Endothelin (ET)-1 and ET-3 inhibit estrogen and cAMP production by rat granulosa cells *in vitro*

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Abstract

Endothelin (ET)-1 and ET-3, two peptides with a potent vasoconstrictive property, produce a variety of biological effects in different tissues by acting through two different receptors, the ET-1 selective ET_A receptor and the non-selective ET_B receptor. An increasing body of literature suggests that ET-1 acts as a paracrine/autocrine regulator of ovarian function. Indeed, ET_B receptors have been identified in rat granulosa cells and ET-1 is a potent inhibitor of progesterone production. In contrast, inconsistent data have been reported about the role of ET-1 on estrogen production and the effects of ET-3 are not known. Therefore, the present study was undertaken to evaluate the effects of ET-1 and ET-3 on estrogen and cAMP production, and the receptor type involved. Given that prostanoids modulate ovarian steroidogenesis and that many actions of ETs are mediated by these compounds, we also evaluated whether the effects of ETs on estrogen and cAMP production might be prostanoid-mediated. ET-1, ET-3, and safarotoxin-S6c (SFX-S6c), a selective ET_B receptor agonist, inhibited basal estrogen production by granulosa cells obtained from immature, estrogen-primed female rats, in a concentration-dependent manner. All three peptides were also capable of inhibiting the production of estrogen stimulated by a half-maximal (1 mIU/ml)

and a maximally stimulatory (3 mIU/ml) concentration of FSH. ET-1 and ET-3 dose-dependently suppressed basal and FSH (1 mIU/ml)-stimulated cAMP production. ET-3 and SFX-S6c were significantly more potent than ET-1 in suppressing estrogen production, suggesting that this effect was not mediated by the ET_A receptor. Indeed, BQ-123, a selective ET_A receptor antagonist, did not influence the inhibitory effects of ET-1 and ET-3 on basal and FSH-stimulated estrogen release. To determine a possible involvement of prostanoids, we evaluated the effects of maximally effective concentrations of ET-1 and ET-3 on estrogen and cAMP production in the presence of indomethacin, a prostanoid synthesis inhibitor. This compound did not have any effect on the suppressive effects of ETs on basal or FSH (1 mIU/ml)-stimulated estrogen or cAMP production.

In conclusion, ET-1 and ET-3 were able to inhibit estrogen and cAMP production by rat granulosa cells, indicating that the inhibitory effects of ETs on ovarian steroidogenesis are not limited to progesterone biosynthesis. This effect does not appear to be mediated by prostanoids or by the classical ET_A and ET_B receptors, at least under these experimental conditions.

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Introduction

Endothelins (ETs), a family of 21-amino acid peptides, are present in three different isoforms coded by separate genes, endothelin (ET)-1, ET-2, and ET-3 respectively (Inoue *et al.* 1989). They were first isolated from endothelial cells by virtue of their potent vasoconstrictor properties (Yanagisawa *et al.* 1988). However, it soon became apparent that these peptides produce a variety of biological effects (Masaki 1993) mediated by two distinct G-protein-coupled receptors named ET_A and ET_B (Watanabe *et al.* 1989). Although these receptors show about 63% amino acid homology, they bind ET isopeptides and analogs with different affinities. ET_A has a more elevated affinity for ET-1 than for ET-3 and ET_A-mediated cellular effects are

blocked by the peptide antagonist, BQ-123. Conversely, the ET_B receptor binds ET-1 and ET-3 with equal affinity and it is selectively activated by safarotoxin-S6c (SFX-S6c) (Bax & Saxena 1994).

A number of studies have shown that ETs participate in the regulation of the endocrine system (Hirai *et al.* 1991, Masaki 1993, Calogero *et al.* 1994) and recent evidence suggests that ETs may act as paracrine/autocrine regulators of ovarian endocrine function. Indeed, it has been shown that ETs are able to inhibit basal and gonadotropin-stimulated progesterone production, cAMP accumulation, and morphological transformation of granulosa cells in culture (Iwai *et al.* 1991, Flores *et al.* 1992, Tedeschi *et al.* 1992, 1994, Kamada *et al.* 1993). Although the effects of ETs on progesterone production are well known,

inconsistent data have been reported on the role of ET-1 in estrogen production by granulosa cells (Kamada *et al.* 1992, Tedeschi *et al.* 1994), and the effects of ET-3 are not known. Hence, this study was undertaken to evaluate the role of ET-1 and ET-3 on estrogen and cAMP production by granulosa cells obtained from immature, estrogen-primed rats, and to identify the ET receptor which mediates this effect. In consideration of the fact that prostanoids exert inhibitory effects on ovarian steroidogenesis (Behrman & Caldwell 1986) and that a number of ET-1 actions are prostanoid-mediated (Anderson *et al.* 1995, Howarth *et al.* 1995), we also evaluated whether indomethacin, a prostanoid synthesis inhibitor, modulates the effects of ET-1 or ET-3 on estrogen or cAMP production.

