Acute vascular effects of the selective estrogen receptor modulator EM-652 (SCH 57068) in the rat mesenteric vascular bed

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Received 27 May 2002; accepted 11 September 2002

Abstract

Objective: Selective estrogen receptor modulators (SERMs) represent a class of compounds that act as either estrogen receptor agonist or antagonist in a tissue-selective manner. SERMs exert beneficial effects on bone and lipids but are not associated with an increased risk of breast or uterine carcinoma. 17β-estradiol (E2) and SERMs such as raloxifene and tamoxifen acutely relax coronary arteries. EM-652 (SCH 57068) is a 4th generation SERM acting as pure antiestrogen in the mammary gland and endometrium. The effects of SERMs on the mesenteric vasculature are unknown. In the present study, the vascular effects of EM-652 and E2 on the rat mesentery were investigated.

Methods: Isolated perfused (5 ml/min) mesenteric vascular bed (MVB) was preconstricted with methoxamine. Increasing doses (0.1–10 μM) of EM-652 or E2 were infused into the perfusate.

Results: EM-652 and E2 relaxed MVBs removed from intact and gonadectomized female and male rats. The amplitude of EM-652 responses was consistently greater than those of E2 and its potency was similar or greater than that of other SERMs. EM-652 and E2 relaxed MVB by an endothelium-independent mechanism. The estrogen receptor (ER) antagonist ICI 182,780 attenuated E2-induced relaxations but only partially block the effects of EM-652. Inhibition of the nitric oxide synthase/cGMP pathway with L-nitro-arginine-methyl-ester (L-NAME) and 1H-(1,2,4)oxadiazolo(4,3-a)quinoxaline-1-one (ODQ) or of prostanoid synthesis with indomethacin failed to reduce EM-652 responses. The vascular effects of EM-652 were also unaffected by potassium channels blockers or inhibitors/scavengers of reactive oxygen species. EM-652 attenuated the vasoconstrictor responses induced by adrenergic agonists and endothelin-1. Conclusions: EM-652 acutely relaxes the mesenteric vasculature by an endothelium-independent pathway which is partly mediated by ER, providing a novel mechanism by which this SERM may exert beneficial actions on the vascular system.

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Keywords: Arteries; Hormones; Receptors; Vasoconstriction/dilation

1. Introduction

Epidemiological studies have shown that premenopausal women are at lower risk for developing cardiovascular disease (CVD) than men of similar age [1]. The cardioprotective effect of estrogens has traditionally been attributed to favourable changes in blood lipids and lipoproteins. However, much attention has focussed recently on the acute vascular effects of estrogen [2,3]. Indeed, there is now considerable evidence that the vascular endothelium is the target of estradiol [4,5]. Although long-term hormone replacement therapy (HRT) reduces the risk of CVD [6] and osteoporotic fracture in postmenopausal women, it is also associated with several potential risks including breast and endometrial cancer. The increased risk of cancer, together with the occurrence of undesirable side effects of HRT has prompted the development of pharmacological tools that modulate estrogen biosynthesis and action [7,8].

Selective estrogen receptor modulators (SERMs) represent a growing class of compounds that act as either estrogen receptor agonist or antagonist in a tissue-selective manner [9]. SERMs are currently used for breast cancer...
prevention and treatment. The potential of SERMs for reducing the risks of CVD has also been documented [10]. SERMs such as raloxifene [11] and tamoxifen [12] have been shown to relax coronary arteries in vitro by an ER-dependent mechanism suggesting that the estrogen-like effects of SERMs are preserved in coronary arteries. However, the in vitro effects of SERMs in the mesenteric vascular bed, a tissue that contributes substantially to the regulation of total peripheral resistance and blood pressure [13] are unknown.

We have synthesized an orally active non-steroidal antiestrogen EM-652 (SCH 57068) and the prodrug EM-800 which are the most potent of the known antiestrogens [7,8]. EM-652 is a 4th generation SERM acting as a pure antiestrogen in the mammary gland and endometrium [7,8,14]. Whether EM-652 shares the vascular actions of other SERMs is still unknown. The present study was therefore designed to investigate the acute vascular effects of EM-652 on the rat isolated perfused mesentery and the possible modulation by endothelial factors.

