Hydroxyestrogens inhibit angiogenesis in swine ovarian follicles

G Basini¹, S Bussolati¹, S E Santini¹, F Bianchi², M Careri², A Mangia², M Musci² and F Grasselli¹

¹Dipartimento di Produzioni Animali, Biotecnologie Veterinarie, Qualità e Sicurezza degli Alimenti - Sezione di Fisiologia Veterinaria and ²Dipartimento di Chimica Generale ed Inorganica, Chimica Analitica, Chimica Fisica, Università degli Studi di Parma, Via del Taglio, 8, Viale Uberti 17/A, 43100 Parma, Italy

(Correspondence should be addressed to G Basini; Email: basini@unipr.it)

Abstract

The rapid, controlled, and cyclical nature of angiogenesis in the ovarian follicle suggests the potential for sex steroids to influence neovascularization. Angiogenesis is regulated by a local balance between the levels of endogenous stimulators and inhibitors. Multiple lines of evidence suggest that estrogens stimulate angiogenesis via effects on endothelial cells. However, it is of outstanding value to investigate the negative control of this process. Since the main estrogen metabolites, 2-hydroxyestradiol and 4-hydroxyestradiol (4-OHE2) have been demonstrated to function as anti-estrogen in several estrogen-dependent organs; the aim of this study was to investigate their potential involvement in the modulation of follicular angiogenesis. Hydroxyestrogens were quantified in swine follicular fluid and their effects were studied on granulosa cell vascular endothelial growth factor (VEGFA) production and tested in an angiogenesis bioassay. Our study documents that these molecules are physiologically present in swine follicular fluid and their concentrations significantly (P<0.001) increase during follicle development. Moreover, angiogenesis bioassay revealed that both hydroxyestrogens significantly (P<0.001) inhibited new vessel growth. We evidenced that the most potent negative effect is mediated by 4-OHE2. The anti-angiogenic potential of this molecule is also supported by its ability to inhibit (P<0.001) VEGFA production by granulosa cells. Increased knowledge in this area is of utmost importance for future therapeutic options to contrast infertility disorders associated with aberrant angiogenesis; this would be also very useful for the treatment of diseases characterized by deregulated angiogenesis and vascular regression.


Introduction

In adult organisms, angiogenesis is virtually absent under normal conditions. An exception takes place in the ovaries where neovascularization is imposed recurrently by cyclic development of transient structure and cyclic repair of damaged tissues (Fraser 2006). This observation suggests the potential for sex steroids to influence vessel growth. In particular, increased incidence in premenopausal females of diseases involving endothelial cell proliferation, such as Takayasu's arteritis and lupus, suggests a possible role for estrogens in angiogenesis (Ahmed et al. 1985, Shelhamer et al. 1985). Experimental evidence further supports the involvement of these steroids in physiological and pathological vascularization; among these, the finding that angiogenesis is impaired in estrogen receptor knockout mice and also the observation that estrogen receptor antagonists can inhibit endothelial cell proliferation (Losordo & Isner 2001).