Materials and Methods

Rat granulosa cell culture

Rat granulosa cells were cultured as previously reported (Jia & Hsueh 1986, Calogero *et al.* 1996). Briefly, intact female immature (25 days old) Sprague-Dawley rats (Charles River, Calco, CO, Italy) were implanted subcutaneously with a 10 mm Silastic brand capsule (Dow Corning, Midland, MI, USA) filled with diethylstilbestrol (DES; Sigma Chemical Co., St Louis, MO, USA). Four days after implantation, rats were sacrificed by cervical dislocation, and ovaries were removed for granulosa cell collection. Ovaries were decapsulated, follicles were punctured with 27-gauge hypodermic needles, and granulosa cells were carefully expressed into McCoy's 5a medium (Life Technologies, Paisley, Strathclyde, UK) to make a single cell suspension that was used for control and experimental cultures. An aliquot was diluted with trypan blue stain, and viable cells were counted with a hemocytometer. Cells were plated into 16 mm, 24-multiwell plates (Corning, Milan, Italy) at a density of 50 000 viable cells/well/0.5 ml at 37 °C in a humidified, 95% air-5% CO₂ water-jacketed incubator in the presence of graded concentrations of ET-1 (Peninsula Laboratories Inc., Belmont, CA, USA), ET-3 (Peninsula), SFX-S6c (Sigma), BQ-123 (Peninsula), and/or indomethacin (Sigma) for six days. BQ-123 or indomethacin were added simultaneously with ETs. Time-course studies showed that maximal estrogen production in response to increasing concentrations of follicle-stimulating hormone (FSH) (NIDDK hFSH B-1, AFP-8792B) was achieved after six days of incubation (Calogero *et al.* 1996). Each treatment was applied in triplicate within each experiment and each experiment was replicated at least three times. Incubation was carried out using McCoy's 5a medium supplemented with 2 mM L-glutamine (Life Technologies), 200 U/ml penicillin, 200 µg/ml streptomycin sulfate, 0.5 µg/ml fungizone (Life Technologies), 10⁻⁶ M androstenedione (Sigma), 10⁻⁷ M DES (Sigma), 37.5 ng/ml human chorionic gonadotropin (hCG, CR-127; NIDDK,

Bethesda, MD, USA), 35 µg/ml methyl-isobutylxanthine (MIX; Sigma), and 1.25 µg/ml insulin (Eli Lilly Co., Indianapolis, IN, USA). DES and MIX were added to enhance the sensitivity of granulosa cells to FSH (Jia & Hsueh 1986). At the end of the culture period, medium samples were frozen at -20 °C until assayed for estrogen.

Estrogen radioimmunoassay

The concentration of estrogen in the incubation medium was measured directly, without extraction, by radioimmunoassay, as previously reported (Calogero *et al.* 1996). Aliquots (200 µl) of medium, diluted 1:40–1:100, or standard solutions were incubated with 100 µl anti-serum (final dilution 1:10 000), and 100 µl [³H]estradiol (E₂) (about 9000 c.p.m.) at 4 °C for 18–20 h. Ice-cold charcoal-dextran was then added to achieve separation of bound from free labeled hormone. Tubes were centrifuged at 1500 g at 4 °C for 11 min, the supernatants were collected and counted in a β-counter after the addition of scintillation fluid. The anti-estrogen serum (RD/94/84; Analytical Antibodies, Segrate, MI, Italy) bound 40.7 ± 2.5% (*n*=11) of [³H]E₂. Non-specific binding was 3.1 ± 0.4%. The intra- and interassay coefficients of variation were 3.6 ± 0.3% and 13.8% respectively.

cAMP measurement

At the end of the incubation, the medium was collected for estrogen measurements and 0.5 ml ice-cold ethanol (90%) was added to each well. The plate was placed at -20 °C for 30 min and subsequently at 4 °C for 60–90 min. Thereafter, each sample was collected and centrifuged in a microfuge, the supernatant collected, dried, and stored at -20 °C until the day of the RIA. cAMP was measured by RIA using a commercially available kit (Amersham International plc, Amersham, Bucks, UK). cAMP antiserum bound 59.8 ± 2.8% (*n*=2) of ¹²⁵I-cAMP (about 7000 c.p.m./100 µl). The detection limit (ED₉₀) of the assay was 19.6 fmol/tube (49.0 fmol/well). The intra- and interassay coefficients of variation were 1.3 ± 0.3% and 5.7% respectively.

