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Equilin displays similar endothelium-independent vasodilator potential to 17 β -estradiol regardless of lower potential to inhibit calcium entry

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Abstract

Conjugated equine estrogens (CEE) have been widely used by women who seek to relieve symptoms of menopause. Despite evidence describing protective effects against risk factors for cardiovascular diseases by naturally occurring estrogens, little is known about the vascular effects of equilin, one of the main components of CEE and not physiologically present in women. In this regard, the present study aims to compare the vascular effects of equilin in an experimental model of hypertension with those induced by 17 β -estradiol. Resistance mesenteric arteries from female spontaneously hypertensive rats (SHR) were used for recording isometric tension in a small vessel myograph. As effectively as 17 β -estradiol, equilin evoked a concentration-dependent relaxation in mesenteric arteries from female SHRs contracted with KCl, U46619, PDBu or ET-1. Equilin-induced vasodilation does not involve classical estrogen receptor activation, since the estrogen receptor antagonist (ICI 182,780) failed to inhibit relaxation in U46619-precontracted mesenteric arteries. Vasorelaxation was not affected by either endothelium removal or by inhibiting the

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Disclosure
None

release or action of endothelium-derived factors. Incubation with L-NAME (NOS inhibitor), ODC (guanylyl cyclase inhibitor) or KT5823 (inhibitor of protein kinase G) did not affect equilin-induced relaxation. Similarly, indomethacin (COX inhibitor) or blockage of potassium channels with tetraethylammonium, glibenclamide, 4-aminopyridine, or ouabain did not affect equilin-induced relaxation. Inhibitors of adenylyl cyclase SQ22536 or protein kinase A (KT5720) also had no effects on equilin-induced relaxation. While 17β -estradiol inhibited calcium (Ca^{2+})-induced contractions in high- K^+ depolarization medium in a concentration-dependent manner, equilin induced a slight rightward-shift in the contractile responses to Ca^{2+} . Comparable pattern of responses were observed in the concentration-response curves to (S)-(-)-Bay K 8644, a L-type Ca^{2+} channel activator. Equilin was unable to block the transitory contraction produced by caffeine-induced Ca^{2+} release from intracellular stores. In conclusion, equilin blocks L-type Ca^{2+} channels less effectively than 17β -estradiol. Despite its lower effectiveness, equilin equally relaxes resistance mesenteric arteries by blocking Ca^{2+} entry on smooth muscle.

Keywords

Equilin; 17β -Estradiol; Vasorelaxation; Calcium channel; Mesenteric arteries

1. Introduction

Epidemiological studies have revealed that premenopausal women have a lower risk of developing hypertension than men or post-menopausal women [2,33,60]. The premenopausal protection has long been attributed to female hormones, more specifically to 17β -estradiol, which may prevent or delay the onset of hypertension in women. Similarly, gender differences in the development of hypertension are also found in experimental models such as the spontaneously hypertensive rat (SHR). In female SHR, estrogens lower blood pressure and protect against hypertension [41,59], providing a useful model to study the beneficial effects of estrogens in essential hypertension-associated vascular dysfunction.

Despite the large amount of observational and experimental studies suggesting that estrogens replacement therapy reduce the cardiovascular risk in postmenopausal women, the Women's Health Initiative (WHI) and the Heart and Estrogen/Progestin Replacement Study (HERS) reported negative results for prevention of cardiovascular disease by treatment with conjugated equine estrogens (CEE) [21,44]. Several explanations have been proposed for these discrepancies, including the fact that, in those trials, patients were treated with Premarin®, a preparation of CEEs, whereas the majority of animal and clinical studies evaluated the effects of 17β -estradiol, the most abundant circulating estrogen in humans. CEE is a complex formulation containing multiple estrogens, including the sulfate esters of the ring B saturated estrogens (classical estrogens), estrone, 17α -estradiol and 17β -estradiol, and the ring B unsaturated estrogens, equilin, 17α -dihydroequilin, 17β -dihydroequilin, equilenin, 17α -dihydroequilenin, 17β -dihydroequilenin and 8,9-dehydroestrone [62].

Estrogens can regulate vascular function through estrogen receptors (ERs)-dependent and independent pathways [57]. Binding of estrogen to its receptors (membrane or nuclear) triggers vascular effects through the regulation of gene transcription (genomic pathway) and

the activation of several different signaling cascades that alter cellular functions of proteins and ion channels [30,57]. Receptor-independent signaling mediated by estrogen include the direct interaction with a broad range of ion channels. Big conductance calcium (Ca^{2+}) (BK)- and voltage-activated K^{+} channels (Kv) as well as L-type Ca^{2+} channels (LTCCs) are the two most investigated channels under the direct influence of estrogens [26].

