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Bazedoxifene-Induced Vasodilation and Inhibition of Vasoconstriction is Significantly Greater than Estradiol

Margaret A Zimmerman, Ph.D.¹, Dillion D Hutson, B.S.¹, Franck Mauvais-Jarvis, M.D Ph.D.², and Sarah H. Lindsey, Ph.D.¹

¹Department of Pharmacology Tulane University School of Medicine, New Orleans, LA, United States of America

²Department of Medicine, Section of Endocrinology, Tulane University School of Medicine, New Orleans, LA, United States of America

Abstract

Objective: A new strategy for menopausal hormone therapy replaces medroxyprogesterone with the selective estrogen receptor modulator bazedoxifene. While the agonist or antagonist activity of bazedoxifene has been examined in other tissues, the current study explored the impact of bazedoxifene on resistance artery reactivity. We hypothesized that bazedoxifene may induce greater vasoprotective effects than estradiol due to enhanced activation of the G protein-coupled estrogen receptor.

Methods: We measured the vasodilation of mesenteric resistance arteries from adult male and female wildtype and G protein-coupled estrogen receptor knockout mice (N=58) in response to increasing concentrations of bazedoxifene, medroxyprogesterone, and estradiol as well as the impact of these compounds on the responses to phenylephrine and sodium nitroprusside.

Results: Bazedoxifene-induced vasorelaxation was greater than estradiol and blunted phenylephrine-induced contraction, an effect not observed with estradiol. Neither estradiol nor bazedoxifene altered relaxation to sodium nitroprusside. The combination of bazedoxifene + estradiol promoted greater vasodilation than medroxyprogesterone + estradiol and opposed phenylephrine-induced contraction, while medroxyprogesterone + estradiol failed to attenuate this response. Both bazedoxifene + estradiol and medroxyprogesterone + estradiol enhanced sodium nitroprusside-induced relaxation in females. Vascular responses were similar in both sexes in wildtype and G protein-coupled estrogen receptor knockout mice.

Conclusion: Bazedoxifene and bazedoxifene + estradiol relaxed mesenteric arteries and opposed vasoconstriction to a greater degree than estradiol or medroxyprogesterone + estradiol. These effects were independent of sex and G protein-coupled estrogen receptor expression. We conclude that bazedoxifene may provide vascular benefits over estrogen alone or estrogen plus progestogen combinations in postmenopausal women.

Corresponding Author: Margaret Zimmerman, 1430 Tulane Avenue, mailbox 8683, New Orleans, LA 70112, Phone: 504-988-2581, Fax: 504-988-5283, mzimmer5@tulane.edu.

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Keywords

G protein-coupled estrogen receptor; Vessels; Bazedoxifene; Estradiol; Medroxyprogesterone; Selective Estrogen Receptor Modulators.

Introduction

The combination of estrogen therapy with the selective estrogen receptor modulator (SERM) bazedoxifene (BZA) is a novel progestogen-free therapy that alleviates menopausal symptoms and negates the proliferative effects of estrogen on the breast and uterus (1, 2). Activation of nuclear estrogen receptors induces a conformational shift and recruitment of coregulators to selectively alter gene transcription (3). The expression of coregulators varies by tissue, thereby allowing tissue-specific responses to the same ligand-receptor complex. Moreover, the conformation of the estrogen receptor is important for coregulator recruitment and can be influenced by the chemical structure of the ligand. SERMs such as BZA capitalize on the unique complexity of this system to produce agonist or antagonist responses in each tissue. In addition, BZA induces the degradation of estrogen receptor alpha (ER α) via the ubiquitin proteasome system to further oppose proliferation of uterine and breast tissue (4).

In comparison with estrogen therapy, hormone therapy containing medroxyprogesterone (MPA) is associated with a higher risk of breast cancer and cardiovascular disease (5). MPA antagonizes the protective effects of estrogen on flow-mediated dilation in menopausal women (6, 7) and on atherosclerosis in ovariectomized monkeys (8). Therefore, replacing MPA with another drug to antagonize the proliferative effects of estrogen in the uterus and breast may provide a safer option (9). The first progestogen-free hormone therapy for women combines estrogen therapy with BZA instead of a progestogen (10). The Selective Estrogens, Menopause, and Response to Therapy (SMART) trial shows that BZA combined with estrogen therapy for up to two years improves menopause-related symptoms with an acceptable cardiovascular safety profile (11, 12).

The loss of estrogens that occurs with menopause is associated with an increase in blood pressure (13) and a reduction in endothelial function (14). Conversely, acute administration of 17 β -estradiol (E2) induces vasorelaxation in postmenopausal women (15) as well as in female animals (16). Previous generation SERMs, such as raloxifene and 4OH-tamoxifen, improve acetylcholine-induced relaxation in mesenteric arteries from ovariectomized female Wistar rats (17) and stimulate nitric oxide production to a similar extent as E2 in cultured endothelial cells (18). In postmenopausal women, raloxifene treatment significantly increases brachial artery flow-mediated dilation compared with placebo (6, 14). These studies indicate that SERMs may provide similar vascular benefits as E2. However, whether the substitution of MPA with BZA in hormone therapy is beneficial for the vasculature remains to be determined.

Estrogen receptors alpha and beta (ER α /ER β) and the G protein-coupled estrogen receptor (GPER) are expressed in endothelial and vascular smooth muscle cells and contribute to vascular tone (19, 20). SERMs are commonly characterized according to their impact on

nuclear ER activation, but SERMs such as tamoxifen also have affinity for membrane-bound estrogen receptors (21). Moreover, GPER mediates the effects of both tamoxifen and raloxifene in endometrial cancer, suggesting activation of membrane-initiated signaling pathways (22, 23). Therefore, the goals of the current study were to assess 1) the impact of BZA on vascular function, 2) the differences in vascular reactivity between mono and dual hormone therapies, 3) sex differences, and 4) the contribution of GPER. Our overall hypothesis was that BZA would induce greater vascular responses due to activation of GPER.