Data analysis

Results, reported as means ± S.E.M. throughout the study, are expressed as percentage of basal estrogen production. Statistical evaluation was performed by analysis of variance (ANOVA) followed by Duncan's multiple range test. The effects of ET-1, ET-3, or SFX-S6c on basal and FSH-stimulated estrogen dose-response curves were analyzed applying the four parameter logistic equation; a computer program, 'Allfit', was employed for the computation (DeLean *et al.* 1978). Significance was accepted at a *P* value below 0.05.

Figure 1 Effects of graded concentrations of ET-1 (○), ET-3 (●), and SFX-S6c, a selective ET_B receptor agonist (△), on basal estrogen production by rat granulosa cells. Results (means $\pm$ S.E.M.) are expressed as percentage of estrogen accumulation by granulosa cells incubated without any test substance for six days. Each treatment was applied in triplicate in each of at least three different experiments. * $P < 0.05$ vs concentration zero.

Results

Basal estrogen accumulation over six days was 1.25 ± 0.04 ng/well. ET-1, ET-3, and SFX-S6c suppressed basal estrogen production in a concentration-dependent fashion ($P < 0.001$, one-way ANOVA) (Fig. 1). ET-3 and the ET_B receptor activator SFX-S6c were more potent than ET-1. Indeed, 10^{-11} M was the lowest effective concentration tested, whereas it was 10^{-10} M for ET-1. Accordingly, the half-maximal inhibitory concentrations (IC_{50} s) of ET-3 and SFX-S6c were significantly lower than that of ET-1 and not statistically different from each other ($P < 0.05$; see Table 1).

FSH stimulated the production of estrogen by about 7- to 8-fold at a concentration of 1 mIU/ml (half maximal) and by about 16- to 17-fold at a concentration of 3 mIU/ml (maximally effective). ET-1, ET-3, and SFX-S6c were able to inhibit the stimulation produced by 1 and 3 mIU/ml FSH in a concentration-dependent fashion ($P < 0.005$, one-way ANOVA) (Fig. 2). Their lowest effective concentration was about 10^{-11} M, whereas that of ET-1 was about 10-fold higher. Similar to basal estrogen production, the IC_{50} s of ET-3 and SFX-S6c were significantly lower

Figure 2 Effects of graded concentrations of ET-1 (○), ET-3 (●), and SFX-S6c, a selective ET_B receptor agonist (△), on estrogen production by rat granulosa cells exposed to a half-maximal (A) and a maximally (B) stimulatory concentration of FSH. Results (means $\pm$ S.E.M.) are expressed as percentage of estrogen accumulation by granulosa cells incubated without any test substance for six days. Each treatment was applied in triplicate in each of at least three different experiments. * $P < 0.05$ vs concentration zero.

than that of ET-1 and not statistically different from each other ($P < 0.05$; see Table 1).

Basal intracellular cAMP content was 82.4 ± 7.2 fmol/well. ET-1 and ET-3 inhibited, in a concentration-dependent manner, the intracellular content of cAMP both in basal conditions and in response to 1 mIU/ml FSH (Table 2). Both peptides were equipotent in suppressing basal and FSH-stimulated cAMP accumulation and, at the highest concentration tested (10^{-9} M), they were able to overcome completely the stimulation produced by FSH (1 mIU/ml). Overall, the suppressive effect of ET-1 and

Table 1 Half maximal inhibitory concentrations (IC_{50}) of ET-1, ET-3, and SFX-S6c on basal and FSH-stimulated estrogen production by rat granulosa cells incubated for six days. Results are means $\pm$ S.E.M.

	ET-1 (pM)	ET-3 (pM)	SFX-S6c (pM)
Basal	107.5 ± 20.2	$1.3 \pm 0.6^*$	$2.9 \pm 2.0^*$
FSH 1 mIU/ml	50.0 ± 10.0	$19.0 \pm 5.0^*$	$5.0 \pm 2.0^*$
FSH 3 mIU/ml	23.0 ± 3.0	$3.8 \pm 0.6^*$	$11.0 \pm 1.2^*$

* $P < 0.05$ vs IC_{50} of ET-1.

