Oestrogen and progesterone inhibit the stimulated production of endothelin-1

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Important vascular proteins such as endothelin-1 (ET-1) promote the development of cardiovascular diseases. Oestrogen, and perhaps progesterone, prevent the development of vascular disease in women through incompletely understood cellular mechanisms. We hypothesized that oestradiol or progesterone might regulate the production of ET-1 as a potential novel mechanism. We found that serum and angiotensin II (AII) significantly stimulated ET-1 secretion from cultured bovine aortic endothelial cells, inhibited 50–75 % by oestradiol or by progesterone. Serum and AII stimulated ET-1 mRNA levels, inhibited at least 70 % by oestradiol and by progesterone. Serum stimulated ET-1 transcription mainly through the first 43 nucleotides of the ET-1 promoter, but oestradiol and progesterone did

not inhibit this. In contrast, AII stimulated ET-1 transcription through nucleotides –143 to –98, specifically involving an activator protein-1 (AP-1) site at –102. Oestradiol and progesterone caused a 60–70 % inhibition of AII-stimulated wild-type construct –143ET-1/CAT activity (CAT is chloramphenicol acyltransferase). AII-stimulation of ET-1 transcription was critically dependent on stimulation of mitogen-activated protein kinase (erk) activity, inhibited by oestradiol and progesterone. In summary, we found that sex steroids inhibit AII-induced erk signalling to the ET-1 transcriptional programme. This novel mechanism of negative transcriptional regulation by oestradiol and progesterone decreases the production of ET-1, potentially contributing to the vascular protective effects of these steroids.

INTRODUCTION

In the vasculature, the female sex steroid oestradiol (OE) has been shown to protect women from developing cardiovascular disease [7]. It is unclear whether progesterone (Prog) has similar protective actions, but overall the data suggest that Prog is neutral or protective [2] in this regard. The cellular mechanisms by which sex steroids accomplish this are only partially understood. One possibility is that OE and Prog inhibit the production of important vascular proteins, such as endothelins [3], which are strongly implicated in the pathogenesis of cardiovascular disease.

The endothelins are a family of 21-amino-acid peptides consisting of three known members: endothelin-1, endothelin-2 and endothelin-3 [4]. Endothelin-1 (ET-1) is produced mainly by vascular endothelial cells and is a potent vasoconstrictor and mitogen for vascular-smooth-muscle cells (VSMC). ET-1 often acts as a paracrine hormone through transmembrane ET_A receptors expressed on VSMC (reviewed in [3]). As a result of its vasoconstrictive and proliferative effects, ET-1 has been implicated in various pathogenic processes, including atherosclerosis, acute myocardial infarction and congestive heart failure [5–7]. It is thought that cardiac hypertrophy induced either by the important vasoactive peptide angiotensin II (AII), or by haemodynamic overload, may be mediated, at least in part, through ET-1 production and action [8,9]. Increased ET-1 transcription, translation and secretion from endothelial cells (EC) occurs in response to a variety of conditions, and is often rapid (reviewed in [3]). It is possible that ET-1 production in EC could be modulated by OE or Prog, since specific receptors for these steroids have been shown to exist in vascular endothelial cells [10,11]. Little is understood, however, about the regulatory mechanisms of ET-1 production.

In humans, preproET-1 is processed to a 38-amino-acid prohormone (Big ET-1), subsequently acted on by endothelin-converting enzyme, resulting in mature ET-1 [12,13]. It has been shown in bovine aortic endothelial cells (BAEC) that two specific promoter regions bind the *trans*-activating proteins GATA-2 and AP-1 which are necessary for basal transcriptional activity [14–17]. The mechanisms underlying the control of stimulated ET-1 transcription are not known.

In the present study we examined two important and possibly interactive issues. We sought the determinants of positive transcriptional regulation of ET-1, comparing the important vascular protein AII to the more complex stimulus, serum. We also determined whether and how the sex steroids OE and Prog inhibit the stimulation of ET-1 production. We report that OE and Prog inhibit ET-1 production, pointing to a potential means of protecting the vasculature. Further, we found that the two positive regulators utilized different transcriptional molecular determinants, and that OE and Prog have previously unappreciated actions to negatively regulate transcription through interrupting signalling to the nucleus.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle medium (DMEM), Hanks balanced salt solution (HBSS) and LipofectAMINE reagent were from Gibco-BRL (Grand Island, NY, U.S.A.); 17 β -OE, Prog, testosterone, AII, thrombin from human plasma, *o*-nitrophenyl β -D-galactopyranoside and actinomycin D were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); ET-1 antiserum and

Abbreviations used: AII, angiotensin II; AP-1, activator protein-1; BAEC, bovine aortic endothelial cell(s); CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; OE, oestradiol; EC, endothelial cell(s); OER, oestrogen receptor; erk, mitogen-activated protein (MAP) kinase; ET-1, endothelin-1; HBSS, Hanks balanced salt solution; HDL, high-density lipoprotein; MBP, myelin basic protein; Prog, progesterone; Thr, thrombin; VSMC, vascular-smooth-muscle cell; MEK, MAP kinase kinase; Actino, actinomycin D.

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Prog were from Peninsula Laboratories (Belmont, CA, U.S.A.); chloramphenicol acetyltransferase (CAT) assay reagents were from Promega (Madison, WI, U.S.A.); TRI reagent was from Molecular Research Center Inc. (Cincinnati, OH, U.S.A.); pTRI-Actin-mouse was from Ambion (Austin, TX, U.S.A.); the OE-receptor (OER) antagonist ICI 182,780 was a gift from Dr. Alan Wakeling (Zeneca Pharmaceuticals, Alderley Park, Macclesfield, Cheshire, U.K.).