Methods

Animals.

Male and female wildtype (WT) and GPER knockout (KO) mice (N=58) were generated and genotyped as previously described (24, 25). Mice were housed in an AAALAC-accredited vivarium with a temperature-controlled 12h light-dark cycle. Animals had *ad libitum* access to standard chow diet and water. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved and monitored by the Tulane University Institutional Animal Care and Use Committee.

Vascular Reactivity.

Animals were euthanized using isoflurane, and first-order mesenteric arteries were isolated and removed of surrounding adipose tissue. These resistance vessels were chosen because of their established role in the maintenance of blood pressure (26). Four vessels from each animal (male WT N=5, male KO N=9, female WT N=8, female KO N=8) were cut into 2 mm segments and mounted on a wire myograph (DMT 620M, Danish Myotechnology, Denmark). Internal circumference was normalized to 0.9·IC100, where IC100 is the internal circumference at a transmural pressure of 100 mmHg (20). Baseline vascular dynamics were assessed in response to 80 mM KCl, 10^{-5} M phenylephrine (PE), and 10^{-6} M acetylcholine (ACh), and vessels that relaxed less than 50% to ACh were considered denuded and excluded from the study. Three different protocols were used for the study and are illustrated in Supplementary Figure 1.

Protocol 1: Viable vessels were pre-contracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 10^{-5} M; Tocris, Bristol, United Kingdom) and assigned to receive increasing concentrations of bazedoxifene acetate (BZA; Pfizer, New York, NY), E2 (Calbiochem, Darmstadt, Germany), or dimethyl sulfoxide (DMSO; 1:5000 to 1:1000) as the vehicle. Vessels from the same animal were assigned to different drug treatments, and each vessel was exposed to only one drug for the duration of the study. For the direct vasodilatory effects of these drugs, data was expressed as a % of $PGF_{2\alpha}$ tension. After repeated 5 min washings, mesenteric arteries were pre-treated for 3 min with 10^{-9} M BZA, E2, or DMSO and then constricted to increasing concentrations of PE (10^{-7} to 10^{-4} M). PE contraction curves were expressed as a % of the contraction induced by 80 mM KCl. Vessels were washed and pre-treated again for 3 min with 10^{-9} M BZA, E2, or DMSO. After pre-constriction with $PGF_{2\alpha}$ (10^{-5} M), the

mesenteric arteries were relaxed with increasing concentrations of sodium nitroprusside (SNP; 10^{-10} to 10^{-5} M). SNP relaxation was expressed as a % of PGF_{2α} tension.

Protocol 2: A second set of female mice (WT N=5, KO N=11) was used to determine vascular reactivity in response to the combination of E2 with BZA or medroxyprogesterone (MPA; Pfizer). Mesenteric arteries were pre-constricted to PGF_{2α} (10^{-5} M) and relaxed with increasing concentrations of 1) DMSO, 2) MPA+E2, or 3) BZA+E2 (10^{-9} to $10^{-5.5}$ M). Vessels were washed and pre-treated for 3 mins with 10^{-9} M of the same drugs administered previously: 1) DMSO, 2) MPA+E2, or 3) BZA+E2 before being exposed to increasing concentrations of PE (10^{-7} to 10^{-4} M). Vessels were washed and pre-treated again before pre-constriction with PGF_{2α} (10^{-5} M) and relaxation with increasing SNP concentrations (10^{-10} to 10^{-5} M).

Protocol 3: Vascular responses to the GPER agonist G-1 and E2 were assessed in an additional set of female WT and KO (N=6 per group) animals. Mesenteric arteries were isolated and pre-constricted with PGF_{2α} (10^{-5} M) before relaxing to increasing concentrations of E2 and G-1 (Azano Pharmaceuticals, Albuquerque, NM).

Droplet Digital PCR.

Tissue was collected from a subset of animals used for vascular reactivity (N=6–8 per group) to confirm genetic deletion of GPER and potential compensatory changes in ERα. Animals were euthanized using isoflurane, and kidneys were collected and stored in RNAlater solution (ThermoFisher Scientific, Waltham, MA). Harvested tissue (30 mg) was mechanically homogenized in 600 μL of QIAGEN RLT Buffer and purified RNA was collected via the QIAGEN RNeasy Mini Kit (QIAGEN, Germantown, MD). After droplet generation, one-step reverse transcription was performed with the Bio-rad One-Step RT-ddPCR Advanced Kit with primers and dual-labeled fluorescent probes for GPER (Assay ID: dMmuCPE5103031) and ERα (sequence: dMmuCPE5092740; Bio-rad, Hercules, CA). Droplets were analyzed using the Bio-Rad QX200 system and QuantaSoft software as previously described (27).

Statistical Analysis.

All data are presented as mean ± standard error of the mean. A power analysis was completed for each figure using α=0.05 and 90% power to detect a 20% change in vascular response to confirm the appropriate sample size. Statistical significance for vascular dose response curves was determined using repeated measures two-way ANOVA (concentration X treatment) since each vessel was exposed to increasing concentrations of the same drug. ddPCR data was analyzed by two-way ANOVA (sex X genotype). Statistical tests were followed by Tukey's multiple comparisons and accepted at P<0.05. Analyses were performed using Prism Version 6.0 software (GraphPad Software Inc, San Diego, CA).

Results

BZA Induces Greater Vasorelaxation than E2

In mesenteric arteries from male WT animals, both E2 and BZA significantly relaxed vessels at $10^{-5.5}$ M (Figure 1A; $P < 0.001$ vs. vehicle). Maximum relaxation was significantly greater with BZA ($18 \pm 8\%$) compared with E2 ($50 \pm 5\%$; $P < 0.001$, Cohen's $d = 4.9$). In female WT vessels, E2 and BZA induced significant relaxation starting at 10^{-6} M (Figure 1B; $P = 0.002$ and $P < 0.001$ vs. vehicle, respectively). Maximal vasorelaxation was significantly greater for BZA ($6 \pm 1\%$) compared with E2 ($37 \pm 3\%$; $P < 0.001$, Cohen's $d = 1.7$). Vascular responses to E2 and BZA were comparable between male and female WT mice ($P = 0.818$ and $P = 0.069$, respectively).

