Acute effects of testosterone on intracellular Ca\(^{2+}\) kinetics in rat coronary endothelial cells are exerted via aromatization to estrogens

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Sierra-Ramirez, Alfredo, Tomás Morato, Rafael Campos, Iván Rubio, Claudia Calzada, Enrique Méndez, and Guillermo Ceballos. Acute effects of testosterone on intracellular Ca\(^{2+}\) kinetics in rat coronary endothelial cells are exerted via aromatization to estrogens. Am J Physiol Heart Circ Physiol 286: H63–H71, 2004. First published August 15, 2004; 10.1152/ajpheart.00784.2003.—The objective of this work was to evaluate the effects of testosterone (T) and 17β-estradiol (E\(_2\)) on coronary microvascular endothelial cells (CMECs) of male and female rats. To analyze the short-term effects of such sex steroid hormones on intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) kinetics, we used the chelating agent fura-2 acetoxyethyl ester. We also explored the possibility of testosterone aromatization by using selective inhibitors of the aromatase enzyme cytochrome P-450 aromatase (P450arom), aminoglutethimide (4 μM), and hydroxysteroid localization (4 μM). The presence of P450arom was investigated by immunocytochemical and immunoblot assays using peptide-generated polyclonal antibodies raised against a 20-aminocaprylic acid synthetic fragment of rat P450arom and by in situ hybridization to locate the aromatase mRNA in such cells. The activity of P450arom was demonstrated by the stereospecific loss of the tritium atom of [\(^{3}H\)androstenedione]. Our results indicate that both T and E\(_2\) induced a rapid increase in [Ca\(^{2+}\)]\(_i\). The fact that the effects of E\(_2\) and T were carried out within milliseconds suggests that they were exerted at the membrane level and not through intracellular receptors. The possibility of involvement of PLC-β in these effects is suggested because U-73122 (a PLC inhibitor) blocked the effects of both T and E\(_2\). Immunocytochemical assays indicated the presence of androgenic and estrogenic receptors in these cells. The effects of T were blocked by the selective aromatase inhibitors. We also demonstrated membrane association of P450arom, expression of the ovary-specific mRNA after in situ hybridization, and E\(_2\) formation resulting from a significant activity of P450arom in CMECs. There were no gender-based differences.

phospholipase C-β; cytochrome P-450 aromatase; stereospecific MUSE have a greater incidence of cardiovascular disease than women during their reproductive years, and these gender differences diminish after menopause (30, 35). There are many experimental and epidemiological studies suggesting the cardio-vascular protective effects of estrogen (7, 19, 37, 55, 57). These beneficial effects are exerted by direct and indirect actions on blood vessels including a positive effect on plasma lipoproteins by a decrease in low-density lipoprotein cholesterol and an increase in high-density lipoprotein cholesterol (57), an antioxidant effect (19, 37), inhibition of fibrosis (19), and a vasodilating effect that is achieved by increased production of nitric oxide (NO) and prostacyclin and by a direct inhibitory effect on vascular smooth muscle (19, 37).

On the other hand, the vascular effects of testosterone (T) are not well defined, and reported effects of androgens on the cardiovascular system are conflicting. Androgens are associated with an increased risk of cardiovascular disease in men (20, 30, 35). The literature reveals numerous deleterious cardiovascular associations of androgenic steroids (2, 36, 48). Other reports support beneficial effects of T on the cardiovascular system such as antiangiinal effects (33), significant antiatherosclerotic effects (1), induction of coronary artery dilation, and increased coronary blood flow (58).

Sex steroid hormones are involved in many physiological and pathophysiological processes. Steroid hormones have been described as modulators of nuclear transcription. However, other effects of these hormones have been recognized as nongenomic in origin because they occur in a very short time. These effects may modulate intracellular signalization pathways and perhaps influence genomic actions of steroids and in this way integrate genomic and nongenomic pathways (14).

In fact, the mechanism(s) behind sex steroid hormone effects at the cardiovascular level is controversial and may include several processes. T and 17β-estradiol (E\(_2\)) play an important role in Ca\(^{2+}\) flux in several cell types. Data from our laboratory obtained from male rat aorta endothelial cells in culture showed that E\(_2\) induces an increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and acts as an agonist of endothelial activity in a nongenomic manner (42), whereas T has no direct effects on [Ca\(^{2+}\)]\(_i\), kinetic but blocks bradykinin-induced increases in [Ca\(^{2+}\)]\(_i\). These results suggest direct steroid stimulation of an intracellular second-messenger pathway (41). However, aorta is a conduit artery, and due to endothelial heterogeneity, it is necessary to explore the steroidal effects on the endothelium of other arterial sources such as coronary vasculature.