Cell culture

BAEC were cultured as previously described [18,19]. Cells formed a monolayer with a typical cobblestone appearance, exhibited contact inhibition and tested positive for Factor VIII-related antigen. Cells were maintained in tissue-culture medium (DMEM with foetal-bovine serum) until 24 h prior to experimentation, at which time cells were serum-deprived for most experiments. All experiments were done with second-passage BAEC, with the exception of transient transfection assays, which required BAEC in the fifth passage for efficient transfection.

Secretion experiments

As previously described [19], BAEC were grown to near confluence on 12-well culture plates, containing approx. 5×10^5 cells/well. Cells were serum-deprived for 24 h prior to experimentation, followed by treatment for 4 h with various known and physiologically relevant stimulators of ET-1 production {foetal-bovine serum (serum), AII, high-density lipoprotein (HDL) or thrombin [20–22]} in the absence or presence of OE or Prog (1–100 nM). The period of incubation was based upon time-course experiments with AII and serum. Some experiments were also done in the presence of ICI 182,780 (1 μ M), an OER antagonist. At the end of the treatment period, medium was collected and the ET-1 concentration was measured using a sensitive double-antibody radioimmunoassay [19,23]. Data from three experiments were combined for analysis (12 wells/condition).

Solution hybridization and S1-nuclease protection

BAEC were grown to near confluence on 100 mm-diameter Petri dishes. After 24 h of synchronization in serum-free DMEM, cells were treated with the representative ET-1 stimulators, serum (3%) or AII (100 nM), with or without OE or Prog (100 nM) for 1–4 h. Cells were then washed with PBS, and total RNA was extracted using TRI reagent. The RNA was solution-hybridized with a 32 P-labelled antisense cRNA probe for ET-1 as previously described [24,25]. Hybridized RNA was separated by electrophoresis, and loading differences were adjusted by a hybridization to a 32 P-labelled antisense cRNA probe for β -actin (Ambion). The gel was exposed to film for 24–72 h, followed by densitometric analysis of the autoradiographic bands. Sense probes produced no hybridization. Data were combined from six experiments.

Transient transfection and reporter-gene studies

BAEC (passage 5) were grown to 40–50% confluence on 60 mm-diameter Petri dishes. Cells were then transiently transfected with 0.3–1.5 μ g of fusion plasmids containing various lengths of 5'-flanking ET-1 promoter sequence and the prokaryotic CAT gene. Constructs included –1.7 kb nt ET-1/CAT, –.143, –.098, and –.043ET-1/CAT (kindly provided by Dr. M. E. Lee, Harvard Medical School) [16]. We also used wild-type –.204 ET-1/CAT and a –.204ET-1 mutant AP-1/CAT containing a

two-nucleotide substitution in the wild-type activator protein-1 (AP-1) consensus sequence which disrupts AP-1 protein binding to the ET-1 promoter (kindly provided by Dr. Thomas Quertermous, Vanderbilt University, Nashville, TN, U.S.A. [17]. PCAT (Promega) was used as a positive control for CAT expression. To control for transfection efficiency, cells were co-transfected with 1 μ g of pON249, a β -galactosidase expression vector [26]. Data were usually normalized for transfection efficiency; when the experimental condition induced β -galactosidase expression, total protein was instead quantified in this setting. Transient transfection of BAEC was carried out using LipofectAMINE reagent (GIBCO-BRL) (2–4 μ l/tube). Immediately prior to transfection the cell culture medium was aspirated and the cells were washed with DMEM lacking serum or antibiotic/antimycotic. Cells were then incubated with liposome–DNA complexes for 4 h at 37 °C, followed by recovery for 7 h in DMEM containing 10% (v/v) foetal-bovine serum. Then, prior to experimental treatment, cells were synchronized for 36 h in serum-free DMEM. Cells were then treated with serum (5%) or AII (100 nM) with or without OE or Prog (100 nM), or OE or Prog alone, for 6 h at 37 °C, followed by harvesting. In several experiments, the soluble MAP kinase kinase (MEK) inhibitor PD 98059 (20 μ M) [27] was added 15 min prior to AII addition. Cell extracts underwent three rounds of freezing and thawing, and supernatants were assayed for β -galactosidase or CAT activity. For CAT activity determination, the conversion of [3 H]chloramphenicol into acetylated chloramphenicol was determined as described previously [23]. β -Galactosidase activity was determined as previously described [23]. The units for CAT activity were normalized for transfection efficiency by dividing by respective β -galactosidase values and data are expressed relative to the wild type –.204 or –.143 ET-1/CAT activity. Experiment were carried out at least three separate times, each condition in triplicate in each experiment, and all data were combined for graphical representation. In additional experiments, pCMV5-erk2, an active erk-2 expression plasmid [28–30], was co-transfected with –.143 ET-1/CAT in cells incubated with AII and OE, OE alone or in the absence of steroid or peptide.

MAP kinase (erk) studies

Erk activity was determined as previously described [31] in an *in vitro* tube assay using erk activity immunoprecipitated from cells incubated with AII, 100 nM with or without OE or Prog (100 nM) for 8 min. Phosphorylation of myelin basic protein by erk was quantified by densitometry after SDS/PAGE separation. Equal amounts of protein were loaded on the gel under all conditions, and the study was repeated twice.