Equilin is one of the major components of CEE, comprising approximately 20–30% of the estrogenic steroid content [3]. Despite its natural origin, equilin and 17β -estradiol have different chemical structures, estrogen receptor binding affinity, selectivity, and agonistic properties and therefore, might not provide effects comparable to 17β -estradiol [12,39]. For exemplo, equilin exhibits low affinity for estrogen receptors. Even so, this estrogen compound displays higher antioxidant potency than estrone and 17β -estradiol [52]. In human aortic vascular smooth muscle cells (VSMC), equilin is less potent than 17β -estradiol in inhibiting mitogen-induced VSMC growth and MAPK activity [12]. Thus, we hypothesized that equilin displays cardiovascular protective effects by means of an ER-independent mechanism. Based on these observations, we sought to investigate the effects of equilin and its mechanism of action in isolated mesenteric arteries from female spontaneously hypertensive rats (SHR). Equilin effects were compared to those induced by 17β -estradiol.

2. Methods

2.1. Animals

All experimental protocols were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Protocols were approved by the Committee for Animal Research of the University of Sao Paulo, Sao Paulo, Brazil (Protocol n° 145/2010) and by the Augusta University Committee on the Use of Animals in Research and Education. Female SHR (16–18 week-old) were purchased from Harlan Laboratories (Indianapolis, IN) and were maintained on a 12-hour light/dark cycle under controlled temperature (22 ± 1 °C), with access to food and water *ad libitum*.

2.2. Vascular function studies

Force development in response to a specific experimental protocol was evaluated in mesenteric arteries from rats, as previously described [35]. After carbon dioxide (CO_2) euthanasia, the mesenteric vascular bed was removed and placed in Krebs-Henseleit solution (KHS) of the following composition (in mM): 130 NaCl, 14.9 NaHCO_3 , 4.7 KCl, 1.18 KH_2PO_4 , 1.17 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.5 glucose, 1.56 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.026 EDTA. Segments (2 mm in length) of the mesenteric arteries were mounted on 40- μm wires in a small vessel myograph for isometric tension recording. The vessels were allowed to equilibrate for about 30 min in modified Krebs-Henseleit solution, which was gassed with 5% CO_2 in O_2 to maintain a pH of 7.4. The relationship between resting wall tension and internal circumference was determined, and the internal circumference, L100, corresponding to a transmural pressure of 100 mmHg for a relaxed vessel in situ, was calculated. The vessels were set to the internal circumference L1, given by $L1 = 0.9 \times L100$. The effective internal

lumen diameter was determined as $L1 = L1/\pi$, and was between 200 and 300 μm . After stabilization, arterial integrity was assessed by stimulation of vessels with 120 mM KCl. Endothelial function was assessed by testing the relaxant effect of acetylcholine (ACh, 1 μM) on vessels precontracted with phenylephrine (1 μM). The failure of ACh to elicit relaxation of mesenteric arteries (which were previously subjected to rubbing of the intimal surface with a human hair) was taken as proof of endothelium removal.

2.3. Experimental protocols

Concentration-responses curves to equilin (10 nM–100 μM) and 17 β -estradiol (10 nM–100 μM) were performed in endothelium-intact mesenteric arteries on basal tonus to evaluate vascular contractile effects of these estrogens. Considering that treatment of resistance mesenteric arteries on passive basal tonus with equilin did not produce changes in force levels, the relaxant effect of equilin and 17 β -estradiol were evaluated after contraction induced by different agonists, as follows: KCl (120 mM), U46619 (1 μM – a thromboxane A_2 agonist), endothelin-1 (ET-1, 10 nM) and PDBu (protein kinase C activator, 0.3 μM).

The role of classic ERs on equilin-induced relaxation was examined after incubation of mesenteric arteries with the ER antagonist ICI 182,780 (10 μM) for 30 min before U46619-induced precontraction [15].

To determine whether vasorelaxation to equilin was dependent on the endothelium, responses were also determined in endothelium-denuded arteries precontracted with U46619. The role of the NO/cGMP/protein kinase G signaling pathway on equilin-induced relaxation was evaluated after incubation (30 min) of intact mesenteric arteries with L-NAME (100 μM), a non-specific NO synthase inhibitor, ODQ (10 μM), a guanylyl cyclase inhibitor, or KT 5823 (1 μM), a protein kinase G inhibitor [24,54]. In order to determine the role of prostanoids on equilin-induced relaxation, indomethacin (10 μM), a cyclooxygenase inhibitor, or indomethacin plus L-NAME were used [31].

The involvement of adenylyl cyclase/cAMP/protein kinase A signaling pathway on equilin-induced relaxation was evaluated after incubation of intact mesenteric arteries with the adenylyl cyclase inhibitor (SQ 22536, 100 μM) or the protein kinase A inhibitor (KT 5720, 1 μM) 30 min prior to U46619-induced precontraction [25].

To investigate the contribution of K^+ channels to equilin vasodilation, mesenteric arteries were incubated with tetraethylammonium (TEA, 1 mM), a non-selective Ca^{2+} -activated K^+ channel blocker; glibenclamide (10 μM), an ATP-dependent K^+ channel blocker; 4-aminopyridine (4-AP, 1 mM), a voltage-dependent K^+ channel blocker, or ouabain, a Na/K-ATPase inhibitor (100 μM), 30 min prior to U46619-induced precontraction [7,31,55].