Endothelium is a dynamic tissue that secretes and modifies vasoactive substances. These cell type responses influence and are influenced by the cells they are in relation with (14, 23), such that it is expected that endothelial cells would show functional specialization depending on the origin of the vascular bed. We hypothesized that there are differential sex steroid-induced effects in aortic endothelium and in endothelial cells of the microvascular coronary area. Therefore, the purpose of the

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present study was to analyze the steroid hormone-induced increase in [Ca\textsuperscript{2+}], kinetics in cultured rat coronary microvascular endothelial cells (CMECs). We also analyzed the intracellular pathways involved in these effects, the possibility of T metabolism in the short-term effects of [Ca\textsuperscript{2+}], kinetics, as well as immunoneexpression of the \( \alpha \)-estrogenic (ER-\( \alpha \)) and androgenic (AR) receptors in CMECs. Furthermore, to investigate the possible gender differences in response to sex steroids, this study included male and female rat CMECs.

Our results show that E\( \text{2} \) and T induce an increase in [Ca\textsuperscript{2+}], and that the effects of T are exerted through its aromatization. Male and female rat CMECs also show mRNA and protein expression and activity of cytochrome P-450 aromatase (\( P450_{\text{arom}} \)), which shows that T metabolism could have an important role in cardiovascular response to this sex steroid.

**MATERIALS AND METHODS**

The protocols for this study were approved by institutional ethical and research committees.

**Isolation and culture of male and female rat CMECs.** Cultures were obtained according to a previously described method (40). Briefly, adult male and female Wistar rats (body wt, 250–300 g; handled separately) were anesthetized with pentobarbital sodium (50 mg/kg ip) and heparin (150 U/kg). Hearts were rapidly excised, washed in Hank's balanced salt solution, and dissected to discard atria, right ventricle, and connective and valvular tissues. Left ventricles were then opened by the anterior wall, washed, and immersed in 70% ethanol for 40 s to devitalize epicardic mesothelial and endocardial endothelial cells. The remaining tissue was chopped and placed in types IA and IV collagenase solution (2 mg/ml) for 40 min at 37°C. Digested tissue was passed through a 70-μm metallic mesh. Dissociated cells in the filtrate were centrifuged at 3,000 rpm for 3 min in a 30% Percoll gradient. Finally, cells were resuspended in DMEM supplemented with 20% FBS, endothelial growth factor (2 ml/100 ml; GIBCO-BRL). Cells were then plated in fibronectin-covered flasks and incubated in a humidified chamber at 37°C in a 5% CO\textsubscript{2} atmosphere.

**Characterization.** Criteria for characterization of this cell type included identification of a typical “cobblestone” cell morphology and expression of factor VIII-related antigen (von Willebrand) using immunofluorescence cell staining (42). More than 99% of cells expressed the von Willebrand factor. The protocols for this study were approved by institutional ethical and research committees.

**Experiments for [Ca\textsuperscript{2+}] \text{measurement.}** CMECs were trypsinized and resuspended in culture medium (DMEM supplemented with 2% PBS and antibiotic/antimicotic solution (2 ml/100 ml; GIBCO-BRL)) to a final concentration of 2.4 × 10\textsuperscript{5} cells/ml. A droplet of ~25 μl was placed in the center of a coverslip dish (no. 1, glued to a perforated plastic petri dish). After the cells were attached to the slide, additional medium was added to the well to a final volume of 2 ml and cells were incubated. The cell cycle was synchronized by serum deprivation. CMECs were loaded with 3 μM fura-2 acetoxymethyl ester (AM) for 2 h in HEPES-Krebs-Henseleit solution (pH 7.4 at 37°C) composed of (in mM) 117.8 NaCl, 6 KCl, 1.75 CaCl\textsubscript{2}, 1.2 NaHPO\textsubscript{4}, 1.2 MgSO\textsubscript{4}, 5 glucose, 5 sodium pyruvate, and 20 HEPES at room temperature in the dark. The cells were washed and postincubated in the same buffer for 1 h. The experimental dishes were placed on an inverted microscope (dual-wavelength fluorescence-imaging system InCyt Im2; Intracellular Imaging; Cincinnati, OH) to observe the fluorescence emitted by the fura-2 Ca\textsuperscript{2+} complex when stimulated by UV light. The fura-2 fluorescence response to the Ca\textsuperscript{2+} was calibrated from the ratio of 340:380-nm-wavelength fluorescence values after subtraction of background fluorescence using Ca\textsuperscript{2+} standards (Kit II; Molecular Probes; Eugene, OR) in the absence of cells and as described by Grynkiewicz et al. (25). The dissociation constant for the fura-2/Ca\textsuperscript{2+} complex was taken as 224 nM. The values for maximal and minimal rates (\( R_{\text{max}} \) and \( R_{\text{min}} \), respectively) were calculated from measurements using 25 μM digitonin and 4 mM EGTA and raising the pH to 8.3.