ET-1 mRNA half-life studies

Half-life studies were carried out as described previously [21]. Briefly, after 24 h of synchronization in serum-free DMEM, BAEC were incubated with serum (3%) or AII (100 nM) with actinomycin D (1 μ M) with or without OE or Prog (100 nM) or DMEM alone (control condition) for 15, 30 or 60 min, at which time total RNA was extracted. Solution hybridization and S1-nuclease protection were carried out, and the half-life of the ET-1 transcript was calculated from the density of the autoradiographic bands at each time point by a curve-fitting equation (CA-Cricket Graph 3; Islandia, Star, New York, NY, U.S.A.). Loading was normalized based on hybridization of the RNA to a 32 P-labelled antisense cRNA β -actin probe. Data from four

experiments were combined. The time points selected were based on the known half-life of ET-1 in BAEC [19].

Metabolic labelling studies

Studies of new ET-1 protein synthesis (translation) were carried out as previously described [19]. Briefly, following synchronization for 24 h, cultured BAEC were incubated in methionine-free DMEM with 10% dialysed fetal-bovine serum for 1 h prior to experimentation. Cells were then incubated with serum (3%) or AII (100 nM) with or without OE or Prog (100 nM) or OE or Prog alone, and 250 μ Ci of [35 S]methionine for 4 h. The cells were washed and then lysed in buffer containing 1% Triton X-100/1% bovine haemoglobin/1 mM iodoacetamide/1 mM PMSF/2 μ g/ml leupeptin/aprotinin (0.2 units) for 1 h at 4 °C. The lysate was pre-cleared and ET-1 was immunoprecipitated using polyclonal antibody. Antibody preabsorbed with ET-1 (1 μ M) for 12 h before use in the immunoprecipitation procedure served as a specificity control. The immunoprecipitated protein was then resolved by SDS/PAGE [19]. The gel was then fixed, subjected to fluorography and then autoradiography. The size of the protein bands were compared with molecular-mass markers resolved under the same conditions. Data from three experiments were combined and quantified by laser densitometry.

Statistics

Data from three secretion studies were combined ($n = 12$ wells/condition), a mean $\pm$ S.D. for each condition was calculated, and the various conditions were compared by analysis of variance, followed by a multiple-range test (Fisher PLSD) for significant F values ($P < 0.05$). For the half-life studies, the ET-1 transcript half-life for each treatment was derived from the equation describing the corresponding best-fit curve with relative density (y) set at 50%.

RESULTS

Secretion studies

We first determined the time courses for AII and serum-stimulation of ET-1 secretion from the BAEC and found that each maximally stimulated ET-1 secretion above control at 4 h by greater than 100%. Subsequent studies were then carried out for 4 h. Serum (3%) increased ET-1 secretion by a mean of 112 ± 9 (S.D.)% above control after 4 h of incubation (Table 1). OE and Prog (100 nM) respectively decreased this stimulation to $53 \pm 7\%$ and $37 \pm 4\%$, whereas lower concentrations of OE or Prog had no significant effect. AII (100 nM) increased ET-1 secretion $104 \pm 9\%$ above control, and this increase was significantly decreased by OE and Prog (100 nM) to only 31 ± 5 and $37 \pm 5\%$ (Table 1). Inhibition was also significant at 10 nM sex steroid (to 57 ± 6 and $41 \pm 6\%$ respectively). Neither OE nor Prog by themselves had a significant effect on basal ET-1 secretion, and 10 nM 17 α -OE did not inhibit either the serum or AII stimulation of ET-1 secretion (results not shown).

To show that the inhibitory effects of OE are mediated through an OER, ICI 182,780, an OER antagonist lacking agonistic properties [32], was used. In the presence of ICI 182,780, OE modestly inhibited the stimulatory effects of serum or AII to 89 ± 7 and $78 \pm 4\%$ above control respectively. These data indicate that OE inhibits stimulated ET-1 secretion through an OER. ICI 182,780 had no effect on progesterone's ability to inhibit serum or AII-stimulated ET-1 secretion, suggesting that OE and Prog are acting through two separate steroid receptors, both coupled to the inhibition of stimulated ET-1 secretion. ICI 182,780 by itself, or with AII or serum, had no effect on ET-1 secretion (results not shown).

We also determined whether OE or Prog might inhibit the stimulatory action of other known and relevant secretagogues

Table 1 Effects of OE or Prog on stimulated ET-1 secretion from cultured bovine aortic endothelial cells at 4 h

Data are the means $\pm$ S.E.M. values from three experiments combined ($n = 12$ per condition), except for ICI 182,789 ($n = 9$). OE (E), Prog (P) or ICI 182,780 individually had no effect on secretion of ET-1 from control cells (no protein added to incubation medium). * $P < 0.05$ for control versus secretagogue by analysis of variance plus Fisher PLSD; + $P < 0.05$ for secretagogue plus steroid versus secretagogue; ++ $P < 0.05$ for serum + OE or AII (A) + OE versus serum + OE (or AII + OE) plus ICI 182,780. Other abbreviations: H, HDL; T, thrombin.