To evaluate the Ca^{2+} antagonistic effect of equilin on mesenteric arteries, three different protocols were performed. In the first protocol, endothelium-denuded mesenteric arteries were contracted with KCl (120 mM). When the contraction reached a plateau, the tissues were washed repeatedly with normal KHS for 30 min. Afterwards, the solution was replaced by a Ca^{2+} -free KHS containing EGTA (1 mM). Concentration-dependent contraction curves to CaCl_2 (10 μM –10 mM) were then performed in K^+ -depolarization medium (60 mM K^+).

Mesenteric arteries were incubated (1, 10 or 100 μM) with equilin, 17 β -estradiol or vehicle (ethanol), 30 min before the CaCl_2 curves were obtained [6].

In the second protocol, endothelium-denuded mesenteric arteries were contracted with KCl (120 mM). When the contraction reached a plateau, the tissues were washed repeatedly with normal KHS for 30 min. Afterwards, the solution was replaced by a Ca^{2+} -free KHS containing EGTA (1 mM). Concentration-dependent contraction curves to Bay K 8644 (10 nM–0.3 μM) were then performed in K^+ -depolarization medium (10 mM K^+). Mesenteric arteries were incubated (1, 10 or 100 μM) with equilin, 17 β -estradiol or vehicle (ethanol), 30 min before the Bay K 8644 curves were obtained [6].

In the third protocol, to evaluate equilin effects on Ca^{2+} release from the sarcoplasmic reticulum, endothelium-denuded mesenteric arteries were contracted with phenylephrine (1 μM) in normal KHS. When the contraction reached a plateau, the tissues were washed with Ca^{2+} -free KHS containing EGTA (1 mM), to deplete intracellular Ca^{2+} stores. The intracellular Ca^{2+} stores were refilled by exposing the vessels to normal KHS. After a wash in a Ca^{2+} -free solution, a transient contraction due to a release of the accumulated Ca^{2+} was induced by caffeine (20 mM). Mesenteric arteries were incubated (1, 10 or 100 μM) with equilin, 17 β -estradiol or vehicle (ethanol), 15 min before the caffeine contraction was induced. In experiments using high- K^+ solution, an equimolar concentration of Na^+ was replaced by K^+ to maintain normal ion strength [6].

To ensure that the vehicle into which the drugs were dissolved did not alter vascular reactivity, experiments were also performed using ethanol in the absence of drug. Additions of the vehicles (final ethanol and DMSO concentration in each bath 0.2%) instead of equilin and estradiol did not influence either contraction or relaxation of the mesenteric arteries.

2.4. Drugs

Equilin, 17 β -estradiol, phenylephrine, acetylcholine, indomethacin, L-NAME (N^ω -nitro-L-arginine methyl ester), and KCl were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tetraethylammonium, glibenclamide, 4-aminopyridine, ouabain, (S)-(–)-Bay K 8644, U46619 (9,11-dideoxy-11 α ,9 α -epoxymethanoprostaglandin F2 α), ICI 182,780, KT 5720, KT 5823, SQ 22,536 (9-(tetrahydro-2-furanyl)-9H-purin-6-amine), and ODQ (1H-[1–2,4]oxadiazolo [4,3- α]-quinoxalin-1-one) were obtained from Tocris Bioscience (Bristol, UK). Caffeine was purchased from Roth (Karlsruhe, Germany).

Equilin and 17 β -estradiol were dissolved in 70% v/v ethanol with further dilution in distilled water before use. Working concentrations of ethanol in the bath were < 0.01% (v/v).

2.5. Statistical analysis

Relaxation is represented as a percentage of the maximal response to the contractile agents. The individual curves were fitted into a curve by non-linear regression analysis. pD_2 (defined as the negative logarithm of the EC_{50} values) and maximal response (R_{MAX}) were compared by analysis of variance (one-way ANOVA) followed by *post hoc* Dunnett's test or Tukey, when appropriated. The Prism software, version 5.0 (GraphPad Software Inc., San

Diego, CA, USA) was used to perform the analysis of these parameters as well as to fit the sigmoidal curves. Data are presented as mean $\pm$ SEM. N represents the number of animals used. P values < 0.05 were considered significant.

3. Results

3.1. Relaxant effects of equilin in resistance mesenteric arteries

Equilin did not produce changes in force levels of mesenteric arteries on passive basal tonus (data not shown). However, after contraction with U46619, ET-1, PDBu or KCl, equilin (10 nM–100 μ M) evoked a concentration-dependent relaxation in mesenteric arteries from female SHR (Table 1). No significant differences were observed in the effects of equilin among vessels precontracted with the different agents. These effects were equivalent to the vasorelaxant effects of 17 β -estradiol (10 nM–100 μ M) (Table 1).