**Effects of E\( \text{2} \) on [Ca\textsuperscript{2+}].** A series of experiments were carried out to characterize the short-term effects of E\( \text{2} \) (0.01 nM to 1 μM) on [Ca\textsuperscript{2+}], in both male and female rat CMECs in culture.

**Effects of T on [Ca\textsuperscript{2+}].** Dose-response curves to T (0.01 nM to 1 μM) were performed to evaluate the effects of T on [Ca\textsuperscript{2+}], in both male and female rat CMECs in culture.

**Immunoneexpression of androgenic and \( \alpha \)-estrogenic receptors.** To determine the expression of T (AR) and E\( \text{2} \) (ER-\( \alpha \)) receptors in male and female rat CMECs, we used an immunocytochemical process and confocal microscopy as follows: after 48–72 h of serum deprivation, cells were washed with ice-cold 0.1 M PBS and fixed in 4% paraformaldehyde for 30 min. The cells were washed and incubated for 30 min with a blocking solution (0.5% bovine albumin free of IgG). The cells were then incubated for 24 h at 4°C with AR or AR antibodies (1:100 dilution, developed in rabbit; Santa Cruz Biotechnology). After this period, cells were washed and postincubated for 1 h at room temperature in a dark chamber with fluorescein-conjugated or rhodamine secondary antibodies (goat anti-rabbit Ig, 1:250 dilution). To evaluate the coexpression of ER-\( \alpha \) and ARs after incubation with the ER-\( \alpha \) antibodies (including a FITC-labeled secondary antibody), we performed a second incubation, this time with the anti-AR antibodies and using a rhodamine-labeled secondary antibody. Finally, the immunoneexpression was evaluated by confocal microscopy (confocal TCS SP2; Leica).

**Effects of U-73122 on T and E\( \text{2} \) responses.** In another set of experiments to determine the participation of intracellular Ca\textsuperscript{2+} stores in T- and E\( \text{2} \)-induced effects, CMECs were incubated with the PLC inhibitor U-73122 (1 μM).

**Effects of aromatase inhibitors on T response.** We explored the participation of the aromatase enzyme \( P450_{\text{arom}} \) in the T-induced effects by preincubating the cells for 10 min with the selective inhibitors aminoglutethimide (4 μM) and 4-hydroxyandrostenedione (4 μM).

**Immunocytochemical assay of aromatase.** The expression of \( P450_{\text{arom}} \) in male and female rat CMECs was evaluated by the use of immunocytochemical assays and confocal microscopy. A 20-aminoc acid peptide corresponding to residues 379–398 of the rat \( P450_{\text{arom}} \) protein was synthesized. The peptide was coupled to the hemocyanin protein carrier (GIBCO-BRL). Antibody preparation was performed by immunizing adult male New Zealand White rabbits using a standard protocol as described by Sanghera et al. (44).

The obtained serum was treated as follows: IgG fractions were removed using affinity chromatography, peak fractions were dialyzed, and the immunoreactive titer was determined by ELISA. The F(ab')\textsubscript{2} fragment was obtained by enzymatic digestion of IgG (immobilized pepsin; Pierce). Polyclonal antibodies were stored at ~20°C.

CMECs were plated on coverslips, fixed in 4% paraformaldehyde for 30 min, washed, incubated for 30 min with blocking solution (0.5% bovine albumin free of IgG), and then incubated for 24 h at 4°C with the F(ab')\textsubscript{2} fragment of peptide-generated polyclonal antibodies to rat \( P450_{\text{arom}} \) (1:100 dilution). Cells were washed and postincubated for 1 h at room temperature in a dark chamber with FITC secondary antibody (goat anti-rabbit Ig, 1:250 dilution; Santa Cruz Biotechnology). Finally, \( P450_{\text{arom}} \) expression was evaluated by fluorescence and confocal microscopy (confocal TCS SP2; Leica).

**Preparation of rat CMEC homogenates that contain aromatase.** Homogenates of male and female rat CMECs were prepared via mechanical separation from plates with a cell scraper and low-speed centrifugation at room temperature for 5 min. The pellet was washed with PBS, added to an ice-cold radioimmunoprecipitation assay buffer (that contained PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate,
and 0.1% SDS) with freshly added protease inhibitors (Sigma), incubated on ice for 30 min at 4°C, disrupted, and homogenized further by sonication and Polytron homogenization (Daigger). The protein content of the homogenates was measured using a standard Bradford assay (6, 50). Homogenates were aliquoted and stored at -70°C until use for P450arom activity assay.