Addition (concn.)	Secretion (% above control)	Addition (concn.)	Secretion (% above control)
Control	0		
Serum (3%)	$112 \pm 9^*$	—	—
S + E (100 nM)	$53 \pm 7^+$	S + P (100 nM)	$37 \pm 4^+$
S + E (10 nM)	83 ± 8	S + P (10 nM)	76 ± 6
S + E (1 nM)	85 ± 7	S + P (1 nM)	101 ± 10
S + E (100 nM) + ICI 182,780	$89 \pm 7^{++}$	S + P (100 nM) + ICI 182,780	39 ± 6
A (100 nM)	$104 \pm 9^*$		
A + E (100 nM)	$31 \pm 5^+$	A + P (100 nM)	$37 \pm 5^+$
A + E (10 nM)	$57 \pm 6^+$	A + P (10 nM)	$41 \pm 6^+$
A + E (1 nM)	92 ± 8	A + P (1 nM)	82 ± 7
A + E (100 nM) + ICI 182,780	$78 \pm 4^{++}$	A + P (100 nM) + ICI 182,780	35 ± 3
H (10 μ g/ml)	$88 \pm 8^*$		
H + E (100 nM)	$22 \pm 4^+$	H + P (100 nM)	$26 \pm 3^+$
H + E (10 nM)	62 ± 6	H + P (10 nM)	58 ± 6
H + E (1 nM)	77 ± 6	H + P (1 nM)	77 ± 8
T (5 units/ml)	$100 \pm 12^*$		
T + E (100 nM)	$35 \pm 3^+$	T + P (100 nM)	$36 \pm 4^+$
T + E (10 nM)	72 ± 4	T + P (10 nM)	78 ± 8
T + E (1 nM)	90 ± 7	T + P (1 nM)	92 ± 6

Figure 1 Effect of OE (E) Prog (P) on serum (S) or AII-induced ET-1 steady-state mRNA levels in cultured BAEC over time, as determined by solution hybridization and S1-nuclease protection (upper panel)

Loading differences for all experiments were adjusted by parallel hybridization of identical amounts of RNA to a ^{32}P -labelled antisense cRNA probe for β -actin (lower panel). Each steroid or peptide was present at 100 nM. Each lane represents the signal from 20 μg of total RNA, solution-hybridized with a ^{32}P -labelled antisense cRNA probe for ET-1, digested with S1 nuclease, and then separated by denaturing PAGE. Lane —, radiolabelled ET-1 cRNA probe alone; lane +, S1-nuclease-digested probe; lanes 1–21, control cells and BAEC incubated under the indicated conditions. The protected band is 196 bases, as determined by comparison with ^{32}P -labelled, *Hinf*I-digested pBR 322. The gel is representative of five additional experiments.

Figure 2 Schematic diagram of the proximal ET-1 5'-flanking promoter sequence

GATA-2 and AP-1 DNA-binding sites are indicated, as are the CAAT and TATAAA consensus sequences. Most of the ET-1 5'-flanking promoter constructs used in the present study are indicated.

for ET-1, in order to generalize the effects of OE or Prog. Incubation of the BAEC with physiologically relevant concentrations of HDL (10 $\mu\text{g}/\text{ml}$) [21] resulted in an $88 \pm 8\%$ increase in ET-1 secretion above control, and this was maximally inhibited to only $22 \pm 4\%$ by OE and $26 \pm 3\%$ by Prog (Table 1). Finally, thrombin (5 units/ml) stimulated ET-1 secretion $100 \pm 12\%$ above control, and this was maximally inhibited to approx. 35% only by both OE and Prog at the highest concentration examined (Table 1). A limited number of studies were done using various concentrations of OE and Prog combined, in the setting of stimulation by AII or thrombin. No additive or synergistic effects were found. We also determined the effects of testosterone on ET-1 secretion. Testosterone by itself, or with serum or AII, had no effect on ET-1 secretion, indicating the specificity of OE and Prog action (results not shown).

mRNA levels

We determined whether OE and/or Prog have an inhibitory effect on ET-1 mRNA in the setting of stimulation by AII or serum. We selected AII because it is an important vasoactive peptide and potent modulator of ET-1 production [20] and because important actions of AII are mediated, at least in part, through stimulation of ET-1 production [8,9]. Serum was selected because it is a complex, but physiologically relevant, stimulator

of ET-1 production [22]. Cultured BAEC were incubated with AII (100 nM) or serum (3%) with or without OE or Prog (100 nM), or OE or Prog alone for 1, 2 or 4 h. On the basis of six experiments combined, serum treatment stimulated ET-1 steady-state mRNA levels approx. 2.5 ± 0.45 -fold above control at 4 h (Figure 1). This increase was significantly inhibited by OE and Prog ($80 \pm 4\%$ and $78 \pm 6\%$ respectively). AII also stimulated ET-1 steady-state mRNA maximally at 4 h (3.2 ± 0.59 -fold above control). This increase was significantly inhibited $81 \pm 5\%$ and $73 \pm 5\%$ by OE and Prog respectively. Neither OE nor Prog alone had an effect on basal ET-1 steady-state mRNA (results not shown). These experiments demonstrate that OE and Prog inhibit steady-state ET-1 mRNA in the setting of stimulation by serum or AII. However, this could result from an inhibitory effect on ET-1 transcription and/or an increase in transcript degradation.