3.2. Role of estrogen receptors and endothelium on equilin and 17 β -estradiol-evoked relaxation

The effects of ERs and endothelium removal on equilin and 17 β -estradiol-evoked relaxations are depicted in Fig. 1A and B, respectively. The relaxation to equilin ($R_{MAX} = 99.94 \pm 0.57\%$, $pD_2 = 4.79 \pm 0.02$, $n = 6$) and 17 β -estradiol ($R_{MAX} = 92.67 \pm 2.05\%$, $pD_2 = 4.75 \pm 0.08$, $n = 5$) in U46619-precontracted mesenteric arteries was not inhibited by the ER antagonist (ICI 182,780) (Fig. 1A). Moreover, there were no differences in the relaxing responses to either equilin or 17 β -estradiol in preparations with or without endothelium (Fig. 1B).

3.3. Role of endogenous NO/cGMP/protein kinase G and prostanoids on equilin and 17 β -estradiol-evoked relaxation

To investigate the role of endogenous NO, cGMP production and protein kinase G activation in equilin and 17 β -estradiol-induced vasorelaxant responses, concentration-response curves were generated in the absence or presence of the nitric oxide synthase inhibitor L-NAME, the guanylyl cyclase inhibitor ODQ, or the protein kinase G inhibitor KT 5823. None of these inhibitors were found to affect the relaxing responses to either equilin or 17 β -estradiol (Fig. 2A and B).

The contribution of vasodilator prostanoids to equilin and 17 β -estradiol-induced relaxation was evaluated by testing the effects of the cyclooxygenase inhibitor indomethacin. The relaxing effects of both estrogens were not affected by indomethacin (Table 2). Furthermore, the combined inhibition of prostanoid and NO pathways by indomethacin plus L-NAME had no effect on relaxation induced by either equilin or 17 β -estradiol (Table 2).

3.4. Role of adenylyl cyclase/cAMP/protein kinase A signaling pathway on equilin and 17 β -estradiol-evoked relaxation

The purpose of these experiments was to elucidate the signaling pathways involved in the vasorelaxant response to equilin. Inhibition of adenylyl cyclase with SQ 22,536 or protein kinase A with KT 5720 failed to reduce the relaxation to equilin in mesenteric arteries with endothelium (Table 2).

3.5. Role of potassium channels on equilin and 17 β -estradiol-evoked relaxation

The involvement of K⁺ channels in mediating equilin-evoked relaxations was determined by evaluating the effects of the non-selective Ca²⁺-activated K⁺ channel blocker TEA, the ATP-sensitive K⁺ channel blocker glibenclamide, the voltage-dependent K⁺ channel blocker 4-AP channel, as well as the Na/K-ATPase inhibitor ouabain. None of these blockers were found to affect either equilin- or 17 β -estradiol-evoked vasorelaxation responses (Fig. 3A and B).

3.6. Effect of equilin on calcium channels

The force development in response to Ca²⁺ influx and after caffeine stimulation was evaluated in mesenteric arteries incubated with equilin or 17 β -estradiol. Fig. 4A and B show that CaCl₂ evoked vasoconstriction in a concentration-dependent manner in endothelium-denuded resistance mesenteric arteries depolarized by 60 mM KCl. Pre-incubation with equilin at 1 μ M ($109.50 \pm 3.07\%$, Fig. 4A) had no significant effect on CaCl₂-induced contraction when compared to control preparations ($101.30 \pm 2.29\%$, Fig. 4A). However, incubation with equilin at 10 μ M slightly, but significantly, decreased CaCl₂-induced contraction in resistance mesenteric arteries ($85.50 \pm 5.27\%$, Fig. 4A). An equivalent effect was observed with equilin incubation at 100 μ M ($80.46 \pm 4.20\%$, Fig. 4A). On the other hand, the magnitude of 17 β -estradiol effects on CaCl₂-induced contraction was greater when compared to those observed with equilin (Fig. 4A and B). Upon 17 β -estradiol incubation, the force development in response to CaCl₂ was decreased in a concentration-dependent manner [at 10 μ M ($55.47 \pm 3.71\%$, Fig. 4B) and 100 μ M ($12.08 \pm 1.50\%$, Fig. 4B) when compared to untreated preparations ($101.30 \pm 2.74\%$, Fig. 4B)].

Contraction of vascular smooth muscle in response to the administration of the L-type Ca²⁺ channel agonist Bay K 8644 in K⁺ (10 mM) depolarizing medium was concentration-dependently inhibited by both equilin (Fig. 4C) and 17 β -estradiol (Fig. 4D), [at 10 μ M (equilin = $28.61 \pm 3.87\%$; 17 β -estradiol = $9.78 \pm 1.64\%$) and 100 μ M (equilin = $10.77 \pm 1.78\%$; 17 β -estradiol = $1.92 \pm 0.28\%$) when compared to control preparations (equilin = $49.79 \pm 4.79\%$; 17 β -estradiol = $52.89 \pm 6.87\%$, Fig. 4C and D)]. Similarly to the observed with CaCl₂, equilin produced a lower degree of inhibition of Bay K 8644 responses when compared to 17 β -estradiol (Fig. 4C and D).