Immunoblotting. Lysates of male and female rat CMECs were obtained as previously described. The homogenate was transferred to microcentrifuge tubes and centrifuged at 10,000 g for 10 min at 4°C. The supernatant fluid (particle size, < 2.5 μm) was centrifuged at 100,000 g for 30 min at 4°C to isolate plasma membrane vesicles. Both the pellet and supernatant from the 10,000 and 100,000 g centrifugations were tested with P450arom immunoblotting. Protein content was measured using a standard Bradford assay (6, 50). The tissue homogenates (120 μg) mixed with Laemmli sample buffer (that contained 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.04% bromphenol blue, and 0.125 M Tris-HCl, pH 6.8; Bio-Rad) were boiled for 5 min. Samples were loaded into wells (30 μg/lane), and proteins were resolved by electrophoresis via SDS-PAGE in a 10% gel and then transferred to 0.45-μm nitrocellulose membranes (Bio-Rad). The nonspecific binding was blocked by incubating the membranes in Blotto (that contained PBS, 5% milk, and 0.05% Tween 20) for 1 h. Blocked membranes were incubated in primary antibody [F(ab')2 fragment of peptide-generated polyclonal antibodies to 450 arom] diluted in Blotto (1:250 dilution) for 1 h, washed, and incubated for 2 h with F(ab')2-peroxidase conjugate (HRP)-conjugated secondary antibody (Sigma) diluted in Blotto (1:500 dilution), washed three times with PBS-0.05% Tween 20 and once with PBS, and finally stained by the use of a diaminobenzidine substrate kit (Vector Laboratories). We used purified goat polyclonal antibody raised against the ribosomal protein S6 (molecular mass, 27.5 kDa) as control.

In situ hybridization. Aromatase antisense fluorescein-conjugated oligonucleotide probes were commercially obtained (Acesolab) from three major species of aromatase cDNA sequences on the 5’ region corresponding to exon I: placenta (5’-TTCCATCATTTCTGGTTT-GCCT-3’), ovary (5’-TTACAGTCAAACAACAGGAGCC-3’), and prostate/testis (5’-CAAAAGG-GACAGGAAAAATTACAGA-3’). These were used in in situ hybridization analyses of P450arom mRNA in male and female rat CMECs as previously described (8, 27). Briefly, CMECs were plated on coverslips and fixed in 4% paraformaldehyde, washed, treated with 0.01% Triton X-100 for 90 s, washed, incubated with 100 μg/ml proteinase K for 10 min at 37°C, and then washed and incubated for 5 min with 2 mg/ml glycine. Later, slides were preincubated with hybridization buffer that contained 5 ml of deionized formamide, 2 ml of 20× SSC, 0.2 ml of 1× Denhardt’s solution, 0.5 ml of salmon sperm DNA (10 mg/ml), 0.25 ml of yeast tRNA (10 mg/ml), and 2 ml of dextran sulfate at 70°C for 20 min. Probes were applied in hybridization buffer (10 μl/200 μl of buffer) and incubated in a humidified chamber at 37°C for 8–12 h. After the hybridization, slides were washed with 0.1% of 1× SSC-SDS at room temperature and were visualized using fluorescence microscopy. Sense oligonucleotide probes and RNase I treatment (0.5 mg/ml) were used as negative controls, and DNase I treatment (0.5 mg/ml) was used as the positive control.

Preparation of placenta microsomes that contain aromatase. Human placenta, obtained immediately after delivery, was processed at 4°C according to a published method (43). Briefly, to obtain partially purified P450arom, tissue was washed with cold 0.9% saline solution, dissected free of fetal membranes and large blood vessels, and weighed. The remaining tissue was homogenized in a blender for 1 min in buffer that contained (in M) 0.25 sucrose, 0.05 phosphate, and 0.04 nicotinamide, pH 7.0. One volume of buffer to three parts of tissue by weight was used to provide optimal solutions of optimal activity. The homogenates were centrifuged at 800 g for 15 min at 4°C, and the supernatant was recovered and centrifuged at 10,000 g for 15 min at 4°C. Again the supernatant was recovered and centrifuged at 80,000 g for 1 h at 4°C to obtain microsomal pellets. The pellets were resuspended in 12 ml of 0.05 M phosphate buffer, pH 7.0, and the protein contents of the homogenates were measured using Coomassie brilliant blue G250 (Sigma) and human serum albumin as a standard (6, 50). Homogenates were aliquoted and stored at -70°C until use as a positive control for P450arom activity.

