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Idoxifene causes endothelium-dependent, nitric oxide-mediated vasorelaxation in male rats

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Abstract

Selective estrogen receptor modulators are estrogen-like compounds that lack the deleterious effects of estrogen. The present study was designed to determine whether idoxifene, a new selective estrogen receptor modulator, may have a vasodilatory effect on aortic vessels from male animals, and if so, to investigate the mechanism (i.e., endothelium-independent, direct vasorelaxation vs. endothelium-dependent, nitric oxide mediated vasorelaxation) by which idoxifene may exert its vasodilatory effect. Superior mesenteric arterial rings from adult male Sprague–Dawley rats were suspended in Krebs–Henseleit ring baths. Rings were contracted with 50 nM U-46619 (9,11-epoxymethano-PGH_2), a thromboxane A_2 mimetic. Cumulative dose–response vasorelaxation to idoxifene (0.01 to 3 μM) was studied in the presence and absence of 200 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME, a nitric oxide synthase (NOS) inhibitor). The results obtained from idoxifene were compared with those from 17β-estradiol. Our experimental results demonstrated that addition of idoxifene to superior mesenteric arterial rings isolated from male rats resulted in a dose-dependent vasorelaxation in the range of 0.1 μM (minimal vasodilatory concentration) to 3 μM (maximal vasodilatory concentration). Pre-treatment with L-NAME to block nitric oxide (NO) production virtually abolished idoxifene-induced vasodilatation, indicating that idoxifene caused an NO-mediated vasorelaxation in vessels from male animals. Addition of 17β-estradiol also resulted in an endothelium-dependent vasorelaxation in aortic rings from male rats. However, these vessels were 30-fold less sensitive to 17β-estradiol than to idoxifene in their vasorelaxation responses. Taken together, these results demonstrate that selective estrogen receptor modulators are superior to traditional estrogen in their vascular protection and may thus have potential therapeutic use in protection against cardiovascular disease, especially in male patients.

Keywords: Idoxifene; Estrogen; Nitric oxide (NO)

1. Introduction

Experimental and clinical studies have provided ample evidence that estrogen exerts a significant antiatherosclerotic effect and reduces morbidity and mortality from cardiovascular diseases. The mechanisms by which estrogen evokes its protective effects are not fully understood. Earlier studies have suggested that estrogen may exert its cardiovascular protection by improving plasma lipid profiles (Blum and Cannon, 1998). However, this change accounts for only 25–50% of the protective effect of estrogen against cardiovascular diseases (Gruchow et al., 1988). Accumulating evidence now indicates that estrogen has a direct effect on the vascular endothelium resulting in increased nitric oxide (NO) bioactivity, which may contribute significantly to its cardiovascular protective effects (Kauser and Rubanyi, 1997; Miller, 1999).

Despite the apparent beneficial effects of estrogen in preventing cardiovascular diseases, it is estimated that $<10\%$ of women who might benefit from this therapy are actually taking estrogen (Harris et al., 1990). The major reasons for this are fear of estrogen-induced breast and uterine cancer (Judd et al., 1983). Although epidemiological observations and clinical studies have demonstrated that the morbidity and mortality of cardiovascular diseases are much higher in males than in females, side effects of estrogen such as gynecomastia prevent its routine use in males. The search for more acceptable and safer hormonal therapies has led to

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the evaluation of compounds known as selective estrogen receptor modulators. Previous pharmacological studies have demonstrated that idoxifene is a novel selective estrogen receptor modulator that has estrogen agonism on one or more desired target tissues such as bone and liver, and estrogen antagonism and/or minimal estrogen agonism in reproductive tissues such as the breast or uterus (Mitlak and Cohen, 1999; Nuttall et al., 1998; Treinen et al., 1998). However, the effect of idoxifene on vascular tissues (i.e., estrogen agonism or antagonism) remains elusive. In particular, whether or not idoxifene may exert significant vasodilatory effects on male animals, as it has been shown to do in vessels from female animals, has not been previously investigated.