Pre-treatment with BZA but not E2 attenuates vasoconstriction

To assess the impact of E2 and BZA on vasoconstriction, mesenteric arteries were incubated with 10^{-9} M BZA, E2, or vehicle prior to increasing concentrations of PE. In male WT arteries, pre-treatment with E2 did not alter the PE response (maximum E2: $190 \pm 20\%$ vs. maximum vehicle: $207 \pm 20\%$, $P = 0.81$; Figure 2A). In contrast, BZA significantly blunted PE-induced vasoconstriction at 10^{-6} M and 10^{-5} M ($P < 0.001$ vs. vehicle). In female WT arteries, pre-treatment with E2 again failed to alter PE-induced contraction ($182 \pm 9\%$ vs. $171 \pm 7\%$, $P = 0.61$; Figure 2B). However, BZA significantly blunted the PE response up to 10^{-4} M PE ($64 \pm 6\%$; $P < 0.001$ vs. vehicle, Cohen's $d = 4.5$). The impact of E2 on vasoconstriction was comparable between sexes ($P = 0.71$), but BZA attenuated the PE response to a greater degree in females versus males ($P = 0.008$).

To assess whether BZA enhanced the smooth muscle response to nitric oxide, male and female WT arteries were pre-treated with BZA, E2, or vehicle prior to the SNP relaxation curve. In male WT vessels, the maximal SNP relaxation was comparable between groups (E2: $10 \pm 1\%$, BZA: $10 \pm 2\%$, vehicle: $6 \pm 1\%$, $P = 0.46$; Figure 2C). Similar to males, the SNP response in female WT mice was similar (E2: $11 \pm 1\%$, BZA: $18 \pm 3\%$, vehicle: $20 \pm 4\%$, $P = 0.23$; Figure 2D).

Dual Hormone Therapy Containing BZA Influences Vascular Reactivity to a Greater Degree than MPA

Clinical treatment of menopausal symptoms combines estrogen with MPA, but a novel alternative is to replace this progestogen with BZA. Therefore, studies were conducted in a separate set of females to assess the impact of these hormone combinations on vascular reactivity. In female WT arteries, both BZA+E2 and MPA+E2 significantly relaxed vessels starting at 10^{-6} M (Figure 3A; $P < 0.001$ vs. vehicle). Maximum dilation was significantly greater for BZA+E2 compared with MPA+E2 ($8 \pm 1\%$ vs. $31 \pm 5\%$; $P < 0.001$, respectively).

Additional studies compared the influence of dual hormone therapy on PE-induced contraction and SNP-induced relaxation. Mesenteric arteries were pre-treated with BZA+E2, MPA+E2, or vehicle prior to PE and SNP response curves. PE-induced contraction in female WT mice was similar between pre-treated MPA+E2 and vehicle (Figure 3B; $P = 0.89$). However, BZA+E2 significantly blunted constriction even at the maximum PE concentration

of 10^{-4} M ($P<0.001$ vs. vehicle). In female WT mice, there was a significantly greater SNP response in vessels pre-treated with either BZA+E2 or MPA+E2 (Figure 3C; $P<0.05$). In summary, dual hormone therapy with either BZA or MPA enhanced vasorelaxation to SNP, but only therapy containing BZA opposed PE-induced contraction.

GPER Deletion Confirmed in KO animals

Genomic DNA isolated using tail biopsies was used to genotype animals for use in additional studies (Figure 4A). A reduction in GPER function was confirmed by assessing relaxation to the selective agonist G-1 (Figure 4B). Vascular responses were only assessed in females due to our previous work showing a reduced response in males (28). E2-induced relaxation was not different between female KO and WT arteries, and the response to E2 and G-1 was similar in female WT arteries. However, G-1-induced relaxation was significantly blunted in vessels from female KO mice ($P=0.02$). Droplet digital PCR was used to confirm a lack of GPER at the mRNA level and to assess potential compensatory upregulation of $ER\alpha$ due to developmental deletion of GPER. GPER transcript was abolished in kidney samples from both male and female KO mice (Figure 4C; $P<0.001$). No alterations in $ER\alpha$ transcript levels were found between WT and KO animals (Figure 4D; $P=0.28$). However, renal $ER\alpha$ mRNA was significantly greater in males compared to females in both groups ($P<0.001$).

GPER Deletion Does Not Alter Vascular Responses to BZA

Vessels from KO animals were used to determine whether GPER mediates the vascular response to BZA. In male GPER KO mice, both E2 and BZA significantly relaxed vessels at 10^{-6} M (Figure 5A, $P=0.123$ and $P<0.001$, respectively). Similar to male WT mice, BZA induced a greater maximal relaxation than E2 ($28 \pm 11\%$ vs. $45 \pm 4\%$; $P=0.005$). There were no differences in E2 and BZA responses between male WT and KO mice ($P=0.64$ and $P=0.75$, respectively). In female GPER KO animals, vasorelaxation reached statistical significance for BZA starting at 10^{-6} M (Figure 5B, $P<0.001$), while the E2 response was delayed until $10^{-5.5}$ M ($P<0.001$). The maximum dilation was $39 \pm 5\%$ for E2 and $10 \pm 4\%$ for BZA ($P<0.001$ vs. vehicle). There were no differences in E2 and BZA responses between female WT and KO mice ($P=0.63$ and $P=0.43$, respectively).

