examined by RT-PCR. mRNA was extracted from VSMC (Ao, Rad, Cor) treated with vehicle, E<sub>2</sub> (10 nM), or LPS (100  $\mu$ g/ml) for 24 h respectively. (D) RT-PCR analysis for estrogen receptors and NOS2A mRNA expression in aortic vascular smooth muscle cells. Values are expressed as means  $\pm$  S.E.M. of three individual experiments, performed in triplicate. \* $P$  < 0.05, significantly different from the vehicle.

ERs among various VSMC (Ao, Cor, and Rad) was studied using semi-quantitative RT-PCR (Fig. 3A). In Ao, ESR1 mRNA was significantly more abundant than ESR2 mRNA. In Rad arterial VSMC, the converse was seen: ESR2 mRNA was significantly more abundant. However, in Cor arterial smooth muscle cells, no significant difference was detected between ESR1 and ESR2 mRNA expressions (Fig. 3). Potential differences in NOS2A expression following E<sub>2</sub> were next evaluated (Fig. 3B–D). In Ao, which express mainly ESR1, NOS2A mRNA expression was significantly suppressed by 10 nM E<sub>2</sub>. By contrast, NOS2A mRNA expression was increased by E<sub>2</sub> in Rad artery VSMC, which express predominantly ESR2. No significant changes in NOS2A expression were detected following E<sub>2</sub> treatment of Cor VSMC. These data support the hypothesis that the relative level of ESR1 and ESR2 expressions may regulate expression of NOS2A.

#### NOS2A reporter activity in aortic VSMC and Rad arterial VSMC

To confirm whether the difference of distribution of ERs affects transcriptional regulation of NOS2A, NOS2A reporter activities were examined in Ao and Rad VSMC. Luciferase assay of the NOS2A reporter construct pGL3-hNOS2A 8296 bp was performed (Fig. 4). In Ao, NOS2A reporter activity was suppressed by 10 nM E<sub>2</sub>, and 1  $\mu$ M ICI 182 780 reversed the effect. By contrast, in Rad arterial VSMC, NOS2A reporter activity was increased by E<sub>2</sub>, and the effect of E<sub>2</sub> was reversed by ICI 182 780. These results support that NOS2A reporter activity is negatively regulated by ESR1 and positively regulated by ESR2.



**Figure 4** NOS2A reporter activity in aortic vascular smooth muscle cells and radial arterial vascular smooth muscle cells. Luciferase assay of the NOS2A reporter construct pGL3-hNOS2A 8296 bp in aortic vascular smooth muscle cells (Ao) and radial arterial vascular smooth muscle cells (Rad) with vehicle, E<sub>2</sub> (10 nM), and ICI 182 780 (1  $\mu$ M) for 24 h. Values are expressed as means  $\pm$  S.E.M. of three individual experiments, performed in triplicate. \* $P$  < 0.05, \*\* $P$  < 0.01, significantly different from the vehicle.

### NOS2A reporter activity in COS-7 cells

To further explore the role of ERs on NOS2A transcriptional regulation, we also conducted experiments in COS-7 cells, which lack endogenous ER, by cotransfecting NOS2A reporter plasmid and ER expression plasmid. E<sub>2</sub> treatment of COS-7 cells did not alter NOS2A reporter activity in the presence of ESR1 coexpression, while NOS2A reporter activity increased 2.3-fold when ESR2 was coexpressed with the NOS2A reporter (Fig. 5A). We performed similar experiments using the selective estrogen receptor modulator raloxifene (Ral; Fig. 5B). 10 nM Ral led to a reduction in NOS2A reporter activity to 40% of control in the presence of ESR1 expression, while NOS2A reporter activity was significantly increased in the presence of ESR2 coexpression in COS-7 cells.