Table 2 Concentration-dependent effects of ET-1 and ET-3 on the intracellular cAMP content of rat granulosa cells cultured under basal or FSH (1 mIU/ml)-stimulated conditions. Results (means ± S.E.M.) are expressed as a percentage of cAMP accumulation in granulosa cells incubated without any test substance for six days. Each treatment was replicated three times in each of five different experiments

	ET-1	ET-3
Endothelin concentration		
Basal		
0	100.0 ± 5.8	100.0 ± 5.8
10 ⁻¹⁰ M	91.0 ± 5.1	94.6 ± 6.9
10 ⁻⁹ M	84.5 ± 6.4 ^a	70.4 ± 6.3 ^{a,b}
10 ⁻⁸ M	81.1 ± 4.5 ^a	67.3 ± 6.4 ^{a,b}
FSH (1 mIU/ml)		
0	237.5 ± 18.6	237.5 ± 18.6
10 ⁻¹¹ M	201.6 ± 13.1	197.1 ± 17.8
10 ⁻¹⁰ M	150.9 ± 16.5 ^{a,c}	164.9 ± 20.4 ^{a,c}
10 ⁻⁹ M	114.2 ± 10.4 ^{a,c}	102.8 ± 10.2 ^{a,c}

^a*P*<0.05 vs concentration zero; ^b*P*<0.05 vs concentration 10⁻¹⁰ M; ^c*P*<0.05 vs concentration 10⁻¹¹ M.

ET-3 on intracellular cAMP content reached statistical significance at concentrations higher than those required to suppress estrogen release.

We evaluated whether the selective ET_A receptor antagonist, BQ-123, could antagonize the inhibitory effects of ET-1 or ET-3 on basal or FSH-stimulated estrogen production. The results of these experiments showed that BQ-123 did not have any effect on ET-1- or ET-3-suppressed basal or FSH-stimulated estrogen production (Fig. 3), thus ruling out the involvement of ET_A receptors. To evaluate a possible involvement of prostanoids, we tested the effects of maximally effective concentrations of ET-1 and ET-3 on estrogen and cAMP production in the presence of indomethacin, a cyclooxygenase inhibitor. Indomethacin, employed at concentrations of 10⁻⁷ and 10⁻⁶ M, did not modify the suppressive effects of either peptide on basal or FSH (1 mIU/ml)-stimulated estrogen and cAMP production (Fig. 4).

Discussion

Experimental evidence suggests that ETs are auto/paracrine regulators of ovarian function. First, immunoreactive ET-1 is abundant in ovarian follicular fluid (Iwai *et al.* 1991, Magini *et al.* 1996) where it is partly released from granulosa cells (Iwai *et al.* 1991, Kamada *et al.* 1993). Secondly, ET receptors are localized in granulosa cells. Human luteal-granulosa cells express mainly ET_A receptors (Kamada *et al.* 1995, Mancina *et al.* 1997), whereas porcine and rat granulosa cells express almost exclusively ET_B receptors (Kamada *et al.* 1992, Iwai *et al.* 1993, Tedeschi *et al.* 1994). However, a more recent study showed that cultured porcine granulosa cells express the

ET_A receptor, because ET-1 was more potent than ET-3 in displacing the binding of labelled ET-1 in a radio-receptor assay (Flores *et al.* 1995). Thirdly, ETs promote rat ovarian DNA polymerase activity (Usuki *et al.* 1991) and porcine granulosa cell proliferation (Iwai *et al.* 1991). Lastly, many studies have consistently reported that ET-1, ET-2, and ET-3 suppress the production of progesterone and cAMP basally and following stimulation with gonadotropins (Iwai *et al.* 1991, Flores *et al.* 1992, Kamada *et al.* 1992, 1993, Tedeschi *et al.* 1992, 1994).

The findings of the present study showed that ET-1 and ET-3 are also able to suppress the production of estrogen and cAMP from rat granulosa cells both basally and in response to FSH. This inhibition does not seem related to deleterious effects of ETs on cell attachment or survival in culture since it has been shown that ETs do not have cytotoxic effects on granulosa cells (Tedeschi *et al.* 1992, 1994, Hurwitz *et al.* 1992). To our knowledge, only two other studies have explored the effects of ETs on estrogen production and both of them tested only the effects of ET-1 (Kamada *et al.* 1992, Tedeschi *et al.* 1994); hence this is the first report showing an inhibitory effect of ET-3 on estrogen and cAMP production from rat granulosa cells. Our results are similar to those reported by Tedeschi and co-workers (1994) who showed that ET-1, at a concentration of 10⁻⁷ M, inhibited FSH-stimulated, but not basal, estrogen production by suppressing aromatase activity, as determined by the tritiated water method. On the other hand, Kamada *et al.* (1992) found that ET-1 did not have any effect on estrogen production from porcine granulosa cell cultures. The reason for this discrepancy is not known at the present time. It may relate to species differences, although no species difference has been reported for progesterone production from porcine granulosa