Pre-treatment with E2 did not alter PE-induced contraction in male KO mice compared with vehicle (Figure 5C, $P=0.93$). In contrast, BZA significantly blunted vasoconstriction to 10^{-6} M and 10^{-5} M PE ($P<0.001$ vs. vehicle). Maximum constriction to 10^{-4} M PE was comparable between E2 and BZA ($160 \pm 6\%$ vs. $120 \pm 19\%$, $P=0.07$), and when compared with vehicle ($154 \pm 5\%$, $P=0.93$). PE responses for both E2 and BZA pre-treated vessels were similar between male WT and KO mice ($P=0.36$ and $P=0.98$, respectively). In female GPER KO vessels, pre-treatment with E2 did not alter the PE response (E2: $190 \pm 9\%$ vs. vehicle $176 \pm 5\%$, $P=0.57$; Figure 5D). Similar to the female WT response, in GPER KO vessels, BZA significantly attenuated vasoconstriction even at the maximum PE concentration of 10^{-4} M ($111 \pm 15\%$; $P<0.001$).

Similar to WT vessels, the SNP response in male GPER KO vessels was not altered between treatment groups (E2: $11 \pm 3\%$, BZA: $11 \pm 2\%$, vehicle: $12 \pm 3\%$, $P=0.91$; Figure 5E). SNP

responses in the presence of E2 and BZA were similar between male WT and KO mice ($P=0.97$ and $P=0.93$, respectively). The SNP response in female GPER KO vessels was not altered by treatment (E2: $16 \pm 6\%$, BZA: $11 \pm 2\%$, vehicle: $16 \pm 7\%$, $P=0.43$; Figure 5F). The SNP response between female WT and GPER KO mice was similar for E2 pre-treated vessels ($P=0.97$). However, the SNP response was greater in GPER KO vessels pre-treated with BZA compared with WT females ($P=0.006$).

GPER Deletion Does Not Alter BZA Response in Dual Hormone Treatment

Female GPER KO mice were used to determine the contribution of GPER in the effects of dual hormone therapies using BZA and MPA. Both BZA+E2 and MPA+E2 significantly relaxed pre-constricted vessels in female GPER KO mice starting at 10^{-6} M (Figure 6A; $P<0.001$ vs. vehicle). Maximum dilation was significantly greater for BZA+E2 compared with MPA+E2 in KO vessels ($9 \pm 3\%$ vs. $25 \pm 5\%$; $P<0.001$). The direct vasodilatory response of BZA+E2 and MPA+E2 was not altered when GPER was deleted ($P=0.098$ and $P=0.40$ vs. WT, respectively). Vasoconstriction to PE in female GPER KO animals was significantly blunted by pre-treatment with BZA+E2 (Figure 6B; $P<0.001$ vs. vehicle) but not by MPA+E2 ($P=0.4805$ vs. vehicle). Maximum constriction to PE was significantly different between pre-treatment with BZA+E2 ($73 \pm 19\%$) and MPA+E2 ($175 \pm 7\%$; $P<0.001$). GPER deletion did not alter the PE response in vessels pre-treated with BZA+E2 and MPA+E2 ($P=0.91$ and $P=0.50$ vs. WT, respectively). Female GPER KO artery responses to SNP were similar between treatment groups (BZA+E2: $12 \pm 3\%$, MPA+E2: $12 \pm 5\%$, vehicle $14 \pm 10\%$, $P=0.89$; Figure 6C). GPER deletion did not alter the SNP response in vessels pre-treated with BZA+E2 and MPA+E2 ($P=0.78$ and $P=0.62$ vs. WT, respectively).

Discussion

The major finding from the current study is that BZA is a more potent vasodilator than E2, and unlike E2, also opposes PE-induced contraction. In addition, we showed that the combination of BZA+E2 induced greater responses than MPA+E2 in the mesenteric arteries of female mice. The impact of BZA on vascular reactivity was mostly independent of sex and was not altered by genetic deletion of GPER. These results indicate a potential for enhanced vasoprotection for dual hormone therapies using BZA compared with MPA.

BZA was a more potent vasodilator than E2 in mesenteric arteries even though its binding affinity to $ER\alpha/ER\beta$ is lower (29, 30). Moreover, BZA and BZA+E2, but not E2 or MPA+E2, significantly blunted PE-induced contraction. In cerebral arteries of male rabbits, BZA prevents vasoconstriction via inhibition of L-type Ca^{2+} channels (31). A potential mechanism is estrogen-induced translocation of these ion channels from the membrane to the nucleus, resulting in lower plasma membrane localization in female versus male C57Bl/6J mice (32). However, we did not observe a sex difference in PE-induced contraction in the current study. Loss of circulating estrogen during this *ex vivo* approach could disrupt the translocation of L-type Ca^{2+} channels and promote a similar phenotype in males and females. BZA may be more effective than E2 in reducing the number of membrane-bound L-type Ca^{2+} channels to blunt PE-induced contraction.

Vasorelaxation to the nitric oxide donor SNP was not altered by E2 or BZA but was enhanced by dual treatment containing BZA+E2 or MPA+E2, indicating a cooperative or synergistic mechanism. Similarly, the combination of E2 and progesterone, but neither alone, promotes an increase in the SNP response in cerebral slices from female Sprague Dawley rats (33). Progesterone also augments the response to SNP in myometrial smooth muscle (34). However, in canine coronary arteries (35) and premenopausal women (36), progesterone antagonizes the vasodilatory effects of estradiol. The possibility that MPA attenuates the protective effects of estrogen is often considered when reflecting on the failure of the WHI trials (37), and clinical studies using natural progesterone formulations will hopefully provide additional information (38). However, neither our study nor data from postmenopausal women indicates a detrimental effect of MPA on smooth muscle-mediated vasorelaxation (39).