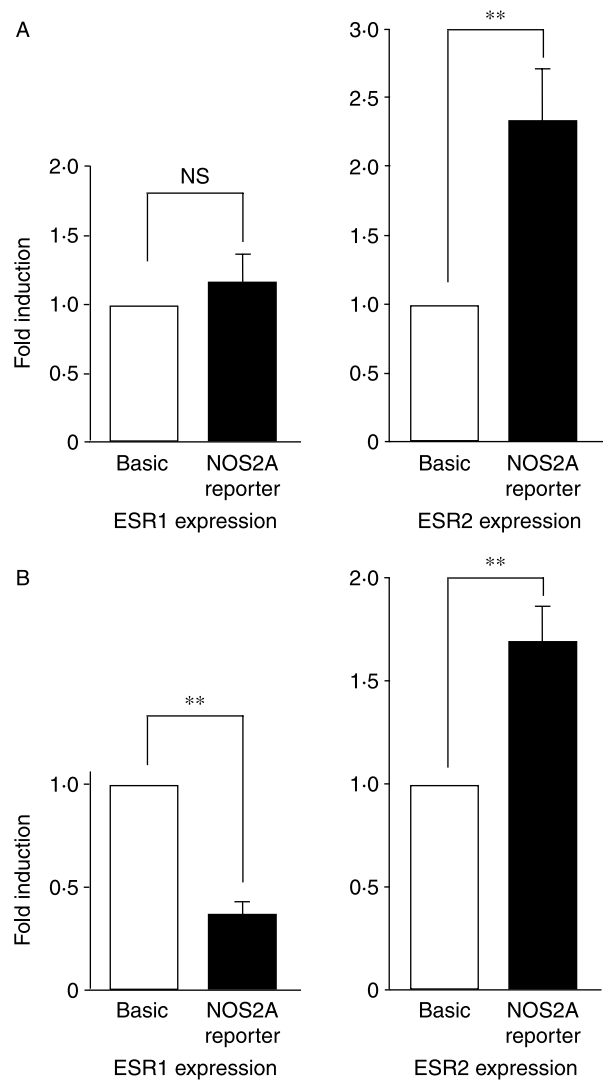
NOS2A reporter activity in COS-7 cells. Rat VSMC (A10 cells), which express ESR2 but not ESR1, were transfected with the reporter construct for full-length hNOS2A promoter (−8296) with or without the expression plasmid for human ESR1. Treatment with 10 nM of E<sub>2</sub> increased NOS2A reporter activity in A10 cells (Fig. 6). Control experiments using ICI 182 780 (1 μM) demonstrated a complete inhibition of E<sub>2</sub>-mediated activation of hNOS2A reporter construct supporting that the effect was mediated by ESR2. Transfection of ESR1 into A10 cells led to inhibition of NOS2A reporter activity in the presence of E<sub>2</sub> (Fig. 7).

### Discussion

This study shows that estrogen-dependent transcriptional regulation of the human NOS2A gene is dependent on the specific subtype present in the cell. The data in both native human VSMC and in heterologous cells, as well as murine VSMC, are consistent with ESR2-mediated increases in NOS2A gene expression and ESR1-mediated suppression of the NOS2A gene.