Within the ovarian follicle, which represents an outstanding system for studying the physiological process of angiogenesis (Basini et al. 2004, 2005, 2007a), it has been demonstrated that estradiol stimulates the expression of vascular endothelial growth factor (VEGFA) mRNA in bovine granulosa cell in vivo and in vitro (Shimizu & Miyamoto 2007). In recent years, the pro-angiogenic mechanisms involved in follicular angiogenesis have been extensively studied while little is known about tempering factors. However, a deeper understanding of the molecular control of angiogenesis is needed in order to provide a novel approach to manipulate reproductive function; moreover, insight should be gained into the changes that precipitate the uncontrolled angiogenesis responsible for the growth of solid tumors. In a previous study (Basini et al. 2007b), we demonstrated that 2-methoxyestradiol is a potential physiological inhibitor of follicular angiogenesis. Since several lines of experimental evidence (Ho 2004, Salih et al. 2007) suggest that estrogen metabolites play an important role in angiogenesis control, our aim was to study the main hydroxyestrogens, 2-hydroxyestradiol (2-OHE2), and 4-hydroxyestradiol (4-OHE2), as factors potentially involved in the modulation of follicular angiogenesis; these metabolites have been demonstrated to function as anti-estrogens in several estrogen-dependent organs (Al-Hendy & Salama 2006). To do so, we first attempted to quantify 2-OHE2 and 4-OHE2 concentrations in swine follicular fluid collected from follicles at different stages of development. Thereafter, the effects of both hydroxyestrogens on VEGFA production from swine granulosa cells were studied. In addition, using an angiogenesis bioassay developed in our laboratory (Basini et al. 2007a), we tested 2-OHE2 and 4-OHE2 on porcine aortic endothelial cell (AOC) growth in a three-dimensional fibrin gel matrix.
Materials and Methods

All the reagents were obtained from Sigma unless otherwise specified.

Follicular fluid and granulosa cell collection

Swine ovaries were collected at a local slaughterhouse, placed in cold PBS (4 °C) supplemented with penicillin (500 IU/ml), streptomycin (500 µg/ml), and amphotericin B (3-75 µg/ml), maintained in a freezer bag, and transported to the laboratory within 1 h. After a series of washings with PBS and ethanol (70%), follicles were divided into three classes corresponding to the diameter: small (<3 mm), medium (3–5 mm), and large follicles (>5 mm). Follicular fluid was collected with a 26 gauge needle from follicles of each class to determine 2-OHE2 and 4-OHE2 contents.

A 26 gauge needle was employed to aseptically harvest granulosa cells from follicles >5 mm, since we previously demonstrated (Basini et al. 2004) that large follicles are mostly involved in the angiogenic events. The cells were released in medium containing heparin (50 IU/ml), centrifuged for involution of the angiogenic events. The cells were seeded in culture medium (CM) composed by M199 supplemented with sodium bicarbonate (2.2 mg/ml), BSA (0.1%), penicillin (100 IU/ml), streptomycin (100 µg/ml), amphotericin B (2.5 µg/ml), selenium (5 ng/ml), and transferrin (5 µg/ml). Once seeded, the cells were incubated at 37 °C in a humidified atmosphere (5% CO2).

Gas chromatograph (GC)–mass spectrometry (MS) analysis

GC-MS analysis was performed using a HP 6890 Series Plus GC (Agilent Technologies, Milan, Italy) equipped with Mass Selective Detector (MSD) 5973 (Agilent Technologies). Helium was used as the carrier gas at a flow rate of 1 ml/min; the GC was operated in splitless mode (injection volume: 0.5 µl) with the PTV injector (Agilent Technologies) equipped with a PTV multi-baffled liner (i.d. 1.5 mm, Agilent Technologies) and operating under the following conditions: 70 °C for 0-5 min and 700 °C/min to 280 °C. Chromatographic separation was performed on a 30 m × 0.25 mm, df 0.25 µm factor four capillary column equipped with a EZ-guard column (Varian, Torino, Italy). The following GC oven temperature program was applied: 70 °C, 30 °C/min to 230 °C, 230 °C for 8 min, 10 °C/min to 250 °C, 30 °C/min to 310 °C, and 310 °C for 15 min. Transfer line and source were maintained at 250 and 230 °C respectively. The mass spectrometer was operated in time selected ion monitoring mode (SIM) by recording the current of the following ions: from 0600 to 1020 min m/z 482, 369, 256 for 2-FE; and from 1020 to 1500 min m/z 576, 463, 421 for 4-OHE and 4-OHE. A solvent delay of 6 min was applied. The molecular ions were used for quantitation, whereas the corresponding ion ratios were used to confirm the identification of the analytes. A dwell time of 100 ms was used for all the ions. Preliminary full scan electron impact data were acquired to determine appropriate masses for SIM under the following conditions: ionization energy, 70 eV; mass range, 50–600 amu; and scan time, 3 scan/s. All the analyses were performed setting the electron multiplier voltage at 1700 V.