Therefore, the aims of the present study were to (1) determine the vasodilatory effect of idoxifene in superior mesenteric arterial rings isolated from male animals and compare its effect with that of traditional estrogen; and (2) investigate the mechanisms by which idoxifene may exert its vasoactive effect in these vessels (i.e., endothelium-independent, direct vasorelaxation vs. endothelium-dependent, NO-mediated vasorelaxation).

2. Materials and methods

2.1. Materials

Idoxifene (pyrrolidino-4-iodotamoxifen) was synthesized by SmithKline Beecham Pharmaceuticals (King of Prussia, PA). 17β-estradiol and N^{ω}-nitro-L-arginine methyl ester (L-NAME) was purchased from Sigma (St. Louis, MO). Stock solutions (10 mM) of idoxifene or 17β-estradiol were made with dimethylsulfoxide (DSMO) (Treinen et al., 1998). Adult male Sprague–Dawley rats were obtained from ACE Animals. The experiments were performed in adherence to National Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by the Thomas Jefferson University Committee on Animal Care.

2.2. Preparation of vascular rings and determination of vasorelaxation activity of idoxifene and 17β-estradiol

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). After a midline laparotomy was performed, the superior mesenteric artery was isolated and placed into ice-cold Krebs–Henseleit (K–H) buffer consisting of (mM) NaCl 118, KCl 4.75, $CaCl_2 \cdot 2H_2O$ 2.54, KH_2PO_4 1.19, $MgSO_4 \cdot 7H_20$ 1.19, $NaHCO_3$ 25, and glucose 10.0. The superior mesenteric artery was carefully cleaned of fat and loose connective tissue, and cut into rings of 2–3 mm length. These rings were then mounted on stainless steel hooks, suspended in 37 °C and aerated (95% O_2 and 5% CO_2) 7.5 ml K–H tissue baths, and connected to FORT-10 force transducers (WPI, Sarasota, FL) to record changes in tension via a MacLab data acquisition system. The rings were then stretched to an optimum preload of 1.0 g of force determined in previous experiments in this laboratory and allowed to equilibrate for 60 min. During this period, the K–H buffer in the tissue bath was replaced every 15 min, and the tension of vascular rings was adjusted until 1.0 g of preload was maintained.

After equilibration, the rings were first exposed to maximally effective concentrations of a contractile agonist (100 nM of U-46619, 9,11-epoxymethano-PGH_2) to ensure stabilization of the vascular smooth muscle. The agonist was then washed out and the rings re-equilibrated. Twenty minutes after the initial washing, 50 nM of U-46619 was added to each ring bath to generate approximately 1.0 g of developed force. Once a stable contraction was obtained, idoxifene (0.01–10 μM) or 17β-estradiol (0.1–100 μM) was added to the bath in 0.5 log increments (Figtree et al., 1999). Segments not exposed to idoxifene or 17β-estradiol, but exposed to DMSO solvent, acted as time-matched controls. The presence of a functionally normal endothelium was always verified by observation of the relaxation response to acetylcholine. Those vessels that had a $<70\%$ vasorelaxation response to 10 μM acetylcholine were excluded from further study.

To determine if the vasodilatory effect of idoxifene was endothelium-dependent, 15 additional superior mesenteric arterial rings were studied in which endothelium was removed by gentle rubbing with a wooden probe. A successful denudation of endothelial cells was confirmed by observing a lack of vasorelaxation to acetylcholine, but a normal vasodilatation response to acidified $NaNO_2$, an endothelium-independent vasodilator. The vasorelaxation response to idoxifene was then performed in these endothelial denuded rings as described above. To investigate whether idoxifene-induced vasorelaxation involved an NO-dependent mechanism, a separate set of rings were first incubated with N^{ω}-nitro-L-arginine methyl ester (L-NAME, 200 μM), a competitive nitric oxide synthase inhibitor. Thirty minutes after L-NAME incubation, a concentration–response curve to increasing concentration of idoxifene or 17β-estradiol was studied as described above.