2. Methods

2.1. Animals and in vitro vasoreactivity in the mesenteric vascular bed

All experiments and protocols were performed in accordance with the regulations established by the NIH council and the Canadian Council on Animal Care, and approved by the Animal Care Committee at Laval University. Adult (12–15 weeks of age) male and female rats (Charles River, Montreal, QC) were sham operated or gonadectomized 3 weeks before the experiments. Animals were exsanguinated under isoflurane anesthesia and the superior mesenteric artery was cannulated and the gut removed as previously described [15]. The isolated mesenteric vascular bed (MVB) was perfused through the cannula at a constant flow rate of 5 ml/min and superfused at 0.2 ml/min using two separate pumps with modified Krebs–Henseleit (in mM: 118 NaCl, 4.7 KCl, 1.2 MgCl$_2$·6H$_2$O, 1.0 NaH$_2$PO$_4$, 2.6 CaCl$_2$·2H$_2$O, 25 NaHCO$_3$, 11.1 glucose; 37 °C; pH 7.35–7.45), oxygenated with a 95% oxygen–5% carbon dioxide gas mixture [15]. Drugs were injected into the perfusate, and arteriolar constriction or dilatation was determined by bolus injection of acetylcholine (ACh) (see Fig. 1).

2.2. Experimental protocols

MVBs were removed and prepared as described in Section 2.1 above to determine the vasodilator responses to 17β-estradiol (E2, 0.1–10 μM), EM-652 (0.1–10 μM) and other SERMs (raloxifene, tamoxifen and ERA-923). MVBs were preconstricted with methoxamine (50–70 μM) for 30 min to increase the perfusion pressure to 80–120 mmHg, and then vehicle (ethanol 0.1%), E2 or SERMs were administered in the perfusate. In a preliminary study (n=4, data not shown), we found that bolus injection of E2 or SERMs has no significant effect, therefore, all substances were infused for 15 min for each dose. The maximal relaxing effects of E2 and SERMs occurred at 5 and 15 min, respectively. In experiments to explore the mechanisms of EM-652-induced relaxing effects, MVBs from intact female rats were used and all

![Graph](https://academic.oup.com/cardiovascres/article-abstract/57/2/535/307850/752535307850)
inhibitors were maintained in the perfusate for at least 30 min prior to the addition of EM-652.

The endothelial-dependence of the vascular effects of EM-652 and E2 was determined by examining E2 and EM-652 dose–response curves in methoxamine-preconstricted MVB of intact female rats after endothelial denudation. Endothelial removal was achieved by injecting air bubbles in the perfusate for 5 min, as previously described [15]. The absence of endothelium was ascertained by the failure of a bolus dose (1 μM) of ACh to relax the MVB in methoxamine-preconstricted endothelium-denuded preparations. The involvement of the ER in the effects of EM-652 and E2 was assessed by performing E2 and EM-652 dose–response curves in the absence and presence of the ER blocker ICI 182, 780 (10 μM). This dose was shown to attenuate raloxifene and tamoxifen relaxing responses in rabbit coronary vasculature [11,12]. ICI 182, 780 was maintained in the perfusate for at least 30 min prior to the addition of EM-652 to the medium.

Further series of experiments to explore the mechanisms of EM-652-induced relaxing effects remained focused to studies in female rats. All inhibitors and vehicle were maintained in the perfusate for at least 30 min prior to the addition of EM-652 to the medium. The doses of different inhibitors were based on preliminary experiments as well as on our previous studies in isolated perfused MVB [15,17], or from studies performed by others [11,12,18,30,31].