We did not assess endothelium-dependent relaxation in the current study because responses to raloxifene are independent of the endothelium and nitric oxide (40, 41). Moreover, the vasodilatory effect of BZA in rabbit basilar arteries is not impacted by denuding, inhibition of nitric oxide synthase, or cyclooxygenase inhibition (31). While this study suggests that the mechanism for BZA's effects include opening of potassium channels, a high potassium buffer or potassium channel inhibitors attenuate the vasodilatory response by only 20–25%. However, our data is similar to this study when considering the impact of BZA on L-type calcium channels. In our hands, BZA significantly attenuated and in some cases abolished the response to phenylephrine, suggesting a significant impact on extracellular calcium entry. Future studies should look more closely at the impact of BZA on calcium mobilization using fluorescent imaging techniques.

We did not observe a sex difference in either E2- or BZA-induced dilation in the mesenteric arteries, but the ability of BZA to blunt PE-induced contraction was greater in females compared with males. Other studies show that E2-mediated vasorelaxation is greater in female ER α knockout aortas (42) and mesenteric arteries from female mRen2 rats (28). In addition, female Sprague Dawley rats have reduced raloxifene-induced dilation in pulmonary veins (43) but greater dilation in mesenteric arteries (44). We also found that males have greater renal ER α mRNA and similar GPER levels compared with females. Our results parallel those in mice showing greater renal ER α transcript in adult males but greater protein expression in females (45). Future studies are aimed at profiling sex differences in estrogen receptor expression in all cardiovascular tissues. Overall, these results indicate that the vascular benefits of BZA may be applicable to both sexes, providing additional options for treating cardiovascular complications.

We have previously shown the importance of GPER in vascular function (16) and remodeling (46), and SERMs activate GPER in endometrial tumor cells (23), monkey kidney fibroblasts (47), and cerebral arteries from male rabbits (31). Therefore, we hypothesized that the protective vascular effects of BZA were mediated by GPER. However, our results show that the vascular response to BZA was independent of this receptor in both males and females. Interestingly, genetic deletion of GPER also failed to alter the vasodilatory response to E2 despite attenuation of the response to the selective GPER agonist. Some overlap may exist in the pathways by which E2 induces vascular effects,

resulting in a lack of phenotype when removing one estrogen receptor. Similarly, in male aortas selective activation of GPER induces the same vasodilation as nonselective E2, but when GPER is deleted the E2 response is attenuated but not abolished (18). Receptor compensation may also occur in response to germline deletion, and studies from ER α , ER β , and double ER knockout mice provide evidence that this compensation occurs when deleting the nuclear estrogen receptors (48). While we found no evidence of renal ER α upregulation in the absence of GPER, nuclear ERs may have the capacity to functionally compensate for GPER in its absence. Alternatively, BZA may activate an estrogen receptor-independent pathway which was not altered in GPER KO mice. The recruitment of additional pathways in parallel with ERs may account for the disparity in vasodilation between E2 and BZA and provide a mechanism by which BZA opposes PE-induced contraction. Moreover, these results indicate a potential for enhanced vasoprotection for dual hormone therapies using BZA compared with MPA.

Since current menopausal hormone treatments using BZA produce circulating concentrations of approximately 6 nM (49), one limitation to our study is the use of micromolar concentrations to induce *ex vivo* vasodilation. This discrepancy is also observed for E2-induced *ex vivo* vasodilation (50–53), and most likely results from supraphysiological pre-constriction of arteries in order to maintain tone during a concentration response curve. In contrast, our results showing that 1 nM BZA and BZA+E2, but not E2 or MPA+E2, opposed PE-induced contraction may more precisely reflect the *in vivo* environment. A second limitation of the current study is the measurement of acute but not chronic vascular response to BZA. Since BZA downregulates ER α (54), the impact of long-term BZA on vascular function remains to be determined. Cardiovascular safety is a primary concern for menopausal hormone therapy, and SERMs may increase the risk for stroke and venous thromboembolism (55, 56). However, these events were considered within acceptable safety profile and should be weighed against the benefits.

Conclusions

The current study showed that BZA had more potent vasodilatory properties than E2 in resistance vessels of both sexes independently of GPER. BZA has beneficial effects on bone, lipid metabolism, and uterine and breast tissue (56, 57), and our study indicates additional benefits in the vasculature. Overall, the findings in the current study support that dual hormone therapy containing BZA may provide vascular benefits over therapies containing MPA.

Potential Clinical Value

In the current manuscript, we showed that BZA has enhanced vascular benefits in murine mesenteric arteries compared with E2 and MPA. These findings indicate that clinical administration of dual hormone therapy containing BZA may offer vascular protection than therapies containing MPA. Despite seeing enhanced vascular benefits in the current study, caution remains with hormone therapies containing SERMs due to the increased risk of venous thromboembolic events (56, 58). Therefore, further studies need to assess the long-term implications of hormone therapy containing BZA on the cardiovascular system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References:

1. Johnson K, Hauck F. Conjugated Estrogens/Bazedoxifene (Duavee) for Menopausal Symptoms. *Am Fam Physician*. 2016;93(4):307–14. [PubMed: 26926819]
2. Archer DF. Tissue-selective estrogen complexes: a promising option for the comprehensive management of menopausal symptoms. *Drugs Aging*. 2010;27(7):533–44. [PubMed: 20583848]
3. Komm BS, Lyttle CR. Developing a SERM: stringent preclinical selection criteria leading to an acceptable candidate (WAY-140424) for clinical evaluation. *Ann N Y Acad Sci*. 2001;949:317–26. [PubMed: 11795370]
4. Buscato M, Fontaine C, Fabre A, Vinel A, Valera MC, Noirrit E, et al. The antagonist properties of Bazedoxifene after acute treatment are shifted to stimulatory action after chronic exposure in the liver but not in the uterus. *Molecular and cellular endocrinology*. 2017.
5. Manson JE, Chlebowski RT, Stefanick ML, Aragaki AK, Rossouw JE, Prentice RL, et al. Menopausal hormone therapy and health outcomes during the intervention and extended poststopping phases of the Women's Health Initiative randomized trials. *JAMA*. 2013;310(13):1353–68. [PubMed: 24084921]
6. Akman L, Duygu H, Akercan F, Ulukus M, Ozerkan F, Akin M. The effects of different hormone treatment on endothelial function in healthy postmenopausal women. *Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology*. 2013;29(9):867–72. [PubMed: 23875965]
7. Henmi N, Takahashi K, Amita M, Takata K, Ohta T, Tsutsumi S, et al. Effect of dienogest on estrogen-induced nitric oxide production in human umbilical vein endothelial cells and endothelium-dependent vasodilatation in postmenopausal women. *Menopause*. 2010;17(3):615–21. [PubMed: 20386344]
8. Adams MR, Register TC, Golden DL, Wagner JD, Williams JK. Medroxyprogesterone acetate antagonizes inhibitory effects of conjugated equine estrogens on coronary artery atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology*. 1997;17(1):217–21.
9. Valera MC, Gourdy P, Tremollieres F, Arnal JF. From the Women's Health Initiative to the combination of estrogen and selective estrogen receptor modulators to avoid progestin addition. *Maturitas*. 2015;82(3):274–7. [PubMed: 26261036]
10. Goldberg T, Fidler B. Conjugated Estrogens/Bazedoxifene (Duavee): A Novel Agent for the Treatment of Moderate-to-Severe Vasomotor Symptoms Associated With Menopause And the Prevention of Postmenopausal Osteoporosis. *P T*. 2015;40(3):178–82. [PubMed: 25798038]
11. Kagan R, Komm BS, Ryan KA, Lavenberg J, Yu CR, Pinkerton JV. Timing and persistence of effect of conjugated estrogens/bazedoxifene in postmenopausal women. *Menopause*. 2016.
12. Komm BS, Thompson JR, Mirkin S. Cardiovascular safety of conjugated estrogens plus bazedoxifene: meta-analysis of the SMART trials. *Climacteric : the journal of the International Menopause Society*. 2015;18(4):503–11. [PubMed: 25511453]
13. Portaluppi F, Pansini F, Manfredini R, Mollica G. Relative influence of menopausal status, age, and body mass index on blood pressure. *Hypertension*. 1997;29(4):976–9. [PubMed: 9095086]

14. Colacurci N, Manzella D, Fornaro F, Carbonella M, Paolisso G. Endothelial function and menopause: effects of raloxifene administration. *J Clin Endocrinol Metab.* 2003;88(5):2135–40. [PubMed: 12727966]
15. Lima SM, Aldrich JM, Consolim-Colombo FM, Mansur Ade P, Rubira MC, Krieger EM, et al. Acute administration of 17beta-estradiol improves endothelium-dependent vasodilation in postmenopausal women. *Maturitas.* 2005;50(4):266–74. [PubMed: 15780525]
16. Lindsey SH, Carver KA, Prossnitz ER, Chappell MC. Vasodilation in response to the GPR30 agonist G-1 is not different from estradiol in the mRen2.Lewis female rat. *Journal of cardiovascular pharmacology.* 2011;57(5):598–603. [PubMed: 21326105]
17. Lamas AZ, Caliman IF, Dalpiaz PL, de Melo AF, Jr, Abreu GR, Lemos EM, et al. Comparative effects of estrogen, raloxifene and tamoxifen on endothelial dysfunction, inflammatory markers and oxidative stress in ovariectomized rats. *Life Sci.* 2015;124:101–9. [PubMed: 25623855]
18. Fredette NC, Meyer MR, Prossnitz ER. Role of GPER in estrogen-dependent nitric oxide formation and vasodilation. *J Steroid Biochem Mol Biol.* 2017.
19. Orshal JM, Khalil RA. Gender, sex hormones, and vascular tone. *American journal of physiology Regulatory, integrative and comparative physiology.* 2004;286(2):R233–49.
20. Lindsey SH, Liu L, Chappell MC. Vasodilation by GPER in mesenteric arteries involves both endothelial nitric oxide and smooth muscle cAMP signaling. *Steroids.* 2014;81:99–102. [PubMed: 24246735]
21. Pang Y, Dong J, Thomas P. Estrogen signaling characteristics of Atlantic croaker G protein-coupled receptor 30 (GPR30) and evidence it is involved in maintenance of oocyte meiotic arrest. *Endocrinology.* 2008;149(7):3410–26. [PubMed: 18420744]
22. Vivacqua A, Bonofiglio D, Recchia AG, Musti AM, Picard D, Ando S, et al. The G protein-coupled receptor GPR30 mediates the proliferative effects induced by 17beta-estradiol and hydroxytamoxifen in endometrial cancer cells. *Mol Endocrinol.* 2006;20(3):631–46. [PubMed: 16239258]
23. Petrie WK, Dennis MK, Hu C, Dai D, Arterburn JB, Smith HO, et al. G protein-coupled estrogen receptor-selective ligands modulate endometrial tumor growth. *Obstet Gynecol Int.* 2013;2013:472720. [PubMed: 24379833]
24. Wang C, Dehghani B, Magrisso IJ, Rick EA, Bonhomme E, Cody DB, et al. GPR30 contributes to estrogen-induced thymic atrophy. *Mol Endocrinol.* 2008;22(3):636–48. [PubMed: 18063692]
25. Liu S, Le May C, Wong WP, Ward RD, Clegg DJ, Marcelli M, et al. Importance of extranuclear estrogen receptor-alpha and membrane G protein-coupled estrogen receptor in pancreatic islet survival. *Diabetes.* 2009;58(10):2292–302. [PubMed: 19587358]
26. Intengan HD, Schiffrin EL. Structure and mechanical properties of resistance arteries in hypertension: role of adhesion molecules and extracellular matrix determinants. *Hypertension.* 2000;36(3):312–8. [PubMed: 10988257]
27. Liu J, Edgington-Giordano F, Dugas C, Abrams A, Katakam P, Satou R, et al. Regulation of Nephron Progenitor Cell Self-Renewal by Intermediary Metabolism. *Journal of the American Society of Nephrology : JASN.* 2017;28(11):3323–35. [PubMed: 28754792]
28. Lindsey SH, da Silva AS, Silva MS, Chappell MC. Reduced vasorelaxation to estradiol and G-1 in aged female and adult male rats is associated with GPR30 downregulation. *American journal of physiology Endocrinology and metabolism.* 2013;305(1):E113–8. [PubMed: 23673155]
29. Miller CP, Collini MD, Tran BD, Harris HA, Kharode YP, Marzolf JT, et al. Design, synthesis, and preclinical characterization of novel, highly selective indole estrogens. *J Med Chem.* 2001;44(11):1654–7. [PubMed: 11356100]
30. Komm BS, Kharode YP, Bodine PV, Harris HA, Miller CP, Lyttle CR. Bazedoxifene acetate: a selective estrogen receptor modulator with improved selectivity. *Endocrinology.* 2005;146(9):3999–4008. [PubMed: 15961563]
31. Castello-Ruiz M, Salom JB, Fernandez-Musoles R, Burguete MC, Lopez-Morales MA, Arduini A, et al. Relaxant Effects of the Selective Estrogen Receptor Modulator, Bazedoxifene, and Estrogen Receptor Agonists in Isolated Rabbit Basilar Artery. *Journal of cardiovascular pharmacology.* 2016;68(4):313–21. [PubMed: 27389095]