The effects of equilin on the functional capacity of the sarcoplasmic reticulum to release Ca²⁺ was evaluated by placing the mesenteric arteries in Ca²⁺-free buffer followed by stimulation with caffeine. As observed in Fig. 4E, equilin incubation did not change caffeine-induced contractions, compared to control preparations. Similar results were obtained with 17 β -estradiol (Fig. 4E).

4. Discussion

The present study demonstrated that equilin, one of the most abundant estrogens in CEE induces vasodilator effects in resistance mesenteric arteries from female SHRs and shares the mechanistic action with 17 β -estradiol of inhibiting Ca²⁺ entry via L-type Ca²⁺ channels. This action is acute, nongenomic, independent of the release or action of endothelium-derived factors and does not involve binding of estrogen to its receptors and the subsequent

activation of signaling cascades involved in the activation of VSMC signaling pathways for vascular relaxation.

Estrogens have long been known to have protective effects against risk factors for the development of cardiovascular diseases and these effects are diminished after menopause, when the prevalence of hypertension and heart disease-related death for women steadily increases. The SHR is a well-known and widely used animal model of hypertension with documented vascular dysfunction [13,41,43,59], providing a suitable model to study the vascular effects of compounds used for replacement therapy. Previous studies have evaluated the vascular actions of estrogens [25,29,37,56]; however, no studies have so far investigated the acute vascular effects of equilin in resistance mesenteric arteries, which substantially contribute to peripheral vascular resistance.

The potency and responsiveness of equilin in inducing relaxation of isolated mesenteric arteries was equivalent to that of 17β -estradiol. The time course of relaxation to equilin in mesenteric arteries was also comparable with 17β -estradiol and indicates that an acute or nongenomic signaling mechanism seems to be involved in the relaxant response to this compound. Although the acute effects of equilin on vascular reactivity have not been demonstrated until now, previous work reported that in vivo chronic treatment with equilin increased mean arterial blood pressure and vasoconstriction to agonists such as KCl, norepinephrine, and serotonin in perfused mesenteric vascular bed from ovariectomized female Sprague-Dawley rats [32]. The divergences between these results and those obtained in the present study could be explained by factors like the experimental model (Sprague-Dawley rats vs. SHR); the vascular bed (perfused mesenteric arterial bed vs. isolated resistance mesenteric arteries); or the treatment (chronic in vivo vs. acute in vitro).

Similarly to 17β -estradiol, the relaxing effects of equilin were not changed after pre-treatment with the ER antagonist ICI 182,780. This finding leads us to postulate that equilin induces acute endothelium-independent relaxation of mesenteric arteries via a mechanism that does not require the ICI 182,780-sensitive ERs. These data also suggest that the beneficial effects of equilin could occur despite of the number of receptors expressed, which becomes of great significance in conditions where endogenous hormones are absent, as in the menopause in women.

The effects of 17β -estradiol are mediated by $ER\alpha$ and $ER\beta$. It has been reported that $ER\alpha$ predominates in the uterus, mammary glands, pituitary gland, skeletal muscle, adipose tissue, and bone. $ER\beta$, in contrast, mediates 17β -estradiol signaling in the ovary, prostate, lung, cardiovascular and central nervous systems. Even within a single tissue, the expression pattern of each subtype is cell type-specific. Accompanying this difference in ER subtype distribution patterns, there is difference in the pharmacology of the different ER ligands, which could be explained by differences in the amount and/or type of the co-regulatory proteins, which show variations in cells from different tissues of origin [61]. Unlike classical estrogens (estrone and 17β -estradiol), equilin belongs to a group of unique ring B unsaturated estrogens. The ring B unsaturated estrogens are formed by an alternate steroidogenic pathway in which cholesterol is not an obligatory intermediate. In contrast to 17β -estradiol, ring B unsaturated estrogens express their biological effects mainly mediated

by the estrogen receptor β and not the estrogen receptor α [4]. This may provide further support to the hypothesis that the beneficial vascular effects of equilin and the tissue-selective pharmacology could be associated with a lesser impact on the mammary gland and uterus and may be one condition where an equilin may be of clinical relevance. Further studies exploring the effects of in vivo treatment with this compound would be of great relevance in this regard.

An important finding of the present study is that the relaxant effect of equilin in mesenteric arteries is completely endothelium-independent, suggesting a direct effect on the vascular smooth muscle. As expected, the relaxant effect of 17β -estradiol was also found to be entirely endothelium-independent in these vessels. This is in agreement with previous data obtained in this tissue [17,45] and also in isolated perfused mesenteric vascular bed [53]. However, our data do not agree with other studies describing an endothelium-dependent and NO-derived vasodilator response induced by 17β -estradiol [9,27,48]. Differences in animal models and vascular bed studied may be a plausible explanation for those conflicting results, but these data interestingly show the complexity of estrogens signaling.