Transient transfection and reporter-gene studies

To determine whether OE or Prog exhibit inhibitory transcriptional effects and to understand the determinants of positive transcriptional regulation of ET-1, reporter-gene studies were carried out. Separate dishes of BAEC were transiently transfected with —.043ET-1/CAT and —.098ET-1/CAT (constructs known to contain insufficient ET-1 promoter elements to drive basal

Figure 3 Effect of OE (E) and Prog (P) on stimulated ET-1 transcription in BAEC, as determined by transfected CAT reporter constructs

Individual dishes of BAEC were transiently transfected and relative CAT activity was calculated as described in the Materials and methods section. Each bar represents the mean $\pm$ S.E.M. from at least three experiments combined, each condition in triplicate/experiment. (A) Serum (Ser) stimulation of the -143 , -098 and -043 ET-1/CAT reporters. Neither OE nor Prog was able to inhibit serum stimulation of the -043 ET-1/CAT reporter. (B) All stimulates ET-1 transcription through promoter elements contained between -098 and -143 nucleotides and is dependent on the AP-1 site at -102 to -109 . Inhibition of MEK activity with PD 98059 abrogated the All-stimulated -143 ET-1 CAT activity. (C) All stimulation of ET-1 transcription is inhibited by OE and Prog. The effect of All on the CAT activity of non-transfected BAEC is shown at the far right as a control. (D) Effects of erk2 expression on OE inhibition of All-stimulated -143 ET-1/CAT activity. All conditions are co-transfected with pCMV5 as a control for the erk2 expression plasmid. Also shown is the lack of effect of 17α -OE compared to 17β -OE on All-stimulated ET-1/CAT activity.

CAT expression) and -143 ET-1/CAT (construct containing sufficient 5'-flanking sequence for basal transcription) [14]. Additionally, in some experiments, a -204 ET-1 mutant AP-1/CAT containing a two-nucleotide substitution in the wild-type AP-1 consensus sequence (TGACTAA mutated to GTACTAA) was used, as well as a wild-type -204 ET-1/CAT construct [17]. Figure 2 is a cartoon of the proximal ET-1 5'-flanking promoter sequence, including critical consensus sequences, and many of the promoter constructs used in the present study. As shown in Figure 3(A) (as well as Figure 3B), sequences between -098 and -143 bp of 5'-flanking ET-1 promoter were required for a high level of basal transcription. Basal CAT activity was substantially decreased in cells transfected with -098 ET-1/CAT or -043 ET-1/CAT, with CAT activity approx. 15% of the -143 ET-1/CAT control. These findings are consistent with previous studies that have indicated two elements that are critical for basal ET-1 transcription: a TATC (GATA) motif spanning nucleotides -135 to -132 , and an AP-1 consensus sequence spanning nucleotides -109 to -102 [14].

Figure 3(A) also shows that serum treatment of BAEC

transfected with the -143 ET-1/CAT construct resulted in a 2.6 ± 0.53 -fold increase in CAT activity over control, basal, expression. Interestingly, serum treatment consistently stimulated CAT activity in cells transfected with -098 ET-1/CAT and -43 ET-1/CAT, 1.5 ± 0.04 and 1.9 ± 0.05 -fold over control respectively. Serum also stimulated CAT activity in cells transfected with the -204 ET-1 mutant AP-1/CAT reporter construct (3.0 ± 0.38 -fold stimulation above basal expression; results not shown). These results suggest that serum does not require the AP-1 consensus-motif-spanning nucleotides -109 to -102 for stimulation of ET-1 transcription. In addition, serum stimulation appears to be mediated mainly through the first 43 bp of the ET-1 promoter, although there is probably a second positive element between nucleotides -98 and -143 .

We then determined whether OE or Prog (100 nM) could inhibit serum-stimulated ET-1 transcription through the first 43 bp of ET-1 promoter sequence. As shown in Figure 4(A), neither OE nor Prog had an inhibitory effect on serum-stimulated ET-1 promoter activity of the -143 ET-1/CAT or -043 ET-1/CAT constructs. We also found that neither OE nor Prog had

Figure 4 OE and Prog inhibit AII-stimulated MAP kinase (erk) activity

BAEC were incubated with AII (100 nM) ± oestrogen or Prog (100 nM) for 8 min, then erk2 activity was immunoprecipitated from cell lysate and used in a tube activity assay, phosphorylating myelin basic protein (MBP). The stimulation of erk2 activity by AII was inhibited 88 and 92% by oestrogen and Prog respectively (lane 2 versus 5 and 6). A representative study from three experiments is shown.

any inhibitory effects on serum-stimulated ET-1 promoter activity in cells transfected with -0.098ET-1/CAT (results not shown). This suggests that, in the setting of serum stimulation, the sex steroids do not inhibit ET-1 gene expression at the transcriptional level. Instead, we postulate that OE and Prog may inhibit ET-1 steady-state mRNA by increasing transcript degradation (see below).

We also found that treatment of BAEC with AII stimulated CAT activity approx. 2.0 ± 0.1 and 2.3 ± 0.3 -fold over control in cells transfected with the -0.204 and -0.143ET-1/CAT constructs respectively (Figure 3B). Unlike serum, the stimulatory effect of AII was completely lost with the -0.098ET-1/CAT reporter, suggesting that the elements critical for AII stimulation of CAT activity lie between nucleotides -143 and -98 . Transfection with a -0.204ET-1 mutant AP-1/CAT reporter resulted in about an 80% decrease in basal activity (absence of AII) compared with basal wild-type -0.204ET-1/CAT activity (Figure 3B). Importantly, AII was also unable to stimulate CAT activity in cells transfected with the -0.204ET-1 mutant AP-1/CAT construct (Figure 3B). This construct differs from the wild type -0.204ET-1/CAT construct by a mutation of two nucleotides in the wild-type AP-1 consensus site [17]. This result indicates that the AP-1 site (-109 to -102) is necessary for AII-induced ET-1 transcription. We also conclude that the AP-1 consensus motif, in particular, is critical for a high level of basal promoter activity, a finding consistent with those of previous reports [17].