Signal acquisition and elaboration were performed using the HP Chemstation (Agilent Technologies).

Solid-phase extraction (SPE)

2-Fluoroestradiol (internal standard 2-FE, 99-9% purity), (2-OHE2 and 4-OHE2, 98% purity) were purchased from Steraloids (London, UK). Stock solutions were prepared in acetone at a concentration of 100 mg/l and stored at −20 °C for up to 2 weeks, whereas standard and working solutions were prepared daily by dilution from the stock solutions. To 1-0 ml follicular fluid spiked with 10 µg/kg (µg/kg refers to the follicle weight) of 2-FE, 1 ml acetone was added drop by drop to obtain protein precipitation. After centrifugation (400 g, 10 min), the supernatant was applied to the SPE C18 cartridges (Supelco, Bellefonte, PA, USA), previously conditioned with 3 ml diethyl ether. Elution was performed with 4 ml ether that was then evaporated to dryness under a nitrogen stream before the derivatization process.

Derivatization

An aliquot of 15 µl dry toluene (reaction solvent) and 5 µl trifluoroacetic anhydride (derivatizing agent) were added to sample extract. The derivatization procedure was carried out for 10 min at 30 °C. At the end of the reaction, the derivatized samples were submitted to GC–MS analysis.

VEGFA production

The 10⁵ granulosa cells in 1 ml CM +1% fetal calf serum (FCS) were seeded in each well of a 24-well plate, treated with 1, 10, or 100 ng/ml of 2-OHE2 or 4-OHE2 and incubated for 48 h. VEGFA content in culture media was quantified by an ELISA (QuantiKine, R&D System, Minneapolis, MI, USA); this assay, developed for human VEGFA detection, has been validated for pig VEGFA (Barboni et al. 2000). The assay sensitivity was 8.74 pg/ml, the inter- and intra-assay CVs were always less than 7%. A Spectra Shell microplate reader (SLT Spectra, Milan, Italy), set to read 450 nm emission, was used to quantify the reaction product.

Angiogenesis bioassay

Endothelial cell culture An immortalized porcine aortic endothelial cell line (AOC; Carrillo et al. 2002) was generously provided by José Yelamos (Hospital Universitario Virgen de la
Arrixaca, El Palmar, 30120 Murcia, Spain). In all experiments, AOC at 19th passage was used and seeded in CM.

Three-dimensional endothelial cell culture on a fibrin gel support The microcarrier-based fibrin gel angiogenesis assay was performed as described by Grasselli et al. (2003) with some modifications. Briefly, 12.5 mg gelatin-coated cytodex-3 microcarriers in 1 ml PBS were incubated for 3 h to hydrate. After two washings in PBS and one in CM, the microcarriers were put in flasks containing 5 ml CM; AOC (5 \times 10^5) were added and cultured for 24 h in order to let the endothelial cells coat the microcarriers. For the fibrin gel preparation, 40 \mu l microcarriers covered by AOC were pipetted into 6-well plates containing a solution of fibrinogen (1 mg/ml PBS, pH 7-6), added with 1250 IU thrombin (250 \mu l). Fibrin gels were allowed to polymerize for 30 min at 37 \degree C, and then they were equilibrated for 60 min with 2 ml M199. After a change of the medium, AOC were treated with VEGFA (100 ng/ml; PeproTech EC Ltd, London, UK) in the presence or absence of 1, 10, or 100 ng/ml 2-OHE2 or 4-OHE2. The plates were incubated at 37 \degree C under humidified atmosphere (5% CO2). AOC was cultured for 96 h, renewing totally the treatment after 48 h as described above.