Analysis of aromatase activity. P450arom activity was quantified by the stereospecific loss of the tritium atom of the [1β-3H]androstenedione substrate in the aromatization reaction. The conversion rate was determined by the isolation and quantification of tritiated water (32). The standard incubation mixture was prepared with the following: 1) a P450arom source (2.5 μg of placenta microsomal fraction or 600 μg of CMEC homogenate); 2) cofactors: 6.25 mM MgCl2, 7.6 mM glucose-6-phosphate, 1.4 U of glucose-6-phosphate dehydrogenase, and 200 μM β-NADPH (Sigma); and 3) a saturating concentration of [1β-3H]androstenedione (New England Nuclear; Boston, MA): 1 pM in a total volume of 1,100 μl of 0.05 M phosphate buffer, pH 7.0. The mixture was incubated under agitation at 37°C for 60 min. The reaction was stopped by the addition of 300 μl of 2.5% activated charcoal and 0.25% Dextran T-70 (Sigma) and was vortexed for 40 s. The mixture was centrifuged at 800 g for 15 min and the residual substrate ([1β-3H]androstenedione) was removed. The H2O formed was quantified by scintillation counting. Control experiments were made including the P450arom source (placenta microsomal fraction or CMEC homogenates) or by substituting it with 100 μM bovine albumin.

**Statistical analysis.** Data on the changes in [Ca2+]i, (maximal concentration reached was indicated by a spikelike component of the curve) are expressed as means ± SE and were analyzed by one-way ANOVA. The individual contrast between treatments was made by the Bonferroni multiple-comparison test. Each experiment was performed in ~25 cells that were randomly chosen; the cell numbers (n) were limited only by the view field on the microscope objective, but they were representative of the total population (6 × 103 cells). P450arom activity assays were repeated at least four times. Results are expressed as means ± SE; data are compared vs. control experiments. Differences were considered significant at P < 0.05.

**RESULTS**

Effects of E2 on [Ca2+]i. It has been reported that agonists of endothelial activity (such as bradykinin) induce an increase in [Ca2+]i, and the Ca2+ kinetics has two components: a spikelike increase in [Ca2+]i, is followed by a plateau-like second phase that slowly returns to the basal state (9, 42).

Our results show that the addition of E2 to CMECs induces an increase in [Ca2+]i. E2-induced effects are similar to those induced by agonists of endothelial activity. However, the second phase did not return to the basal state, at least not during the experimental time.

Figure 1 is a representative tracing of the short-term increase in [Ca2+]i, induced by 1 nM E2. The dose-response curves (analysis of maximal increase of the spikelike component) for E2 (0.01 nM to 1 μM; Fig. 2) show a bell-shaped biphasic pattern in CMECs from male and female rats.

**Effects of T on [Ca2+]i.** The addition of T to the cultures induced a short latency (in milliseconds) increase in [Ca2+]i. Figure 1 is a representative tracing of the increase in [Ca2+]i, induced by 1 nM T. There were no differences in the onset of effects after addition of E2 or T to CMECs (Fig. 1, inset).

Addition of T (0.01 nM to 1 μM) to male and female rat CMECs induced a dose-dependent effect (see Fig. 2). There were no statistical differences between male and female cell responses.
Immunocytochemistry of AR and ER-α. We found intense expression of ER-α (Fig. 3, green areas) in male and female rat CMECs. Fluorescence seemed to be associated with the cell periphery and may include the plasmalemma as well as the cytoplasm. AR immunoreactivity (Fig. 3, red areas) was also observed in male and female cells. However, fluorescence seemed to be associated only with the cytoplasm level. The sites where the receptors were coexpressed showed as yellow (Fig. 3). No staining was observed when male and female rat CMECs were processed without the primary antibody (data not shown).

Effects of U-73122 on E2- and T-induced increases in [Ca2+]i. Preincubation of male and female rat CMECs for 10 min with 1 μM U-73122 (a PLC inhibitor) induced complete blockade of T- or E2-induced increases in [Ca2+]i. These effects were not the result of depletion of intracellular Ca2+ stores, because the addition of 20 nM caffeine 4 min after the steroid stimulation induced a short-term increase in [Ca2+]i (Fig. 4). There were no differences between male and female cell responses. Alone, U-73122 did not modify the [Ca2+]i.

Effects of aromatase-selective inhibitors aminoglutethimide and 4-hydroxyandrostenedione on [Ca2+]i response to T. Pretreatment (10 min) of male or female cells with either 4 μM aminoglutethimide or 4 μM 4-hydroxyandrostenedione (Fig. 5) resulted in blockade of the T-induced increase in [Ca2+]i. These effects were not unspecific, because the addition of 1 nM E2 induced an increase in [Ca2+]i. There were also no differences between male and female cell responses. Aminoglutethimide and 4-hydroxyandrostenedione produced no effects in [Ca2+]i.