In the setting of stimulation by AII, OE and Prog (100 nM) inhibited ET-1/CAT activity $62 \pm 7\%$ and $75 \pm 6\%$ respectively (Figure 3C). Neither OE nor Prog by themselves significantly affected basal ET-1 promoter activity in cells transfected with any of the above-mentioned reporter constructs (results not shown). Thus OE and Prog appear to negatively regulate ET-1 steady-state mRNA levels differentially, depending on the stimulus, acting through inhibitory effects on transcriptional or possibly through post-transcriptional mechanisms.

MAP kinase studies

To understand the signalling mechanism by which AII stimulates ET-1 transcription and whether OE or Prog interrupt this signalling as a mechanism for negative regulation of transcription, we investigated the role of erk. Using an antibody which detects both erk-1 and erk-2, we found that AII caused a 2.75-fold stimulation above control erk activity after 8 min incubation with the BAEC (Figure 4). This was inhibited 88 and 92% respectively by OE or Prog (100 nM).

Figure 5 AII-stimulated expression of *c-fos* is inhibited by OE or Prog

S1-nuclease protection indicates hybridization of an antisense *c-fos* cRNA with 20 μg of total RNA from BAEC incubated with AII with or without OE or Prog. Under some conditions the MEK inhibitor PD 98059 (10 μM) was added 30 min before Ang II. Data are representative of two separate experiments.

To determine the impact of AII-activated erk kinase for the transcription of ET-1, cells transfected with the -0.143ET-1/CAT reporter were incubated with AII and the soluble MEK inhibitor PD 98059. This compound abrogated the AII-stimulation of ET-1/CAT activity by nearly 70% (Figure 3B) and also comparably inhibited AII stimulation of transfected -0.204ET-1 CAT (results not shown). Since OE and Prog were found to inhibit AII-stimulated erk activity, it is likely that the steroids inhibit AII-induced ET-1 transcription at least in part by interrupting signalling through this kinase.

To provide further support for this idea we determined that OE inhibition of AII-stimulated ET-1/CAT activity was reversed 50% by the co-transfection of an active erk2 construct (Figure 3D). Further, we found that expression of erk2, in the absence of AII, was able to stimulate ET-1/CAT activity comparably with AII. $17\beta\text{-OE}$ inhibited erk2-induced stimulation by 50%. These results solidify our contention (i) that erk can activate ET-1/CAT transcription, (ii) that this is necessary for AII-stimulated ET-1/CAT activity and (iii) that OE can significantly inhibit these mechanisms. We previously showed that this erk2 construct, in the absence of other stimuli, augmented *egr-1* [29] and vascular-endothelial-cell growth-factor transcription [30]. We also determined that $17\alpha\text{-OE}$ had no effect on AII-induced ET-1/CAT activity (Figure 3D).

We then determined the potential role of erk effects on *c-fos* mRNA, and regulation by AII and steroids. It is well known that erk augments *c-fos* transcription through phosphorylating the ternary complex protein elk-1 [33]. AII stimulated the steady-state levels of *c-fos* by 2.1 ± 0.3 -fold, and OE or Prog inhibited this stimulation by 70 and 62% respectively (Figure 5). In addition, the soluble MEK inhibitor PD 98059 abrogated AII stimulation of *c-fos* by 84%. These data indicate the necessity of AII stimulation of erk activity, leading to the increased expression of *c-fos* message.

Half-life studies

To investigate whether OE and/or Prog affect ET-1 transcript degradation, we determined the half-life of the mRNA under a variety of circumstances. We were particularly interested in determining whether OE and/or Prog affect ET-1 mRNA half-life in the setting of serum stimulation, to account for changes in steady-state mRNA. Half-life studies were done in the presence of serum (3%) or AII (100 nM) because the steroids inhibited steady-state ET-1 mRNA only in the presence of these stimulators of ET-1; neither OE nor Prog affected basal ET-1 steady-state

Figure 6 ET-1 transcript half-life studies

BAEC were incubated with (top) serum (3%) or (bottom) AII (100 nM) plus Actino (1 μ M) with or without OE or Prog (100 nM) for 15, 30 or 60 min. The cells were then lysed and total RNA was extracted and hybridized with an antisense cRNA probe for ET-1 as described. Loading was normalized on the basis of a parallel hybridization to an antisense cRNA β -actin probe. Data points shown are the means $\pm$ S.E.M. from four experiments combined. The curves shown are computer-generated best-fit exponential regression of the data points. The ET-1 transcript half-life for each treatment was derived from the equation describing the corresponding best fit curve with relative density (y) set at 50%.