Quantification of AOC growth on fibrin gel matrix Endothelial cell proliferation in the fibrin gel matrix was evaluated by means of the public domain NIH Program Scion Image Beta 4.02 (Scion Corporation, Frederick, MA, USA, http://rsb.info.nih.gov/nih-image/). Ten pictures were taken for each gel at 48 and 96 h; images were converted into gray scale, resized to 50% (Paintbrush Software, MS Office), and saved as Bitmap 24 bit format compatible with Scion. The modified images were then imported into the program and the measurements were made drawing the perimeter of the area occupied by AOC expressed as number of pixel. In order to validate the measurement of the area covered by AOC in fibrin gels as a reliable method to evaluate cell proliferation, fibrin gels were stained by the nuclear dye bisbenzimide (Hoechst 33258, 20 \mu g/ml in PBS for 60 min) and examined by the fluorescence microscope. This procedure was performed 20 times; for each experiment, the number of nuclei was counted under fluorescence and pictures of the area covered by AOC were taken in order to measure the surface covered in the fibrin gel. A strong correlation was observed between the area covered by AOC and the number of nuclei found in the same area (r=0.96).

Statistical analysis Method validation was carried out to meet the acceptance criteria for bioanalytical method validation (Guidance for Industry, Bioanalytical Method Validation 2001).

Instrumental detection (LOD) and quantitation (LOQ) limits were calculated according to EURACHEM guidelines (EURACHEM Guide 1998 http://www.eurachem.ul.pt/).

Table 1 Hydroxyestrogens content (\mu g/l) in swine follicular fluid

<table>
<thead>
<tr>
<th>Follicle class</th>
<th>Small</th>
<th>Medium</th>
<th>Large</th>
</tr>
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<tbody>
<tr>
<td>2-OHE2</td>
<td>0.27±0.04^a</td>
<td>1.16±0.36^b</td>
<td>5.05±0.10^c</td>
</tr>
<tr>
<td>4-OHE2</td>
<td>ND</td>
<td>1.65±0.04^a</td>
<td>6.92±0.03^b</td>
</tr>
</tbody>
</table>

Data are expressed as means±S.E.M. Different letters in the same row indicate a significant difference (P<0.001). ND, not detected.

Once calculated, LOQ was tested for accuracy and precision to meet the previously cited international criteria.

The presence of matrix effect was evaluated by comparing the slopes of the regression models (five levels, three replicated measurements for each level) obtained using the external standard and the standard addition method respectively. Homoschedasticity was verified by applying the Bartlett test; lack-of-fit and Mandel’s fitting test were also performed to check the goodness of fit and linearity (Funk et al. 1995).

The significance of the intercept (significance level 5%) was established by a t-test.

Repeatability and inter-day precision were calculated in terms of relative standard deviation (RSD%) on three concentration levels (at the LOQ level, at the final concentration of 5 and 20 \mu g/kg) performing five replicates at each level.

The accuracy was calculated in terms of recovery rate (RR%) as follow:

\[
RR\% = \frac{c_1 - c_2}{c_3} \times 100
\]

where \(c_1\) is the concentration of the fortified sample, \(c_2\) is the concentration of the sample before fortification, and \(c_3\) is the concentration of fortification. Three different concentration

![Figure 1 Effect of the treatment with 4-OHE2 (1, 10, and 100 ng/ml) for 48 h on VEGFA production by swine granulosa cell. Different letters indicate a significant difference (P<0.001) among treatments as calculated by ANOVA and Schéffe’s F test.](http://www.endocrinology-journals.org)
levels (low, medium, and high) with five replicated measurements were analyzed. The extraction yield in terms of percent recovery was calculated by comparing the results obtained from the injection of pure standards \((n = 3)\) with those related to the analysis of follicular fluids containing the same amount of analyte \((n = 3)\).

Stability, expressed as percentage of the initial concentration of the hormone in the follicular fluid samples analyzed the day after the sampling, was evaluated in terms of freeze–thaw stability (storage at \(-80 \, ^\circ \text{C}\)), short-term stability, and long-term stability.