Immunocytochemistry of aromatase. Intense P450 arom immunoreactivity was observed in male and female rat CMECs. Fluorescence was evident in the cytoplasm and cell periphery (Fig. 6). There were no gender-based differences. The P450 arom immunoreactivity was expressed in 96–98% of rat CMECs.

Immunoblotting. We found expression of P450 arom in male and female rat CMECs. Immunoreactivity was made evident in a single protein band with an apparent molecular mass of ~50 kDa in homogenates of male and female rat CMECs. It was
observed in the supernatant fluid from 10,000 g (S1) and 100,000 g (S2) centrifugations as well as in the pellet of 10,000 g (P1) and 100,000 g (P2) centrifugations. The ribosomal protein S6 was used as a control (single band, 30 kDa; Fig. 7). There were no gender-based differences. No bands were detected when the primary antibody was omitted. We also detected a similar single-protein band in human placenta microsomes (data not shown).

In situ hybridization. Tissue-specific expression of aromatase by utilization of specific exon I probes in male and female rat CMECs was investigated at the mRNA level through in situ hybridization. We used antisense fluorescein-conjugated oligonucleotide probes from three major species of aromatase cDNA sequences of the 5' region corresponding to exon I: placenta, ovary, and prostate/testis. As shown in Fig. 8, a fluorescent mRNA hybridization signal by an ovary-specific probe was found mainly at the cytoplasm level. No reactivity was detected by the use of placenta and prostate/testis probes. There were no gender-based differences. Sense fluorescein-
conjugated oligonucleotide probes were used as negative controls.

Assay for $^3$H$_2$O release. Aromatization of androgens to form phenolic estrogens was measured by the isolation and quantification of tritiated water, which originated from stereospecific loss of the tritium atom from the substrate. Incubation of 1 pM $[^1H]$3H]androstenedione with cofactors (NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and MgCl$_2$) and different concentrations of placenta microsomes (0.05–5 µg) resulted in the formation of tritiated water in a concentration-dependent manner (Fig. 9). The incubation of 1 pM $[^1H]$3H]androstenedione with cofactors and homogenates of male and female rat CMECs at different concentrations (130–1,300 µg) also resulted in the formation of tritiated water in a concentration-dependent manner (Fig. 9). As shown in Fig. 10, male and female rat CMEC homogenates significantly induced the formation of tritiated water. Insignificant activity was detected in background control assays that lacked a P450 arom enzyme source or substituted it with albumin (100 µM).

DISCUSSION

Because the functions of the distinct segments of the vascular tree (arteries, arterioles, capillaries, venules, and veins) are different, it might be expected that endothelial cells would also show functional adaptations and even some specializations according to their origin. The environments surrounding endothelial cells appear to be important for their morphology, molecular expression, and function (21, 31).

Endothelium not only influences the function of other cell types but also is influenced by its neighboring cells; endothelium adapts to environmental signals. Therefore, cell-to-cell communication may play an important role in physiological responses. For example, astrocytes have a direct influence on brain homeostasis of various molecules and on the endothelial enzymatic and transport profiles of the brain at the blood-brain barrier (5, 16). However, the effects of astrocytes are not restricted to the endothelial cells of the brain: they can also be exerted on endothelial cells that originate elsewhere (10). Thus the cell origin could allow a single molecule to have different effects within the same cell type. In this work, we proposed...
that the endothelium of conduit vessels may respond differently to sex steroids than the endothelium of resistant vessels. We previously reported that E2 induced increases in [Ca\(^{2+}\)], through a short-term and nongenomic mechanism, and showed a biphasic curve pattern to suggest membrane-associated processes. E2 induced no effects on Ca\(^{2+}\) kinetics, whereasICI-182780, a pure antiestrogen, blocked the E2-induced increase in [Ca\(^{2+}\)], (42). Our present data on male and female rat CMECs are similar to those found in rat aortic endothelium in culture, but interestingly, there were no differences in gender responses (see Fig. 2). Thus E2 seems to affect endothelium of both conduit and resistant vessels perhaps through the same pathway.

Effects of T on male and female rat CMECs are different from those found in rat aorta (41) because T increased [Ca\(^{2+}\)], (see Fig. 1) in concentration-dependent and gender-independent manners by acting directly as an agonist of endothelial activity (see Fig. 2). These effects were obtained with physiological and pharmacological T concentrations (0.01 nM to 1 \(\mu\)M) and were of rapid onset (milliseconds) and nongenomic origin. The initial spikelike phenomena induced by T and E2 in both male and female rat CMECs was totally blocked by U-73122, which is an inhibitor of PLC-dependent processes (9, 29, 42; see Fig. 4); this suggests membrane-associated mechanisms.