2.3. Statistical analysis

All values in the text, tables and figures were presented as mean ± S.E.M. of N independent experiments. All data were subjected to analysis of variance (ANOVA) followed by the Bonferroni correction for post-hoc t-tests. Probabilities of $p \leq 0.05$ were considered to be statistically significant.

3. Results

In U-46619-precontracted superior mesenteric arterial rings, the cumulative addition of DMSO at the same volume as idoxifene (i.e., 7 μl × 6) resulted in a endothelium-

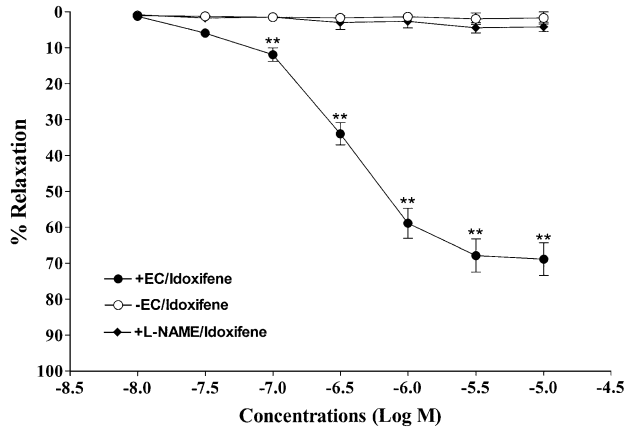

Fig. 1. Concentration-dependent vasorelaxation to idoxifene and its blockade by endothelium denudation and NOS inhibition. $N = 15-18$ rings from at least six animals. $^{**}P < 0.01$ vs. vehicle at the same volume. +EC: endothelium intact; −EC: endothelium denuded.

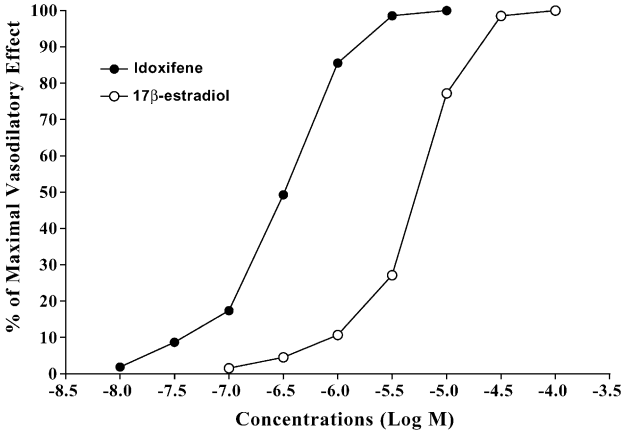

Fig. 3. Comparison of idoxifene- and 17β-estradiol-induced vasodilation in endothelium intact superior mesenteric arterial rings isolated from male rats. The dose–response curves were re-constructed as follows: mean vasodilatory value at concentrations indicated/mean maximal vasodilatory value × 100%.

independent vasorelaxation (2.1 ± 0.8% to 33 ± 1.3% with increasing doses of DMSO). This vehicle-induced, non-specific vasorelaxation was subtracted when calculating idoxifene-induced vasorelaxation. The cumulative addition of idoxifene to U-46619-precontracted superior mesenteric arterial rings resulted in a concentration-dependent vasorelaxation with a minimal effective concentration of 0.1 μM and an EC_{50} of 0.32 μM. The vasorelaxation caused by idoxifene reached a plateau at a concentration above 3 μM, and further increasing the idoxifene concentration to 10 μM only slightly increased its vasorelaxation effect (Fig. 1).