32. Mahmoodzadeh S, Haase H, Sporbert A, Rharass T, Panakova D, Morano I. Nuclear translocation of the cardiac L-type calcium channel C-terminus is regulated by sex and 17beta-estradiol. *J Mol Cell Cardiol.* 2016;97:226–34. [PubMed: 27266387]
33. Chu HP, Sarkar G, Etgen AM. Estradiol and progesterone modulate the nitric oxide/cyclic gmp pathway in the hypothalamus of female rats and in GT1–1 cells. *Endocrine.* 2004;24(2):177–84. [PubMed: 15347845]
34. Orth TA, Shi SQ, Williamson K, Shi L, Chambliss L, Coonrod DV, et al. Additive inhibitory effects of progesterone and sodium nitroprusside on uterine contractility during pregnancy. *Reprod Sci.* 2011;18(9):868–75. [PubMed: 21421898]
35. Miller VM, Vanhoutte PM. Progesterone and modulation of endothelium-dependent responses in canine coronary arteries. *The American journal of physiology.* 1991;261(4 Pt 2):R1022–7. [PubMed: 1681744]
36. Miner JA, Martini ER, Smith MM, Brunt VE, Kaplan PF, Halliwill JR, et al. Short-term oral progesterone administration antagonizes the effect of transdermal estradiol on endothelium-dependent vasodilation in young healthy women. *American journal of physiology Heart and circulatory physiology.* 2011;301(4):H1716–22. [PubMed: 21856917]
37. Miller VM, Harman SM. An update on hormone therapy in postmenopausal women: mini-review for the basic scientist. *American journal of physiology Heart and circulatory physiology.* 2017;313(5):H1013–H21. [PubMed: 28801526]
38. Wharton W, Gleason CE, Miller VM, Asthana S. Rationale and design of the Kronos Early Estrogen Prevention Study (KEEPS) and the KEEPS Cognitive and Affective sub study (KEEPS Cog). *Brain Res.* 2013;1514:12–7. [PubMed: 23603409]
39. Wakatsuki A, Okatani Y, Ikenoue N, Fukaya T. Effect of medroxyprogesterone acetate on endothelium-dependent vasodilation in postmenopausal women receiving estrogen. *Circulation.* 2001;104(15):1773–8. [PubMed: 11591613]
40. Wong CM, Au CL, Tsang SY, Lau CW, Yao X, Cai Z, et al. Role of inducible nitric oxide synthase in endothelium-independent relaxation to raloxifene in rat aorta. *Br J Pharmacol.* 2017;174(8):718–33. [PubMed: 28138957]
41. Tsang SY, Yao X, Essin K, Wong CM, Chan FL, Gollasch M, et al. Raloxifene relaxes rat cerebral arteries in vitro and inhibits L-type voltage-sensitive Ca²⁺ channels. *Stroke; a journal of cerebral circulation.* 2004;35(7):1709–14.
42. Kim SC, Boese AC, Moore MH, Cleland RM, Chang L, Delafontaine P, et al. Rapid estrogen receptor-alpha signaling mediated by ERK activation regulates vascular tone in male and ovary-intact female mice. *American journal of physiology Heart and circulatory physiology.* 2018;314(2):H330–H42. [PubMed: 28887333]
43. Chan YC, Leung FP, Yao X, Lau CW, Vanhoutte PM, Huang Y. Raloxifene relaxes rat pulmonary arteries and veins: roles of gender, endothelium, and antagonism of Ca²⁺ influx. *J Pharmacol Exp Ther.* 2005;312(3):1266–71. [PubMed: 15550571]
44. Chan YC, Leung FP, Wong WT, Tian XY, Yung LM, Lau CW, et al. Therapeutically relevant concentrations of raloxifene dilate pressurized rat resistance arteries via calcium-dependent endothelial nitric oxide synthase activation. *Arteriosclerosis, thrombosis, and vascular biology.* 2010;30(5):992–9.
45. Sharma PK, Thakur MK. Estrogen receptor alpha expression in mice kidney shows sex differences during aging. *Biogerontology.* 2004;5(6):375–81. [PubMed: 15609101]
46. Liu L, Kashyap S, Murphy B, Hutson DD, Budish RA, Trimmer EH, et al. GPER activation ameliorates aortic remodeling induced by salt-sensitive hypertension. *American journal of physiology Heart and circulatory physiology.* 2016;310(8):H953–61. [PubMed: 26873963]
47. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science.* 2005;307(5715):1625–30. [PubMed: 15705806]
48. Hamilton KJ, Arao Y, Korach KS. Estrogen hormone physiology: Reproductive findings from estrogen receptor mutant mice. *Reprod Biol.* 2014;14(1):3–8. [PubMed: 24607249]
49. Pharmacokinetics McKeand W., Proportionality Dose, and Bioavailability of Bazedoxifene in Healthy Postmenopausal Women. *Clin Ther.* 2017;39(9):1769–79. [PubMed: 28756067]