Another set of MVBs (n=5–8 per group) was used to study the effects of incubation with EM-652 on vasoconstrictor responses to depolarization with high potassium chloride (KCl), phenylephrine (Phe), noradrenaline (NE) and endothelin-1 (ET-1). After a 30-min stabilization period, potassium chloride (KCl 50 mM) was perfused over 60 s into the perfusate at 5 min intervals over 30 min, by which time a reproducible constrictor response was obtained. Dose–response curves to Phe, NE and ET-1 were constructed by injecting increasing doses into the perfusion system at 5-min intervals (i.e. when the MVB reached a steady-state). In another set of MVBs, dose–response curves to Phe, NE and ET-1 were constructed in the same manner following 60 min pre-incubation with EM-652 (0.1 and 1 μM).

2.3. Sex hormones analysis

Determination of plasma 17β-estradiol and testosterone levels was performed using high-performance gas chromatography and negative chemical ionization mass spectrometry as described previously [16]. Plasma levels of 17β-estradiol in intact female (29.3±6.6 pg/ml), and testosterone in intact male (1.95±0.31 ng/ml) were higher compared to gonadectomized rats in which sex hormones plasma levels were below the detection limit (5 pg/ml for estradiol, and 0.03 ng/ml for testosterone).

2.4. Drugs used

All agents used except EM-652, raloxifene, tamoxifen and ERA-923 (Chemistry department of Endorecherche, Québec, Canada) were obtained from Sigma (St-Louis, MO. NE was dissolved in 0.1 mM ascorbic acid. ET-1 was dissolved in 1% BSA. Indomethacin was dissolved in sodium bicarbonate. E2, EM-652, raloxifene, tamoxifen and ERA-923, DPI, ODQ, ouabain, glibenclamide were dissolved in ethanol. The final concentration of ethanol in the medium was never greater than 0.1%.

2.5. Statistical analysis

Experiments were performed according to a randomized block design. Results are expressed as means±S.E.M. n refers to the number of animal. Significant differences between mean values for different groups were determined using ANOVA and Fischer’s test. A value of P<0.05 was considered statistically significant.

3. Results

3.1. Effects of EM-652 and E2 on methoxamine-preconstricted MVB

Fig. 1 depicts the original tracing of EM-652 (top) and E2 (bottom) relaxation in methoxamine-preconstricted MVB removed from intact female rats. The integrity of the endothelium in the MVB preparations was first assessed by a bolus injection of ACh. EM-652 and E2 induced significant dose-dependent relaxation of the MVB compared with time-matched ethanol solvent controls (Figs. 1 and 2). However, the course of the relaxation responses to E2 and EM-652 were different. As depicted in Fig. 1, E2 induced sharp and rapid vasodilatory responses with maximal relaxation occurring at 5 min, whereas the relaxing effects of EM-652 were slower and sustained with a maximal effect occurring at 15 min. The relaxing effects of E2 and EM-652 were also observed in MVBs removed from intact male, ovariectomized female and castrated male rats (Fig. 2). In all cases, the maximal relaxing effects of EM-652 were greater than that caused by E2.

3.2. Comparative relaxing effects of EM-652 with other SERMs

Methoxamine-preconstricted MVBs of intact female rats were used to compare the vasodilator responses to EM-652 with that of other SERMs (raloxifene, tamoxifen and ERA-923). Increasing doses (0.1–10 μM) of each SERM was applied for 15 min. All four SERMs induced significant dose-dependent relaxation of endothelium-intact methoxamine-preconstricted MVB (Fig. 3A). The responses to
EM-652, raloxifene and ERA-923 were similar and higher than those induced by tamoxifen.

3.3. Role of endothelium and ER in EM-652 and E2-evoked MVB relaxation

The effect of endothelium removal on EM-652 and E2-evoked relaxations is depicted in Fig. 3B, C. There were no differences in the relaxing responses to either EM-652 or E2 in preparations with or without endothelium. In the presence of the ER blocker ICI 182,780 (10 μM), there was a small but significant \( P<0.05 \) attenuation of relaxation to EM-652 (Fig. 3B). In contrast, ICI 182,780 (10 μM) caused a near-complete inhibition of relaxation to E2 at a dose of 5 μM, whereas a partial inhibition was observed at a higher dose (10 μM) of E2 (Fig. 3C).