The second component of [Ca\(^{2+}\)] kinetics, a sustained plateau induced by T and E2, did not return to basal levels during the experimental time (5 min; see Fig. 1). We do not have a clear explanation for this phenomenon. However, this phase is dependent on Ca\(^{2+}\) influx, and the rate of Ca\(^{2+}\) entry through ion pathways can be modulated by membrane properties. It is possible that steroid hormones may disturb the plasmalemma. Likewise, the mechanisms of action associated with Ca\(^{2+}\) modulation by E2 and T are not fully understood, but modulation of intracellular Ca\(^{2+}\) stores as well as regulation of plasmalemmal Ca\(^{2+}\) channels may be considered (9, 41, 42).

To analyze the possible membrane association and/or coexpression of ER-\(\alpha\) and AR in male and female rat CMECs, we performed immunocytochemical assays and confocal microscopy using a double-stain method. The expression of ER-\(\alpha\) was made evident by the strong (green) fluorescence located in the cell periphery as well as the cytoplasm in both male and female rat CMECs (see Fig. 3). Intense AR expression was also observed in CMECs, but immunoreactivity was located only at the cytoplasm level with no expression at the periphery (see Fig. 3), which suggests no association to the membrane. The coexpression of both receptors resulted in a yellow stain, and this was observed only at the cytoplasm. It is pertinent to emphasize that we used the same conditions and incubation times for both primary and fluorochrome secondary antibodies to avoid differences in IgG diffusion through the cells.

Several studies have reported rapid membrane-initiated estrogen effects linked to ER-\(\alpha\) and the membrane (7, 11, 12, 22, 39). Whereas the vascular effects of T are not well defined, the literature refers to deleterious cardiovascular associations with androgenic steroid use (2, 30, 36, 48). Meanwhile, some reports support beneficial actions of T on the vascular system including antiangiial (33) and antiatherosclerotic (1) effects and coronary artery dilatation (58). Moreover, T causes vasorelaxation in a rapid and nongenomic manner mediated in part by nitric oxide (13, 15) or vascular smooth muscle K\(^+\) channel activation (17, 18, 56, 59). Indeed, T seems to be a modulator of several metabolic processes that is exerted mainly in a regulatory role on [Ca\(^{2+}\)] (34, 41).

Presently there is no evidence that demonstrates a direct association of ARs with the membrane. We were unable to locate ARs at the membrane level. However, another possibility to explain the effects of T on Ca\(^{2+}\) kinetics in CMECs may be related to its metabolism. T is a prohormone converted in situ in active estrogens by steroidogenic tissues.

E2 is synthesized through P450\(_{arom}\) activity in a variety of extragonal sites and acts locally to stimulate adjacent cells or even the cells in which it is produced (51, 53). These sources of P450\(_{arom}\) activity are present throughout the body and may be responsible for local tissue concentrations of estrogens that may mediate important physiological events (47, 52, 60).

On the other hand, local estrogen biosynthesis has been reported in vascular tissues (3, 4, 27, 38, 46). In the present work, using highly selective aromatase inhibitors, we evaluated whether T induced an increase in [Ca\(^{2+}\)] in male and female rat CMECs through its conversion to estrogen. We investigated two types of inhibitors: type 1, 4-hydroxyandrostenedione (4 \(\mu\)M; see Fig. 5), which is a steroidal compound that binds to the androgen substrate-binding site of the enzyme and may be a purely competitive inhibitor or irreversible “suicide substrate,” and type 2, aminoglutethimide (4 \(\mu\)M; see Fig. 5), which is a nonsteroidal compound that interferes with the cytochrome P-450 prosthetic group on the enzyme and binds to the heme group of the aromatase enzyme by coordination through a basic nitrogen atom (26, 45). The Ca\(^{2+}\) kinetics of T were totally blocked by both of these inhibitors. This effect was not the result of intracellular Ca\(^{2+}\) store depletion, because the addition of 1 nM E2 induced an increase in [Ca\(^{2+}\)]. These results show that T in male or female rat CMECs, through its conversion to estrogens, acts by mobilizing Ca\(^{2+}\) from the endoplasmic reticulum.