50. Keung W, Vanhoutte PM, Man RY. Nongenomic responses to 17beta-estradiol in male rat mesenteric arteries abolish intrinsic gender differences in vascular responses. *Br J Pharmacol*. 2005;146(8):1148–55. [PubMed: 16231002]
51. Naderali EK, Walker AB, Doyle P, Williams G. Comparable vasorelaxant effects of 17alpha- and 17beta-oestradiol on rat mesenteric resistance arteries: an action independent of the oestrogen receptor. *Clinical science*. 1999;97(6):649–55. [PubMed: 10585892]
52. Hill BJF, Dalton RJ, Joseph BK, Thakali KM, Rusch NJ. 17beta-estradiol reduces Cav 1.2 channel abundance and attenuates Ca(2+) -dependent contractions in coronary arteries. *Pharmacol Res Perspect*. 2017;5(5).
53. Teoh H, Leung SW, Man RY. Short-term exposure to physiological levels of 17 beta-estradiol enhances endothelium-independent relaxation in porcine coronary artery. *Cardiovascular research*. 1999;42(1):224–31. [PubMed: 10435014]
54. Lewis-Wambi JS, Kim H, Curpan R, Grigg R, Sarker MA, Jordan VC. The selective estrogen receptor modulator bazedoxifene inhibits hormone-independent breast cancer cell growth and down-regulates estrogen receptor alpha and cyclin D1. *Mol Pharmacol*. 2011;80(4):610–20. [PubMed: 21737572]
55. Barrett-Connor E, Mosca L, Collins P, Geiger MJ, Grady D, Kornitzer M, et al. Effects of raloxifene on cardiovascular events and breast cancer in postmenopausal women. *The New England journal of medicine*. 2006;355(2):125–37. [PubMed: 16837676]
56. Christiansen C, Chesnut CH, 3rd, Adachi JD, Brown JP, Fernandes CE, Kung AW, et al. Safety of bazedoxifene in a randomized, double-blind, placebo- and active-controlled Phase 3 study of postmenopausal women with osteoporosis. *BMC Musculoskelet Disord*. 2010;11:130. [PubMed: 20569451]
57. Silverman SL, Christiansen C, Genant HK, Vukicevic S, Zanchetta JR, de Villiers TJ, et al. Efficacy of bazedoxifene in reducing new vertebral fracture risk in postmenopausal women with osteoporosis: results from a 3-year, randomized, placebo-, and active-controlled clinical trial. *J Bone Miner Res*. 2008;23(12):1923–34. [PubMed: 18665787]
58. de Villiers TJ, Chines AA, Palacios S, Lips P, Sawicki AZ, Levine AB, et al. Safety and tolerability of bazedoxifene in postmenopausal women with osteoporosis: results of a 5-year, randomized, placebo-controlled phase 3 trial. *Osteoporos Int*. 2011;22(2):567–76. [PubMed: 20535606]

Figure 1.
BZA Induces Greater Vasorelaxation than E2. Mesenteric artery dilation in response to DMSO, E2, or BZA in (A) male and (B) female WT animals. * $P < 0.05$ versus vehicle; # $P < 0.05$ versus E2.

Figure 2.

Pre-treatment with BZA but not E2 attenuates vasoconstriction. Impact of pre-treatment with DMSO, E2, or BZA on PE-induced contraction in (A) male and (B) female arteries. Impact of pre-treatment on SNP-induced dilation in (C) male and (D) female arteries. * $P < 0.05$ versus vehicle; # $P < 0.05$ versus E2.

Figure 3. Dual Hormone Therapy Containing BZA Influences Vascular Reactivity to a Greater Degree than MPA. (A) Vasodilation in response to DMSO, BZA+E2, and MPA+E2 in female WT animals. (B) PE-induced contraction and (C) SNP-induced dilation in vessels pre-treated with DMSO, BZA+E2, and MPA+E2. * $P < 0.05$ versus vehicle; # $P < 0.05$ versus MPA+E2.

Figure 4. GPER Deletion Confirmed in KO animals. (A) Genotyping for wildtype (WT; 550-bp), heterozygous (HET), and GPER knockout (KO; 730-bp) animals. (B) Mesenteric artery responses to GPER agonist G-1 or E2 in female WT and KO animals. *P<0.05 versus KO G-1. (C) Renal GPER and ER α transcript levels in male and female WT and KO animals. *P<0.05 versus WT; #P<0.05 versus male.

Figure 5.

GPER Deletion Does Not Alter Vascular Responses to BZA. Vasodilation in (A) male and (B) female KO arteries to DMSO, E2, and BZA. Impact of pre-treatment on PE-induced contraction in (C) male and (D) female and on SNP-induced dilation in (E) male and (F) female vessels. * $P < 0.05$ versus vehicle; # $P < 0.05$ versus E2.

Figure 6.

GPER Deletion Does Not Alter BZA Response in Dual Hormone Treatment. (A) Mesenteric artery responses to direct stimulation with DMSO, BZA+E2, and MPA+E2 in female KO animals. (B) Responses to PE-induced contraction and (C) SNP-induced dilation in pre-treated vessels. * $P < 0.05$ versus vehicle; # $P < 0.05$ versus MPA+E2.