To determine whether idoxifene-induced vasorelaxation was dependent on an intact endothelium, the vasoactive effect of idoxifene was determined in endothelium denuded rings. As illustrated in Fig. 1, endothelial denudation completely abolished idoxifene-induced vasodilation, although vasoconstriction induced by U-46619 and vasodilation produced by acidified $NaNO_2$ remained intact (data not shown).

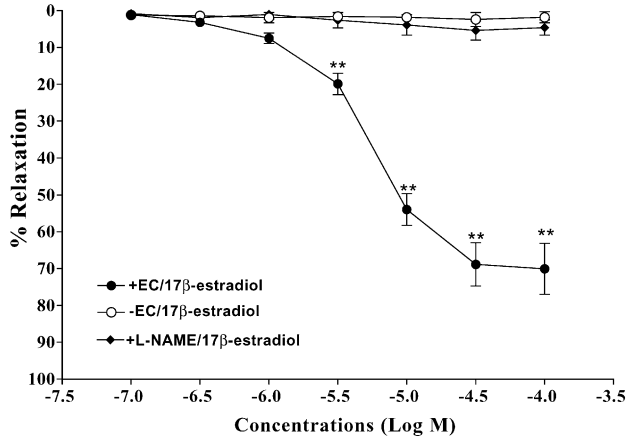

Fig. 2. Concentration-dependent vasorelaxation to 17β-estradiol and its blockade by endothelium denudation and NOS inhibition. $N = 15-18$ rings from at least six animals. $^{**}P < 0.01$ vs. vehicle at the same volume. +EC: endothelium intact; −EC: endothelium denuded.

These results demonstrated that idoxifene is an endothelium-dependent vasodilator.

To further investigate whether idoxifene-induced vasodilation was mediated by NO production from endothelial cells, the effect of L-NAME, a non-selective nitric oxide synthase inhibitor, on idoxifene-induced vasodilation was studied. Similar to experimental effects observed with endothelial denudation, pre-treatment with L-NAME also completely blocked idoxifene's vasodilatory effect (Fig. 1).

Having demonstrated that idoxifene is an endothelium-dependent, nitric oxide-mediated vasodilator in arterial segments isolated from male animals, we further studied the vasodilatory effect of 17β-estradiol and compared the vasodilatory potency of this traditional estrogen with idoxifene in arterial segments from male animals. As illustrated in Fig. 2, addition of 17β-estradiol also resulted in a concentration-dependent vasorelaxation that was endothelium-dependent and nitric oxide mediated. However, the dose–response curve of 17β-estradiol was markedly shifted to the right when compared to that of idoxifene (Fig. 3). Specifically, the minimal and maximal vasodilatory concentrations of 17β-estradiol were 3 and 100 μM, respectively (compared to 0.1 and 3 μM for idoxifene), and the EC_{50} for 17β-estradiol was 6.55 μM (compared to 0.32 μM for idoxifene).

4. Discussion

Endothelial dysfunction, manifested as decreased bioactive NO levels, is one of the most common pathologic changes occurring in various cardiovascular diseases such as ischemia/reperfusion, heart failure and atherosclerosis (Angus, 1996; Harrison, 1994). Endothelial dysfunction contributes significantly to subsequent functional and cellular injury in a variety of pathological pathways. It disturbs the balance between vasorelaxation and vasoconstriction,

and thus may promote vasoconstriction and contribute to the "no reflow phenomena" seen after ischemia and reperfusion. Endothelial dysfunction may also exacerbate tissue injury indirectly by increasing platelet–leukocyte–endothelium interactions. Therapeutic strategies aimed at improving endothelial function, restoring NO production, and enhancing endothelium-dependent vasodilation have been shown to markedly retard the development of atherosclerosis and attenuate vascular and tissue injury associated with ischemia/reperfusion (Lefer et al., 1991).