Activation and up-regulation of eNOS by 17β -estradiol has been reported in the cardiovascular system [46]. More recently, studies have also provided evidence for a role of nNOS in endothelial cells and in the vascular smooth muscle [16,18,27]. The later could provide a mechanism to explain the relaxant effect of estrogens via a NO-dependent, endothelium-independent mechanism. In contrast, we found that in mesenteric arteries, pharmacological blockade of the NO/cGMP/protein kinase G pathway with L-NAME, ODQ or KT 5823 did not affect equilin- or 17β -estradiol-induced relaxation, suggesting that the NO-dependent cascade does not play a role on relaxation to equilin or 17β -estradiol in this vascular tissue. One could argue that we cannot preclude a role for NO-dependent pathways, as pharmacological blockade of one system might upregulate other compensatory mechanisms. Accordingly, it should be noted that simultaneous blockade of NO synthesis and cyclooxygenase with a combination of L-NAME and indomethacin did not attenuate the maximal responses to equilin. Thus, our studies can rule out a role for the NO and/or the prostanoid pathways in equilin-induced vascular actions.

Previous studies have shown that the effects of estrogens are at least partially dependent on the activation of the adenylyl cyclase/cAMP/protein kinase A pathway in neurons [23], rat distal colon [11], kidney cells [51], liver [20] and VSMCs [10,25]. Considering our hypothesis that the action of 17β -estradiol and equilin might underlie the same cellular mechanism in mesenteric arteries, we investigated whether the adenylyl cyclase/cAMP/protein kinase A pathway may also be involved in the relaxant response to equilin and 17β -estradiol in this tissue. However, since SQ 22,536 or KT 5720, inhibitors of adenylyl cyclase and protein kinase A, respectively, failed to affect equilin- and 17β -estradiol-induced relaxation, it seems that, similarly to 17β -estradiol, equilin-induced relaxation in this tissue is not mediated by either adenylyl cyclase or protein kinase A.

It has been demonstrated that potassium channels opening can be involved in NO- and prostanoid-independent vasorelaxation to estrogens compounds [1]. Potassium channels in arterial smooth muscle include Ca^{2+} -activated, ATP-sensitive and voltage-dependent K^{+}

channels [38]. In the present study, blockade of potassium channels with TEA, glibenclamide, or 4-AP did not affect the relaxations to equilin, suggesting that vasorelaxation to this compound is not mediated by: 1) increasing potassium efflux through Ca^{2+} -activated K^+ channels, 2) ATP-sensitive K^+ channels, or 3) voltage-sensitive K^+ channels, respectively. Our findings also show that activation of the smooth muscle Na/K-ATPase is not involved on equilin-induced relaxation. The observation that blockade of K^+ channels also failed to inhibit 17β -estradiol-induced relaxations indicates similar mechanism of action of these compounds.

To gain further insight into the mechanisms that mediate acute vascular relaxation to equilin, we have also investigated the effects of short-term treatment with this estrogen on the vasoconstrictor responses to high K^+ depolarization and G protein-coupled receptor activation by ET-1 and U46619. KCl-induced contraction is mediated by membrane depolarization and Ca^{2+} entry through voltage-operated Ca^{2+} channels (VOCCs), activation of Ca^{2+} -dependent MLC kinase, and increases in MLC phosphorylation. GPCR-induced contraction, on the other hand, is mediated by multiple cell messengers, including inositol 1,4,5-trisphosphate, diacylglycerol, and the low molecular weight GTPase, RhoA, activation of multiple Ca^{2+} channel types, and activation of at least two kinases, RhoA kinase (ROK) and protein kinase C, in addition to Ca^{2+} -dependent MLC kinase [42]. Similarly to the observed with 17β -estradiol, we found that equilin-induced relaxation was not altered in mesenteric arteries pre-constricted with high KCl and following activation by the G protein-coupled receptor agonists (ET-1 and U46619). These results support the notion that equilin exerts a direct effect on the smooth muscle cells from mesenteric arteries via inhibition of Ca^{2+} influx from both VOCCs and receptor-operated Ca^{2+} channels (ROCCs), independently of the signaling pathway for contractile activity.

Although we have not evaluated in our study the effects of in vivo treatment with equilin, this would be an interesting approach for understanding the actions elicited by this compound over other tissues. Aside from the pharmacological differences between equilin and 17β -estradiol in the vasculature demonstrated in our study, these compounds have demonstrated to influence uterine and mammary cell proliferation therefore impacting endometrial carcinoma and breast cancer risk [8,40,49,58]. CEEs, the formulations in which equilin is present, reduced the incidence and mortality from breast cancer. 17β -estradiol and CEE induce apoptosis in long-term estrogen-deprived breast cancer cells; there is also evidence that estrogens may, on the contrary, accelerate the course of mammary cancer in younger women. This effect is different with 17β -estradiol and equilin. For example, it was previously demonstrated that 17β -estradiol exhibits a most proliferatively potent effect on cell proliferation when compared to equilin [34]. The fundamental differences between the effects of equilin and 17β -estradiol observed in the vascular tissues prompted us to speculate that the desirable effects in the uterus and the mammary gland associated with 17β -estradiol could underline an advantage of equilin over 17β -estradiol treatment.