Figure 3 Effects of the selective ET_A receptor antagonist, BQ-123, on basal (upper panel), FSH 1 mIU/ml (middle panel)-stimulated, and FSH 3 mIU/ml (lower panel)-stimulated estrogen production suppressed by ET-1 or ET-3. Results (means $\pm$ S.E.M.) are expressed as percentage of estrogen accumulation by granulosa cells incubated without any test substance for six days. Each treatment was applied in triplicate in each of at least three different experiments. * $P < 0.05$ vs control (CTL).

cell cultures. We found that ET-1 was less effective than ET-3 or SFX-S6c, a selective ET_B receptor agonist, in suppressing estrogen production in all experimental conditions tested, ruling out an involvement of the ET_A receptor. Accordingly, the selective ET_A receptor antagonist, BQ-123, was unable to antagonize the inhibitory effects of ET-1 and ET-3 on estrogen production. The greater potency of ET-3 and SFX-S6c suggests that their effects may be mediated by a non- ET_B receptor. The existence of a non- ET_B receptor-mediated effect of ET-1 has recently been reported on human airway smooth muscle cells (Goldie *et al.* 1995). Alternatively, it may be hypothesized that cell culture conditions may affect the

function of ET receptors, as has recently been reported for human hepatic stellate cells (Pinzani *et al.* 1996). In this regard, Angelova *et al.* (1996) found at least three classes of ET binding sites in sheep choroid plexus cells. One of these is the conventional ET_A receptor that mediates an increase in intracellular levels of inositol 1,4,5-trisphosphate in response to ET-1. The other two have much higher binding affinity and are not coupled to the common signal transduction pathways. However, northern blot analysis showed the presence of only the ET_A receptor mRNA in these cells. Therefore, it remains to be determined whether these multiple binding sites are distinct gene products, multiple affinity states of a single receptor molecule or a result of cooperative association of one site with either the ligand or with other proteins.

Since FSH stimulates aromatase activity in granulosa cells by increasing the generation of cAMP (Catt & Pierce 1986), we evaluated whether ET-1 and ET-3 affected estrogen production by interfering with this ability of FSH. The finding that both ETs are able to suppress basal and FSH-stimulated cAMP formation suggests that their suppressive effects on estrogen accumulation are mediated by the suppressive action of ETs on the cAMP generating mechanism. However, this does not seem to be the only mechanism responsible, since estrogen production was inhibited at lower concentrations of ETs than those necessary to reduce cAMP content significantly. Indeed, ETs are able to interfere with other intracellular signaling pathways in granulosa cells (Flores *et al.* 1992).

It has been shown that ET-1 is able to stimulate the release of prostaglandins and thromboxanes from different cell types in a dose-dependent manner (Wu *et al.* 1993, Markewitz *et al.* 1995, Takimoto *et al.* 1996). Accordingly, a number of ET actions appear to be mediated by prostanoids. For example, indomethacin blunts the vasoconstriction produced by ET-1 on placental blood flow (Howarth *et al.* 1995). Similarly, Anderson *et al.* (1995) reported that the effects of ET-1 on water reabsorption in conscious dogs are enhanced by pretreatment with prostanoid biosynthesis inhibitors. In this study, we tested the hypothesis that the suppression of estrogen and cAMP production induced by ETs on granulosa cells might also be mediated by prostanoids, which *per se* modulate ovarian steroidogenesis (Behrman & Caldwell 1986). However, the lack of indomethacin effects, despite the reported efficacy of the concentration range tested on ovarian prostaglandin production (Band *et al.* 1986, Yoshimura *et al.* 1988), did not support this hypothesis. In conclusion, this study shows that ET-1 and ET-3 suppressed basal and FSH-stimulated estrogen and cAMP production by rat granulosa cells, indicating that the inhibitory effects of ETs on ovarian steroidogenesis are not limited to C_{21} progestin biosynthesis. This effect is not mediated by prostanoids or the classical ET_A or ET_B receptors, suggesting the existence of a novel ET receptor with greater affinity for ET-3 than ET-1.

Figure 4 Effects of indomethacin, a prostanoid synthesis inhibitor, on basal (upper panels) and FSH (1 mIU/ml)-stimulated (lower panels) estrogen (open bars) and cAMP (solid bars) production suppressed by maximally effective concentrations of ET-1 or ET-3. Results (means $\pm$ S.E.M.) are expressed as percentage of estrogen or cAMP accumulation by granulosa cells incubated without any test substance for six days. Each treatment was applied in triplicate in each of at least three different experiments. * $P < 0.05$ vs control (CTL).

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