In the present study, we have demonstrated for the first time that idoxifene, a new selective estrogen receptor modulator, is an endothelium-dependent, nitric oxide-mediated vasodilator in arterial segments isolated from male animals. Although the vasodilatory efficacy of idoxifene is comparable with that of 17β-estradiol (maximal vasodilation: $69 \pm 4.5\%$ for idoxifene, $70.1 \pm 6.9\%$ for 17β-estradiol, $P > 0.1$), idoxifene is approximately 20-fold more potent than 17β-estradiol in superior mesenteric arterial rings isolated from male rats (0.32 vs. 6.55 μM). These results provide direct evidence that this particular selective estrogen receptor modulator not only possesses a favorable tissue-selective profile as demonstrated before (i.e., estrogen agonism on desired target tissues such as bone and liver, and estrogen antagonism in reproductive tissues such as the breast or uterus) (Nuttall et al., 1998), but also a more potent vasodilatory effect in vascular segments from male animals, suggesting that this compound may have potential clinical applications in male patients with cardiovascular diseases.

The exact cellular mechanism by which idoxifene stimulates NO production from endothelial cells in male animals could not be addressed by the present experiment. However, evidence exists suggesting that idoxifene may exert its vasodilatory effect via a novel nongenomic, estrogen receptor-mediated direct enhancement of nitric oxide synthase (NOS) activity (Kim et al., 1999; Mendelsohn, 2000; Simoncini et al., 2000). It is known that estrogen receptors are highly expressed in male vascular endothelial cells (Lindner et al., 1998; Huang et al., 2000). Previous studies have demonstrated that idoxifene binds to estrogen receptors and exerts its estrogen agonist effects on bone and liver tissues in an estrogen receptor-dependent fashion (Mitlak and Cohen, 1999; Nuttall et al., 1998; Treinen et al., 1998). Since idoxifene was added acutely in the tissue bath and the vasodilatory response was observed within minutes, it is unlikely that idoxifene increases NO production by upregulation of NOS-III (endothelial NO synthase) gene expression, as has been reported for estrogen (Kauser and Rubanyi, 1997). Two recent studies have demonstrated that estrogen directly activates NOS-III and increases NO production from bovine aortic endothelial cells (Kim et al., 1999) and fetal lamb pulmonary artery endothelial cells (Chen et al., 1999) via a novel nongenomic pathway. This NOS-III activation effect of estrogen is rapid (< 5 min) and independent of NOS III protein level, is mediated by estrogen receptors localized in cell membrane caveolae rather than classical nuclear receptors (Kim et al., 1999), and is calcium and extracellular signal-regulated kinase-dependent (Chen et al., 1999). In addition to its effect on NO production, idoxifene may also increase levels of bioactive NO through a modulation of NO degrading systems (e.g., reactive oxygen radical generation and antioxidants)(Kauser and Rubanyi, 1997).

In a previous study, we demonstrated that in superior mesenteric arterial rings isolated from ovariectomized female rats, idoxifene also induces an endothelium-dependent, nitric oxide-mediated vasodilation (Ma et al., 2000). However, the vasodilatory potency of idoxifene is comparable to that of 17β-estradiol in these vessels isolated from female rats. In contrast, our present study demonstrated that idoxifene has at least a 20-times more potent vasodilatory effect than 17β-estradiol in vessels isolated from male rats. Our results also differ from those reported by Figtree et al. (1999). Reloxifene, another selective estrogen receptor modulator with a different molecular structure than that of idoxifene, has been reported to acutely relax coronary arteries from male rabbits with a potency comparable to that of estrogen (Figtree et al., 1999). These results suggest that idoxifene may possess unique properties that are not shared by other selective estrogen receptor modulators.

In summary, we have demonstrated in the present study that idoxifene, a novel selective estrogen receptor modulator, acutely relaxes superior mesenteric arterial rings isolated from male animals in an endothelium-dependent, nitric oxide-mediated manner with 20-times greater potency than that of 17β-estradiol. Idoxifene also has known beneficial effects on plasma cardiovascular risk factors. Our data strongly support the clinical evaluation of idoxifene in the treatment of men with cardiovascular diseases where endothelial dysfunction plays a critical role.

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