An important finding of the present study was that both estrogens, 17β -estradiol and equilin, act via a Ca^{2+} antagonist mechanism to induce relaxation in mesenteric arteries. This was supported by our findings that incubation of mesenteric arteries with 17β -estradiol or equilin inhibited Ca^{2+} -induced contractions in high- K^+ depolarization medium. Further evidence for

a Ca^{2+} antagonist effect was provided by experiments showing that the concentration-response curves to the L-type Ca^{2+} channel activator, Bay K 8644 were concentration-dependently inhibited by equilin. A Ca^{2+} antagonist effect also has been demonstrated in the vasorelaxant response to 17β -estradiol and raloxifene in rabbit and porcine coronary arteries [14,22,28,47].

Agonist-induced contraction of vascular smooth muscle may involve Ca^{2+} release from the intracellular stores or Ca^{2+} entry from the extracellular space. Caffeine is known to stimulate Ca^{2+} release from the intracellular stores and to cause transient contraction in vascular smooth muscle. Although the inhibitory effect of equilin on extracellular Ca^{2+} influx has been demonstrated, this compound failed to inhibit Ca^{2+} release from the intracellular stores because it was unable to block the transitory contraction induced by caffeine. Based on this, we can suggest that the functional capacity of the sarcoplasmic reticulum to release Ca^{2+} in VSMC is not involved on equilin effects. Indeed, equilin-induced relaxant effects seem to be mediated by inhibition of Ca^{2+} influx from cellular membrane. This Ca^{2+} antagonist property could constitute one of the main mechanisms of the endothelium-independent relaxation induced by equilin in resistance mesenteric arteries from female SHR.

The lower potency of equilin to inhibit Ca^{2+} influx when compared to 17β -estradiol demonstrated in our study must be viewed from the perspective of cellular regulation of contraction. Two different mechanisms mediate smooth muscle contractions, the thick-filament and the thin-filament regulation [19]. Thick-filament regulation depends on increases in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) and requires phosphorylation of the regulatory myosin light chain. The increases in $[\text{Ca}^{2+}]_i$ in smooth muscle cells leads to the activation of Ca^{2+} /calmodulin-dependent myosin light chain kinase and phosphorylation of the myosin light chains, resulting in increased activity of myosin ATPase and, thus, cross-bridge cycling [36]. On the other hand, thin-filament regulation is Ca^{2+} -independent and triggered by production of diacylglycerol (DAG) through breakdown of phospholipids in the cell membrane. DAG binds to and activates protein kinase C (PKC). Additionally, direct activation of PKC can cause sustained contraction of vascular smooth muscles with no significant change in $[\text{Ca}^{2+}]_i$ [5]. Since smooth muscle contraction is regulated in both Ca^{2+} -dependent and Ca^{2+} -independent manners, there is the possibility that equilin also modulates contractility of vascular smooth muscles in a Ca^{2+} -independent manner.

The different pharmacological profiles exhibited by equilin and 17β -estradiol is also made possible by the existence of additional Ca^{2+} -specific influx, efflux, and sequestration processes and by the existence of specific high-affinity Ca^{2+} binding proteins that serve as intracellular Ca^{2+} receptors, all of which could mediate the effects of equilin. Another important point to be considered is that Ca^{2+} channel blockers acting at the L-type channels may interact at distinct receptor sites. These different receptor interactions underlie, in part, the qualitative and quantitative differences exhibited by channel blockers. These sites are linked to the opening and closing of the channel and to each other by activating or inhibiting allosteric mechanisms. The use dependent activity is consistent with a preferred interaction of the antagonists with the open or inactivated states of the Ca^{2+} channel rather than with the resting state [50]. This activity is not shared equally by all Ca^{2+} blockers and may provide a further basis for the therapeutic differences between equilin and 17β -estradiol.

In conclusion, the present study shows that equilin acts on vascular smooth muscle of mesenteric arteries by inhibiting Ca^{2+} entry via L-type Ca^{2+} channels. Regardless of its lower potential as Ca^{2+} antagonist, equilin relaxes resistance mesenteric arteries with an agonist potency equivalent to the 17β -estradiol. This action is acute, nongenomic, and independent of the endothelium or ICI 182,780-sensitive ERs. Potassium channels do not seem to be involved in the relaxant response to equilin. Although our data suggest a beneficial vascular action by equilin, further studies are required to elucidate whether the in vitro effects of equilin in resistance arteries may be translated into cardiovascular protection.

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Fig. 1. Role of estrogen receptors and endothelium removal on equilin and 17β-estradiol-evoked relaxations in resistance mesenteric arteries from female SHR. (A) Relaxation to either equilin or 17β-estradiol was not inhibited by the estrogen receptor antagonist (ICI 182,780). (B) Endothelium removal did not change the relaxing responses to either equilin or 17β-estradiol. Data are expressed as percentage of precontraction induced by U46619 (1 μM) (mean ± S.E.M. for five to seven animals).

Fig. 2.