3.4. Role of endogenous NO/cGMP and prostanoids in EM-652-evoked MVB relaxation

To investigate the role of endogenous NO and cGMP production in EM-652-induced vasodilatory responses, EM-652 dose–response curves were generated in the absence or presence of either the nitric oxide synthase inhibitor l-NAME (100 μM), or the guanylate cyclase inhibitor, 1H-(1,2,4)oxadiazolo(4,3-a)quinazoline-1-one (ODQ, 5 μM). Neither l-NAME nor ODQ were found to affect the EM-652-evoked relaxation (Fig. 4A). Moreover, preincubation with 2,4-diamino-6 hydroxypyrimidine (DAHP, 5 mM), an inhibitor of the synthesis of tetrahydrobiopterin, a cofactor of nitric oxide synthase, also failed to reduce the EM-652-mediated vasodilatory responses.

The contribution of endogenous vasodilatory prostanooids to EM-652-induced relaxation was evaluated by testing the effects of the cyclooxygenase inhibitors indomethacin (50 μM) and diclofenac (50 μM). The relaxing effects of EM-652 were not affected by either inhibitors (Fig. 4B). However, the combined inhibition of the NO/cGMP and prostanoid pathways by l-NAME + indomethacin caused a slight but significant inhibition of EM-652-mediated relaxation (Fig. 4B).

3.5. Role of potassium channels in EM-652-evoked MVB relaxation

The involvement of potassium \( (K^+ ) \) in mediating EM-652-evoked relaxations were determined by evaluating the effects of the ATP-sensitive \( K^+ \) channel blocker, glibenclamide (5 μM), the calcium-dependent \( K^+ \) channel blocker tetraethylammonium (TEA, 10 mM), the non-specific inhibitor of \( K^+ \) channel, barium chloride (BaCl₂, 75 μM) as well as the Na/K ATPase inhibitor ouabain (5 μM). None of these inhibitors were found to affect EM-652-evoked vasodilatory responses (Fig. 4C).
1,3-benzene-disulfonic acid (Tiron, 10 mM). Neither DPI, nor Tiron pre-treatments affected EM-652 vasodilatory responses (Fig. 4D).

3.7. Effects of EM-652 on MVB vasoconstrictor responses

In the presence of an intact endothelium, perfusion of MVB at a constant flow rate induced a steady basal perfusion pressure (BPP, mmHg) after an initial stabilization period of 60 min. Preincubation with EM-652 for 60 min had no effect on BPP (37±2 in vehicle, 37±1 in EM-652 at 1 μM, and 36±3 in EM-652 at 0.1 μM). Responses to depolarization with high potassium chloride (KCl 50 mM) were attenuated following pretreatment with 1 μM EM-652 (12±3 mmHg) compared to vehicle (20±2 mmHg) and 0.1 μM EM-652 (24±4 mmHg). The vasoconstrictors Phe, NE and ET-1 dose-dependently initiated an increase in PP (i.e. constrictor response) (Fig. 5). Pretreatment with EM-652 (1 and 0.1 μM), at low doses that did not relax pre-contracted MVB (see Fig. 1), significantly blunted the vasoconstrictor responses by 30 to 50%.

4. Discussion

The present study shows for the first time that the 4th generation SERM EM-652 induces acute vasodilator responses in the methoxamine-preconstricted mesenteric vascular bed. While previous studies have addressed the issue of vascular actions of estrogens and estrogen-like molecules, no studies have so far attempted to investigate the acute effects of SERMs in the mesenteric vascular bed, which represents a resistive network that contributes substantially to peripheral vascular resistance. The doses of SERMs and estradiol necessary to induce vasodilation in the MVB are in the same order of magnitude as those used by other groups using different vascular models and role of the endothelium and ER. (A) Maximal vasodilator responses to EM-652, Tamoxifen Raloxifene and ERA-923 in endothelium-intact methoxamine-preconstricted MVB removed from intact female rats. n = 6–10 per group, *, P<0.05: significant differences vs. tamoxifen; ¶, P<0.05: significant differences vs. vehicle-control. Maximal vasodilator responses to EM-652 (B) and E2 (C) (0.1–10 μM) in methoxamine-preconstricted MVB removed from intact female rats in the presence and absence of intact endothelium, and endothelium-intact preincubated with ICI 182,780. n=6–10 per group. *, P<0.05: significant differences vs. vehicle-control.