Given that much of the circulating E2 is bound to sex hormone-binding globulin, it is not likely to have a major impact on transactivation of the estrogen receptor, unlike estrogen produced locally as a consequence of circulating-T metabolism. This supports the idea that local production of E2 by aromatization of T in estrogen-dependent tissues occurs within the same site to affect its functions. The amount of estrogen synthesized by these extragonal sites may be small, but the local tissue concentrations achieved are probably quite high and likely exert significant biological influence locally and play important physiological or pathophysiological roles in paracrine, autocrine, and indeed intracrine fashions (24, 38, 51, 52).

In this work, to examine the presence of P450\(_{arom}\) in CMECs, we developed polyclonal antibodies against a peptide of 20 amino acids that corresponds to residues 379–398 of rat P-450 protein as deduced from the nucleic acid sequence of rat P450\(_{arom}\) cDNA. This segment was chosen because the amino acid sequence has identical homology to the human, rat, and chicken P450\(_{arom}\) proteins but exhibits low homology with the corresponding region of other P-450 proteins (44).

More than 99% of male and female rat CMECs show immunoreactivity, which supplies evidence of the expression of P450\(_{arom}\) in these endothelial cell types (see Fig. 6). Results showed immunopositivity located at the cytoplasm level as
well as the periphery of the cells. This suggests a possible membrane association.

Immunoblotting results revealed the expression of \( P450_{\text{arom}} \) in rat CMECs (see Fig. 7). A single immunoreactive band of \( \sim 50 \text{kDa} \) was detected in male and female rat CMEC homogenates in the supernatant of the 10,000 g S1 centrifugation, which contained subcellular particles <0.4 \( \mu \text{m} \), in the pellet of the 10,000 g P1 centrifugation, which contains subcellular particles >0.4 \( \mu \text{m} \), and in the S2 supernatant and the P2 pellet of the 100,000 g centrifugation. The estimated size of the single band from the homogenate of rat CMECs is similar to the predicted size of the \( P450_{\text{arom}} \) protein as deduced from the respective cDNAs (8, 27, 49) and previous size determinations (32, 44, 54). Aromatase (estrogen synthetase) is a microsomal member of the cytochrome \( P450 \) superfamily (49, 52, 54). We found immunoreactivity in the pellet of the 100,000 g centrifugation (plasma membrane vesicles), and this fact strongly suggests membrane association of aromatase.

In the present study, we assayed three different oligonucleotide probes that correspond to part of exon I of different tissue sources (placenta, ovary, and prostate/testis) to screen aromatase mRNA in rat CMECs by in situ hybridization assays (28). Aromatase mRNA is expressed by alternative splicing using tissue-specific exon I. Sense oligonucleotide probes were used as negative controls. The application of an antisense oligonucleotide probe for ovary resulted in identification of intense reactivity located mainly at the cytoplasm level (see Fig. 8). Hybridization with oligonucleotide probes for placenta and prostate/testis were negative. These data suggest that male and female rat CMECs express ovary-specific mRNA.

We also demonstrated that \( P450_{\text{arom}} \) is catalytically active in rat CMECs and showed indirectly that both male and female rat CMECs produce significant amounts of estrogens. The incubation of the substrate [1\( \beta \]H]androstenedione and cofactors with different concentrations of rat CMECs resulted in the formation of tritiated water in a concentration-dependent manner (see Fig. 9). We could not find gender-linked differences (see Figs. 9 and 10). We used human placenta microsomes in positive control experiments, which showed an active and reproducible aromatization. In background control experiments in which the source of \( P450_{\text{arom}} \) (placenta microsomes or rat CMEC homogenates) was omitted or replaced by bovine serum albumin, no significant aromatization was detected (see Fig. 10), which indicates no significant spontaneous loss of tritium from the substrate. Taken together, our results confer evidence for the presence, expression, and activity of \( P450_{\text{arom}} \) in rat CMECs.

Aromatization of androgens probably represents a complex system. In conclusion, our results show that in male and female rat CMECs in culture, T and E\( _2 \) behave as agonists of endothelial activity by increasing [Ca\(^{2+}\)], through PLC-\( \beta \) activity. These effects are of nongenomic origin and are related to the membrane-associated mechanism. T could have different effects depending on endothelial origin. Male and female rat CMECs in culture express \( P450_{\text{arom}} \) and its activity is blocked with selective inhibitors. \( P450_{\text{arom}} \) in rat CMECs is associated with membrane processes that mediate the rapid and nongenomic increase in [Ca\(^{2+}\)], induced by T. Apparently, exon 1c (ovary specific) was mainly used for the aromatase mRNA of rat CMECs. In rat CMECs, \( P450_{\text{arom}} \) shows significant enzymatic activity. We did not find gender-based differences. CMECs may be a potential site of E\( _2 \) synthesis from circulating T that mediates the [Ca\(^{2+}\)], changes and influences the physiological responses in this endothelial type.

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