Role of NO/cGMP/protein kinase G signaling pathway on equilin and 17β-estradiol-evoked relaxations in resistance mesenteric arteries from female SHR. Relaxant response to equilin was not affected in the presence of the nitric oxide synthase inhibitor L-NAME, the guanylyl cyclase inhibitor ODQ, or the protein kinase G inhibitor KT 5823 (A). Similarly, none of these inhibitors were found to affect the relaxing responses to 17β-estradiol (B). Data are expressed as percentage of contraction induced by U46619 (1 μM) (mean ± S.E.M. for four to six animals).

Fig. 3. Role of potassium channels on equilin and 17β-estradiol-evoked relaxations in resistance mesenteric arteries from female SHR. Relaxation to either Equilin (A) or 17β-estradiol (B) was not inhibited by the non-selective Ca^{2+} -activated K^+ channel blocker TEA, the ATP-sensitive K^+ channel blocker glibenclamide, the voltage-dependent K^+ channel blocker 4-AP channel, or the Na/K-ATPase inhibitor ouabain. Data are expressed as percentage contraction induced by U46619 (1 μM) (mean $\pm$ S.E.M. for four to six animals). TEA = tetraethylammonium, 4-AP = 4-aminopyridine.

Fig. 4.

Effect of equilin on calcium channels in resistance mesenteric arteries from female SHR. (A) Equilin at 1 μM had no significant effect on CaCl_2 -induced contraction when compared to control preparations. However, at 10 μM and 100 μM , equilin significantly decreased CaCl_2 -induced contraction in resistance mesenteric arteries depolarized by 60 mM KCl. Equivalent effect was observed upon 17 β -estradiol incubation (B). Contraction in response to the administration of the L-type calcium channel agonist (S)-(-)-Bay K 8644 in high- K^+ (10 mM) depolarizing medium was concentration-dependently inhibited by both equilin (C) and

17 β -estradiol (D) at 10 μ M and 100 μ M when compared to control preparations. (E) Equilin incubation did not change caffeine-induced contractions. Similar results were obtained with 17 β -estradiol. Data are expressed as percentage of contraction induced by 120 mM KCl (mean $\pm$ S.E.M. for five to seven animals). *P < 0.05 compared with data from the vehicle-treated segments; #P < 0.05 compared with segments incubated with equilin in the same concentration.

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Table 1

Relaxant effects of equilin and 17 β -estradiol in resistance mesenteric arteries from female SHR after contraction induced by different agents: U46619 (1 μ M), Endothelin (ET-1, 10 nM), phorbol 12,13-dibutyrate (PDBu, 0.3 μ M) and potassium chloride (KCl, 120 mM).

Contractile agents	Equilin		17 β -Estradiol	
	R _{MAX}	pD ₂	R _{MAX}	pD ₂
U46619	99.94 $\pm$ 0.57	4.79 $\pm$ 0.02	92.67 $\pm$ 2.05	4.75 $\pm$ 0.08
ET-1	91.84 $\pm$ 1.40	5.15 $\pm$ 0.06	91.13 $\pm$ 1.42	5.17 $\pm$ 0.11
PDBu	96.41 $\pm$ 1.20	5.03 $\pm$ 0.04	94.69 $\pm$ 2.78	5.16 $\pm$ 0.06
KCl	99.15 $\pm$ 0.42	4.95 $\pm$ 0.02	98.24 $\pm$ 0.37	5.15 $\pm$ 0.02

Values are means $\pm$ S.E.M. for four to six animals.

R_{MAX} = Maximum response; pD₂ = $-\log$ EC₅₀.

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Table 2

Relaxant effects induced by equilin and 17 β -estradiol in control conditions and during incubation with the cyclooxygenase inhibitor indomethacin (10 μ M), the indomethacin plus non-specific NO synthase inhibitor L-NAME (100 μ M), the adenylyl cyclase inhibitor SQ 22536 (100 μ M), or the protein kinase A inhibitor (KT 5720, 1 μ M).

	Equilin		17 β -Estradiol	
	R _{MAX}	pD ₂	R _{MAX}	pD ₂
Control	99.94 $\pm$ 0.57	4.79 $\pm$ 0.02	92.67 $\pm$ 2.05	4.75 $\pm$ 0.08
+Indomethacin	99.25 $\pm$ 0.13	5.00 $\pm$ 0.05	86.71 $\pm$ 3.20	4.95 $\pm$ 0.09
+Indomethacin + L-NAME	96.62 $\pm$ 1.22	4.98 $\pm$ 0.06	93.07 $\pm$ 1.46	4.91 $\pm$ 0.10
+SQ 22536	97.47 $\pm$ 1.14	4.83 $\pm$ 0.05	83.92 $\pm$ 2.21	5.01 $\pm$ 0.10
+KT 5720	98.42 $\pm$ 0.12	4.96 $\pm$ 0.06	94.98 $\pm$ 3.21	5.02 $\pm$ 0.09

Values are means $\pm$ S.E.M. for four to six animals. Relaxant effect expressed as percent of contraction elicited by U46619 (1 μ M).

R_{MAX} = Maximum response; pD₂ = $-\log$ EC₅₀.