3.6. Role of reactive oxygen species in EM-652-evoked MVB relaxation

The contribution of reactive oxygen species (ROS) to EM-652-evoked relaxation was determined by the use of the NADPH oxidase inhibitor diphenyleneiodonium (DPI, 5 μM), or the superoxide anion scavenger 4,5-dihydroxy-

Fig. 3. Comparisons of relaxing responses to EM-652 and other SERMs and role of the endothelium and ER. (A) Maximal vasodilator responses to EM-652, Tamoxifen Raloxifene and ERA-923 in endothelium-intact methoxamine-preconstricted MVB removed from intact female rats. n = 6–10 per group, *, P<0.05: significant differences vs. tamoxifen; ¶, P<0.05: significant differences vs. vehicle-control. Maximal vasodilator responses to EM-652 (B) and E2 (C) (0.1–10 μM) in methoxamine-preconstricted MVB removed from intact female rats in the presence and absence of intact endothelium, and endothelium-intact preincubated with ICI 182,780. n=6–10 per group. *, P<0.05: significant differences vs. vehicle-control.
relaxations did not require the presence of gonads. The finding that the relaxing effects of E2 and EM-652 were observed in both males and females is consistent with previous reports using raloxifene and tamoxifen in the rabbit coronary artery [11,12]. These latter SERMs and ERA-923 also induced relaxation in the mesenteric vasculature. In terms of potency, EM-652 was comparable to raloxifene and ERA-923, but greater than tamoxifen.

An important finding of the present study is that the vasodilatory effect of EM-652 in the mesenteric vasculature is completely endothelium-independent. Indeed, we found that the relaxing effects of EM-652 persisted following endothelial removal, suggesting a direct effect on the underlying vascular smooth muscle (VSM) in the mesenteric vasculature. Perhaps less expectedly, the vasodilatory effect of E2 was also found to be endothelium-independent in the MVB. This is in contrast with previous data obtained in coronary arteries showing that the relaxing actions of E2 and SERMs (raloxifene, tamoxifen) are partially endothelium-dependent [11,12]. However, there are also studies supporting the notion that E2-induced relaxing responses are entirely independent from an intact endothelium [21–25] or from NO [20,21,23]. The reasons for these discrepant findings are unknown but many factors such as animal strain, type of vascular bed, the dose and duration of E2 exposure may all contribute to these differences.

The relaxing effects of EM-652 are at least partly mediated by the ER since pre-treatment with the non-specific ER blocker ICI 182,780 significantly inhibited part of the relaxation response. It is therefore conceivable that EM-652 induces relaxation following binding to ER receptors on VSM cells which have been shown to express functional ERs [2]. Thus, in methoxamine-preconstricted mesenteric arteries, part of the endothelium-independent relaxation to EM-652 may be due to its direct actions on smooth muscle membrane-resident ERs.

A large part of EM-652-induced relaxation persisted even in the presence of the ER blockade by ICI 182,780. The same dose of ICI 182,780 (10 μM) was able to completely block relaxation induced by 5 μM E2. The lack
antagonist could also be explained by the fact that EM-652 has a very high affinity for the ER. Although we have not measured the binding characteristics of EM-652 in the MVB, it has been reported that EM-652 has the most potent inhibitory activity on both ER alpha and beta compared to E2 or any of the other antiestrogens tested in human breast cancer cells [7,8]. Indeed, studies in human breast cancer and normal human uterine cells have shown that ICI 182,780 has about 10 times less affinity than EM-652 to displace [3H]E2 [27]. Furthermore, Martel and coworkers reported that EM-652 is seven to eight times as potent as E2 and ICI 182,780 in displacing [3H]E2 from rat uterine ER (IC50 values of 0.52 nM, 4.13 nM and 3.59 nM for EM-652, E2 and ICI 182,780 respectively) [14]. Thus, one could argue that despite the relatively high dose of ICI 182,780 used in this study, it was not sufficient to displace all of the EM-652 bound to the ER.