As for the other parameters, they were examined at least four times (6 replicates/treatment). Experimental data are presented as mean ± S.E.M.; statistical differences between treatments were calculated with Multifactorial ANOVA using Statgraphics package (STSC Inc., Rockville, MD, USA). When significant

<table>
<thead>
<tr>
<th>Hours of incubation</th>
<th>2-Hydroxyestradiol (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>48</td>
<td>53 261 ± 3537^a</td>
</tr>
<tr>
<td>96</td>
<td>95 373 ± 6231^a</td>
</tr>
</tbody>
</table>

Values in the same row with different letters are significantly \((P<0.001)\) different. Data represent the area covered by AOC in the fibrin gel (number of pixel; means ± S.E.M.).

**Figure 2** Phase contrast micrographs showing AOC growth at 48 h in fibrin gel matrix. Cells were cultured in CM or treated with 2-OHE2 at the concentrations of 1, 10, or 100 ng/ml.
differences were found, means were compared by Scheffé’s F test; \( P < 0.05 \) were considered to be statistically significant.

**Results**

Validation of 2-OHE2 and 4-OHE2 assays

The determination of the hydroxyestrogens’ content in follicular fluid samples was carried out after method validation. Instrumental detection and quantitation limits were calculated using standard solutions. Very low LOD and LOQ values were determined, LOQ being equal to 0.2 and 0.8 \( \mu \)g/kg for 2-OHE2 and 4-OHE2 respectively, the LOD values were about three times lower. The presence of the matrix effect was assessed by comparison of the calibration lines calculated using the external standard and the standard addition method, whereas an excellent precision with RSD% lower than 6% characterized the SPE–GC–MS method. Extraction yields higher than 82% and recoveries in the 85(\( G \))–93(\( G \))% (\( n = 3 \)) proved the accuracy of the developed method. Owing

Table 3 Effect of 4-hydroxyestradiol on aortic endothelial cell (AOC) growth

<table>
<thead>
<tr>
<th>Hours of incubation</th>
<th>4-Hydroxyestradiol (ng/ml)</th>
<th>1</th>
<th>10</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>48</td>
<td>53 261 ± 3537( ^{a} )</td>
<td>29 632 ± 3066( ^{b} )</td>
<td>23 412 ± 1550( ^{b} )</td>
<td>23 516 ± 2363( ^{b} )</td>
</tr>
<tr>
<td>96</td>
<td>95 373 ± 6231( ^{a} )</td>
<td>54 764 ± 2790( ^{b} )</td>
<td>45 542 ± 3787( ^{c} )</td>
<td>36 216 ± 1834( ^{c} )</td>
</tr>
</tbody>
</table>

Values in the same row with different letters are significantly \( (P < 0.001) \) different. Data represent the area covered by AOC in the fibrin gel (number of pixel; means ± S.E.M).
to the presence of labile hydroxyl groups, the stability of the investigated hormone in the time was also evaluated. Data obtained after going through three freeze and thaw cycles proved that no degradation of the investigated analytes occurred when both the matrix (storage at $-80\,^\circ$C) and the stock solutions (storage at $-20\,^\circ$C) were maintained at room temperature just for the thawing time. Under these circumstances, no significant differences ($P>0.05$) among the chromatographic responses obtained before, during, and after the freeze and thaw cycles at room temperature were observed. As for the short-term storage, a relevant degradation of the analytes was evidenced by maintaining both the stock solutions and the matrix at room temperature for more than 4 h after thawing, thus observing an evident decrease in the chromatographic responses, with strong differences from the initial concentration levels. Concerning the long-term stability in the case of the stock solutions, it was proved that the recommended storage temperature ($-20\,^\circ$C) could be maintained for up to 2 weeks, whereas shorter times (10 days) and lower temperatures ($-80\,^\circ$C) were required for the preservation of the follicular fluids.

More precisely, samples had to be maintained at $-80\,^\circ$C for a maximum of 10 days, with differences from the initial values always lower than 5%.