mRNA levels. The ET-1 transcript half-life for each treatment was derived from the equation describing the corresponding best-fit curve with relative density (y) set at 50% [serum + actinomycin (Actino): $y = 115 (10^{-0.014x})$; serum + OE + Actino: $y = 88 (10^{-0.017x})$; Serum + Prog + Actino: $y = 110 (10^{-0.019x})$; AII + Actino: $y = 118 (10^{-0.012x})$; AII + OE + Actino: $y = 124 (10^{-0.012x})$; AII + Prog + Actino: $y = 121 (10^{-0.011x})$]. As shown in Figure 6, in the setting of serum incubation, the calculated half-life of ET-1 mRNA is about 26 min. OE (100 nM) decreased the half-life to 14 min (an approx. 46% decrease), and Prog (100 nM) decreased the half-life to 18 min (an approx. 31% decrease). The effects of OE were almost completely reversed in the presence of ICI 182,780 (results not shown). The decrease in ET-1 mRNA half-life caused by Prog may only account for part of the decrease of steady-state mRNA levels; it is currently unclear what might be an additional contributor. In the setting of incubation with AII, the calculated ET-1 mRNA half-life is approx. 31 min. OE and Prog (100 nM) had little effect on ET-1 transcript degradation in this setting (see Figure 6). This is

Figure 7 Inhibition of serum or AII-stimulated ET-1 protein synthesis by OE and Prog

Cultured BAEC were incubated for 4 h with serum (3%) or AII (100 nM) with or without OE or Prog (100 nM) or OE or Prog alone. Metabolic labelling was carried out as described in the Materials and methods section. The specific treatment conditions are indicated. The arrow indicates a labelled protein with an M_r of approx. 24 000, compatible with prepro-ET-1. A representative study is shown, the experiment being repeated a second time.

not surprising, since OE and Prog exert significant inhibitory transcriptional effects in the setting of AII stimulation and do not appear to augment mechanisms underlying transcript degradation.

Protein-synthesis studies

It is possible that OE and/or Prog inhibit the serum (or AII)-stimulated ET-1 secretion in part by post-transcriptional mechanisms, perhaps at the level of translation. As shown in Figure 7, serum stimulated ET-1 synthesis 4.0 ± 1.3 -fold above control, and this increase was inhibited 58% and 70% by OE and Prog respectively. AII stimulated ET-1 production 2.5 ± 0.97 -fold above control, which was inhibited 81% and 72% by OE and Prog respectively. Neither OE nor Prog by itself had a significant effect on basal ET-1 protein synthesis.

DISCUSSION

In the present study we report that the female sex steroids OE and Prog inhibit ET-1 production and secretion from cultured BAEC, in the setting of stimulation by serum or three important plasma proteins: thrombin, AII or HDL. We further showed that the inhibitory effect of OE on ET-1 secretion is mediated through an OER, whereas Prog acts through a distinct receptor. We also found that serum and AII stimulate the transcription of ET-1 through different molecular mechanisms, and that OE and Prog exert both inhibitory transcriptional and translational effects, depending on the positive stimulus.

The regulation of ET-1 secretion is thought to be mediated by alterations in peptide production. Yanagisawa et al. [34] showed that thrombin treatment of cultured porcine aortic endothelial cells increases preproET-1 mRNA within 1 h of treatment, and transforming growth factor- β 1 has also been shown to rapidly increase both ET-1 mRNA levels and peptide secretion [35]. More recent studies have shown that ET-1 production can be regulated by transcriptional as well as post-transcriptional (translational) mechanisms. For instance, the vasodilatory prostaglandins prostaglandin E_2 and prostacyclin significantly inhibit both basal and serum-stimulated ET-1 secretion from cultured BAEC, resulting from transcriptional modulation [31]. In contrast, atrial natriuretic peptide inhibits ET-1 production from cultured BAEC by decreasing ET-1 translation, accompanied by stabilization of ET-1 mRNA (increased transcript half-life) [19]. HDL treatment of BAEC stimulates ET-1 protein translation

and secretion [21]. However, despite these various studies, little is known about the details of positive or negative transcriptional regulation for the ET-1 gene.

We report that the -98 to -143 region of the preproET-1 promoter is important for basal ET-1 transcription in the absence of stimulators; this is in agreement with previous studies that showed that basal ET-1 transcription is dependent on two critical control elements: a TATC(GATA)-motif-spanning nucleotides -135 to -132 and an AP-1 binding consensus sequence spanning nucleotides -109 to -102 [14]. We also found that AII and serum stimulate ET-1 transcription through different molecular mechanisms. Regarding the regulation of ET-1 transcription by AII, the first 143 bp of ET-1 5'-flanking promoter sequence are required, and the necessary positive regulatory elements lie between -98 and -143 nucleotides. Experiments with the -204 ET-1 wild-type and mutant AP-1/CAT constructs showed that the AP-1 site at nucleotides -109 to -102 is critical for AII-induced ET-1 transcriptional activation. Another report demonstrated that AII treatment of VSMC transfected with an AP-1-CAT reporter construct stimulates CAT activity 2.7-fold [36]. Such stimulation is comparable with the degree of induction by AII of the -143 ET-1/CAT reporter used in the present study. Our findings suggested to us that AII might stimulate the transcription of ET-1 through the modification of AP-1 binding proteins, in particular, *c-fos*.