It has been reported that chronic treatment of ovariectomized rats with the SERM LY11708, enhances the release of NO from aortic endothelium [28]. Similarly raloxifene was shown to acutely relax coronary artery via a partial NO-dependent mechanism [11]. Furthermore, Ma et al. [29] reported that both estradiol and the SERM idoxifene have acute stimulatory effects on endothelial NO release in rat superior mesenteric artery rings. In contrast, in our MVB model, we found that pharmacological blockade of the NO/cGMP pathway with L-NAME or ODQ was without effect on EM-652-induced relaxation, suggesting that the NO-dependent cascade plays a minor role in EM-652-induced relaxation in this vascular bed. However, this does not preclude a more subtle role for NO-dependent pathways, as pharmacological blockade of one system might upregulate other compensatory mechanisms. In this regard, it should be noted that simultaneous blockade of NO synthesis and cyclooxygenase with a combination of L-NAME and indomethacin partially attenuated the maximal responses to EM-652. Thus, our studies cannot rule out a minor role for the NO and/or the prostanoid pathways in EM-652 vascular actions.

It has been demonstrated that potassium channels are involved in NO- and prostanoid-independent relaxations to muscarinic agonists [18,30]. In the present study, blockade of potassium channels with glibenclamide, TEA or barium chloride did not affect the relaxations to EM-652, suggesting that potassium channels are not involved in the relaxing effects of this SERM in the MVB. Furthermore, inhibition of the Na/K-ATPase pump with ouabain also failed to inhibit EM-652-induced relaxations. Our findings differ from those of Figtree and colleagues who reported that barium chloride inhibits raloxifene-evoked relaxation in rabbit coronary arteries [11]. The differences in vascular models (rat mesenteric vasculature vs. rabbit coronary arteries) and SERM (EM-652 vs. raloxifene), could explain these apparent divergences.

A decrease in the production of endothelium-derived superoxide anion has been proposed as a mechanism...
accounting for vasculoprotective properties of estrogen [31]. However, our data show that ROS are not involved in EM-652-evoked relaxation as can be judged by the lack of effects of both NADPH oxidase inhibition and superoxide scavenging by preincubation with DPI and Tiron, respectively.

To gain further insight into the acute vascular effects of EM-652, we have also investigated the effect of short-term treatment with the SERM on the vasoconstrictor responses to high potassium depolarization and G protein-coupled receptor activation. We found that an acute pre-incubation with EM-652, at doses which did not relax the pre-contricted mesenteric arteries per se, significantly reduced the vasoconstrictor responses to high KCl and following activation by adrenergic agonists (Phe and NE), and ET-1. These observations are consistent with the finding that short-term incubation with raloxifene attenuated contractile responses in coronary arteries [11]. These results further support the notion that EM-652 exerts a direct effect on the MVB smooth muscle cells, independent of signaling pathway for contractile activity.

In conclusion, our study shows that the new SERM EM-652 acutely relaxes the MVB from female and male rats via an endothelium-independent and partly ER-linked pathway. Although the nature of this pathway remains unknown, our data strongly suggest that the smooth muscle cells are the target of EM-652 in the mesenteric vasculature. As the cardiovascular beneficial effects of HRT based on estrogen and its derivatives have been called into questions [32–34], the present findings raise the potential benefit of using SERMs such as EM-652 against vasculo-degenerative diseases associated with estrogen deficiency, without the known risk of estrogen therapy-related cancer. Further preclinical studies need to be performed in order to evaluate the potential clinical importance of EM-652 in the regulation of arterial blood pressure and in the prevention of cardiovascular complications.

Acknowledgements

The authors wish to thank the URMA staff for technical assistance and excellent care of animals. We thank Dr. Ronald Charbonneau for his continuous support of our work.

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