2-OHE2 and 4-OHE2 contents in follicular fluid

The concentration of 2-OHE2 significantly increased ($P<0.001$) during follicle growth (Table 1), quadruplicating in medium follicles and appearing almost 20 times higher in large follicles than small ones. On the contrary, 4-OHE2 was impossible to quantify in small follicles, detectable in medium follicles, and became four times higher in the large ones.

VEGFA production

Basal VEGFA production by granulosa cells amounted to $960\pm42\,\text{pg/ml (mean}\pm\text{S.E.M.).}$ The treatment with 2-OHE2 did not affect VEGFA production. On the contrary, 4-OHE2 at all concentration tested significantly ($P<0.001$) inhibited VEGFA output ($P<0.001$; Fig. 1).

Figure 4 Phase contrast micrographs showing AOC growth at 48 h in fibrin gel matrix. Cells were cultured in CM or treated with 4-OHE2 at the concentrations of 1, 10, or 100 ng/ml.
Effect of 2-OHE2 and 4-OHE2 on AOC growth

AOC proliferation significantly \( (P<0.001) \) increased with time in control groups; the area covered by AOC almost doubled during the 48-h incubation shifting from 53 261 ± 3537 to 95 373 ± 6231 pixels. AOC incubated with 2-OHE2 showed a growth rate significantly reduced \( (P<0.001) \) both after 48- and 96-h incubation. No differences were found among the different concentrations tested \( (P>0.001; \text{Table 2, Figs 2 and 3}) \). The addition of 4-OHE2 to fibrin gel decreased \( (P<0.001) \) AOC proliferation: all the concentrations were equally effective after 48 h while after 96 h 10 and 100 ng/ml were the most effective. Moreover, the area covered by AOC in fibrin gel was inhibited more efficiently \( (P<0.01) \) by the treatment with 4-OHE2 than with 2-OHE2 at both the end time point for each concentration tested \( (\text{Table 3, Figs 4 and 5}) \).

Discussion

The development of the ovarian follicle is a complex process involving mechanisms that are similar to wound healing and tumor formation. The proliferation rate of endothelial cells in the developing follicle is much higher than that of rapidly growing tumors \( (\text{Neeman et al. 1997}) \), suggesting that this structure resembles a "transitory tumor". However, in contrast to tumor angiogenesis, this process is transient and strictly controlled within the ovary.

Anti-angiogenic factors may represent a potential mechanism to balance angiogenesis; although different anti-angiogenic molecules have been identified \( (\text{Sato 2006}) \), little is known about the negative control of vascularization in the ovary. Several lines of experimental evidence suggest that estrogen and other sex steroids directly modulate angiogenesis \( (\text{Losordo & Isner 2001}) \). In particular, we previously evidenced that 2-methoxyestradiol, a naturally occurred estradiol derivative, inhibits ovarian angiogenesis \( (\text{Basini et al. 2007a,b}) \).

The present study was therefore addressed to explore the potential involvement of other major estradiol metabolites, 2-OHE2 and 4-OHE2, in the control of follicular angiogenesis. First of all, we aimed to assess the physiological presence of the above-mentioned hydroxyestrogens in follicular fluid. Unfortunately, a simple, specific, and sensitive
Concluding remarks

Taken together, our data suggest that hydroxyestrogens 2-OHE2 and 4-OHE2, physiologically present in swine follicular fluid, can potentially exert an inhibitory effect on follicular neoangiogenesis. The most potent negative effect could be mediated by 4-OHE2 that has been shown to be capable of inhibiting the production of the main proangiogenic peptide, VEGFA, by granulosa cells. Since dysfunctional or uncontrolled angiogenesis is involved both in the ovarian failure and in different diseases, clinical medicine may profit from understanding these control mechanisms in order to set up new methods to regulate fertility and to evaluate new therapeutic options for angiogenesis-dependent diseases.

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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