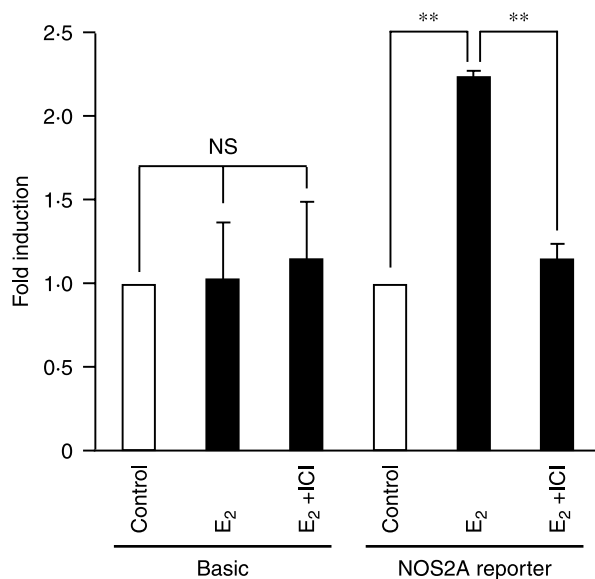
Differential effects of ESR1 and ESR2 have also been observed for the cyclin D1 gene in rat vascular A10 cells, for which estrogen inhibited cyclin D1 expression when ESR1 was expressed but did have an effect in parental A10 cells that express only ESR2 (18). Differential and opposing regulation of promoter activity by estradiol with ESR1 and ESR2 also has been reported recently with the plasminogen activator inhibitor-1 (SERPINE1) promoter in endothelial cells. ESR1 increased SERPINE1 promoter activity in bovine aortic endothelial cells by an estrogen-dependent mechanism, whereas ESR2 suppressed SERPINE1 promoter activity by an estrogen-independent mechanism (Smith *et al.* 2004).

Although both ER subtypes are highly homologous (96%) in their DNA-binding domains, allowing both receptors to, in theory, bind the same EREs within the promoter of the target genes, the transcriptional activity of ERs is mediated by both a ligand-independent transcriptional-activation function (TAF-1) located within the amino-terminal region and a hormone-dependent TAF-2 located within carboxyl-



**Figure 5** (A) NOS2A reporter activity with estrogen receptors 1 and 2 in the presence of E<sub>2</sub> in COS-7 cells. COS-7 cells cotransfected with the NOS2A reporter and estrogen receptor (ER) expression plasmid were incubated for 24 h with or without 10 nM E<sub>2</sub> before assay of luciferase activity. (B) NOS2A reporter activity with estrogen receptors 1 and 2 in the presence of raloxifene in COS-7 cells. COS-7 cells cotransfected with the NOS2A reporter and estrogen receptor (ER) expression plasmid were incubated for 24 h with or without 10 nM raloxifene before assay of luciferase activity. Data are the means of values from four experiments with assays in triplicate, expressed relative to luciferase activity of pGL3-basic. \*\**P* < 0.01, significantly different from basic plasmid.

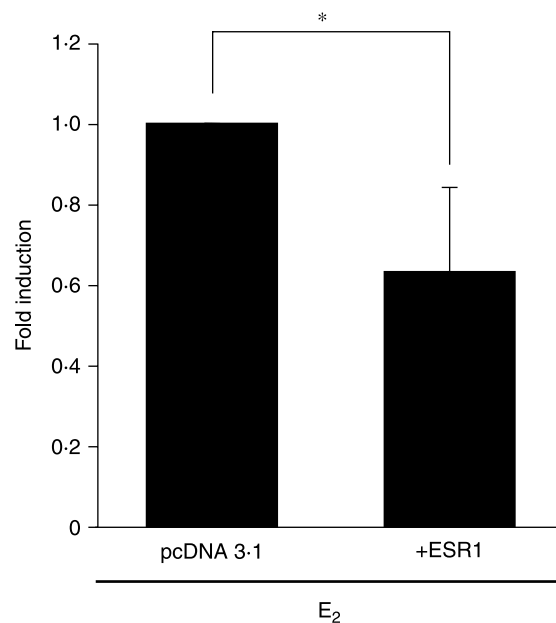
terminal region (Mendelsohn & Karas 1999). Many nuclear receptor coregulators interacting with the TAF-2 domain of various receptors interact equally well with ESR1 and ESR2, including NRIP1, NCOA2, NCOA1, and short heterodimer partner (Seol *et al.* 1998, Smith *et al.* 2004). However, the two receptor isoforms show quite significant differences in their amino-terminal (TAF-1) domains, and it is possible that the