To provide experimental support for this idea, we showed that AII stimulates erk activity, that this is necessary for *c-fos* production, and that erk activity is also necessary for increased ET-1 transcription in response to AII. AII has previously been shown to stimulate erk activity [24], and erk transactivates *c-fos* through phosphorylating the transcription factor Elk-1 [33]. We found that OE or Prog inhibits the ability of AII to stimulate erk and this indicates a novel pathway by which sex steroids can negatively modulate transcription. We also report that estradiol inhibits the ability of erk2 to stimulate ET-1/CAT activity. Although OE or Prog may have additional genomic effects on the prepro ET-1 gene via their cognate nuclear receptors, our results suggest that a substantial part of the negative transcriptional regulation by the sex steroids is through disrupting the positive signalling to the nucleus. Since OE inhibited erk-induced ET-1/CAT activity in the absence of AII, we propose that this steroid can interfere with both the action and activation of this proline-directed kinase. The precise upstream signalling program by which AII stimulate and OE (or Prog) inhibits erk activity is a target for further investigation.

OE or Prog also inhibited the ability of AII to stimulate the production of *c-fos*, the latter being critically dependent on erk activation. Although these studies do not definitively link these two actions of the sex steroids, the results support the idea that they are sequentially related. However, it is possible that sex-steroid inhibition of *fos* could occur through additional mechanisms, or that inhibition of erk signalling alters AP-1-mediated *trans*-activation of ET-1 by another means. Recently, we showed that OE or Prog can inhibit the mitogenic effects of serum or ET-1 on cultured human VSMC [37]. This occurred in part through OE binding a putative membrane OER, resulting in the abrogation of growth-factor signalling through MEK and erk to the nuclear growth program, including *c-fos* induction.

With regard to serum, reporter activity in cells transfected with the -143 , -109 , and -43 ET-1/CAT reporter constructs were all stimulated. Although there may be auxiliary positive and negative regulatory elements between nucleotides -143 and -43 , these data suggests that the element(s) primarily necessary for serum-induced ET-1 transcription lies within the first 43 bp of 5'-flanking promoter sequence. In scanning this sequence, a

few possible binding sites for known transcription factors were found, including LVC, TCF-1 and GMCSF-CS sites [38–43]. However, these factors have only been found in T- and B-lymphocytes. On the basis of the cell specificity of these factors, it appears unlikely that they would function in the regulation of ET-1 gene expression in BAEC. It is more likely that serum is inducing the production of a novel factor which interacts through the first 43 bp of ET-1 promoter sequence. It must be appreciated that a human ET-1 promoter was used in the reporter studies, and hence there may be differences of regulation when transfected into BAEC.

The reporter and half-life experiments indicate that OE and Prog inhibit stimulated steady-state ET-1 mRNA through differential mechanisms, depending on the stimulus. In the setting of AII stimulation, both OE and Prog inhibited ET-1 transcription, most likely via interrupting signalling leading to the formation of the AP-1 complex. Although OER and Prog receptor may also have direct genomic effects as well, the first 143 bp of ET-1 5'-flanking promoter sequence contain no obvious OER or Prog-receptor DNA-binding elements. Perhaps ER or Prog receptor also modify AP-1 effects through a protein–protein interaction, thus interfering with AP-1 binding to its consensus site ([44]; reviewed in [45]). Nevertheless, our data is the first to attribute a negative transcriptional action of sex steroids to the interruption of signalling by a positive *trans*-activating stimulus.

In the setting of stimulation by serum, neither OE nor Prog inhibited transcriptional activity, indicating that neither can interact with the factor(s) involved in serum-induced activation of the ET-1 gene. OE accelerated transcript degradation, whereas Prog had a more modest effect. Transcript degradation by OE can account for the majority of the observed decrease in serum-stimulated steady state ET-1 mRNA, but the effect of Prog may only be partially explained by this observation. It is currently unclear what might be an additional contributor. An interesting issue is how OE and Prog can affect transcript degradation differentially depending on the stimulus. Eukaryotic transcript degradation is complex, involving many different enzyme systems [46]. An individual transcript may simultaneously be the substrate for multiple degradative pathways. Moreover, the rate and mechanism of decay caused by a given pathway can vary depending on cellular conditions. Therefore serum may stimulate a degradative pathway that is selectively enhanced by OE or Prog, one which is distinct from that initiated by AII. This may involve different regulatory elements for mRNA decay, utilized by AII or serum and hence account for the differential effects of the sex steroids.

It was also observed that OE and Prog each decrease ET-1 protein synthesis in the setting of serum or AII stimulation. Inhibition of ET-1 translation by the sex steroids, in the setting of serum stimulation, fully accounts for the decrease in ET-1 secretion. By contrast, the observed inhibition of AII-stimulated ET-1 translation by OE or Prog probably results from inhibition of transcription, followed by translation, although an independent effect on protein synthesis may contribute.

The concentrations of the sex steroids used in the present study, particularly OE, generally exceed serum levels in women. However, following hormone-replacement therapy, serum OE or Prog levels of 30 nM have been measured (reviewed in [47]), and local tissue concentrations are probably even greater [48]. The K_d for the Prog receptor is higher than that of OE for the OER, and serum Prog levels exceed those of OE, so its relevance to physiological is more apparent. One must also be cautious about extrapolating *in vitro* findings to human physiology. However, it has been demonstrated that women given postmenopausal hormone-replacement therapy have significantly decreased

plasma ET-1 levels [49], supporting the idea of direct actions of sex steroids that we have shown here.

In summary, OE and Prog inhibit ET-1 production in cultured BAEC, in the setting of stimulation by several physiologically relevant stimuli. It is known that the incidence of cardiovascular disease in women rapidly rises after the menopause [1], and that replacing the female sex steroids has a vascular-protective effect [50]. Inhibition of ET-1 production from endothelial cells may provide a novel mechanism by which OE and Prog are protective in the vasculature.

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