**Figure 6** Luciferase activity in A10 rat thoracic arterial vascular smooth muscle cells transfected with NOS2A reporter plasmid and treated with 10 nM E<sub>2</sub>, or 10 nM E<sub>2</sub> and 1 μM ICI 182 780 for 24-h reporter activity is shown relative to the vehicle control. Values represent the mean ± s.e.m. of experiments performed in triplicate. \*\**P* < 0.01, compared with vehicle controls.

two receptors interact with different sets of coactivator or corepressor proteins as one explanation for the differential regulation observed in this study. The ligand-binding domains are relatively conserved (55%; Rowan *et al.* 2000) between ESR1 and ESR2, and are reported to display a similar affinity for E<sub>2</sub> (Kuiper *et al.* 1998). It remains to be determined how further varying the ratio of ESR1 and ESR2 could affect the response of the hNOS2A promoter, especially as ERs can form both homodimers and heterodimers.

Differential expression and functions of ESR1 and ESR2 also are observed *in vivo* (Kuiper *et al.* 1998). ESR2 is found in many tissues including the prostate, uterus, ovary, testis, bladder, lungs, and brain, but not in liver. Although ESR2 is also expressed in the mammary gland, it appears that ESR1 is a more important estrogen receptor in this particular tissue. In the uterus, ESR1 is more abundant than ESR2 while ovarian folliculogenesis is predominantly regulated by ESR2. These differences in tissue distribution and function are of potential importance from the pharmaceutical point of view. In syncytiotrophoblast cells transfected with ESR1 expression and corticotropin-releasing hormone promoter-reporter vectors, E<sub>2</sub> significantly decreased the ESR1/CRE-dependent reporter activity. This inhibition of reporter activity via ESR1 might be mediated by a repressor domain in the amino-terminal region of ESR1 comparable with TAF-1 of ESR2 Gustafsson 1999. Alternatively, the ligand-activated ESR1 or ESR2/CRE complex may enhance interactions with coregulator protein(s). Several recent studies have indicated an interaction between the cAMP-mediated signaling and



**Figure 7** E<sub>2</sub> mediates negative transcriptional regulation of the hNOS2A gene via ESR1 in a dose-dependent manner. A10 cells were transfected with the pGL3-hNOS2A reporter construct and ESR1 expression vector or its backbone vector (pcDNA3.1 +). The ratios of reporter construct to expression vector are 1:1, 2:1, and 10:1 respectively. After 24 h, the cells were treated with 10 nM E<sub>2</sub> in ethanol, and incubated for an additional 24 h. Cells were then harvested, and luciferase activity was determined, and normalized to seapansy-luciferase activity. Relative luciferase activity is shown as fold induction, with the mean for the group (backbone vector) taken as 1. Values are expressed as means ± s.e.m. of three individual experiments, which were performed in triplicate. \**P* < 0.05, significantly different from the vehicle.

response to estrogen. Increased cAMP is reported to stimulate the transcriptional activity of ER. The extent of stimulation varied depending on the promoter context (Hall & McDonnell 1999). Phosphorylation of ERs may be involved in this response, because ERs contain a number of potential protein kinase A phosphorylation sites. Alternatively, phosphorylation of the steroid receptor coactivator (NCOA1) has been proposed to promote a more stable interaction between NCOA1 and p300/CBP, facilitating their functional synergism (Coleman *et al.* 2003).

In summary, our findings support that estrogen-mediated effects on the NOS2A gene, a physiologically relevant target of estrogen in vascular smooth muscle, are differentially regulated, induced by ESR2 and inhibited by ESR1. These findings are particularly interesting and somewhat unusual in the context of our recent observation that ESR1 is in general an activator of vascular gene expression in response to estrogen, while the majority (80 percent or more) of genes regulated by ESR2 following estrogen are inhibited in their expression (O'lonne *et al.* 2007). These results suggest that ESR1-selective compounds for ESR1 or ESR2 might have therapeutic

potential for cardiovascular disease. These results may contribute to our understanding of how estrogens can modulate regulation of cardiovascular systems by other effectors.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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