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Endothelium-dependent relaxation by G protein-coupled receptor 30 agonists in rat carotid arteries

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Broughton BR, Miller AA, Sobey CG. Endothelium-dependent relaxation by G protein-coupled receptor 30 agonists in rat carotid arteries. *Am J Physiol Heart Circ Physiol* 298: H1055–H1061, 2010. First published January 8, 2010; doi:10.1152/ajpheart.00878.2009.— Recent studies have identified that the novel membrane estrogen receptor, G protein-coupled receptor 30 (GPR30), is present in blood vessels. However, the signaling mechanisms associated with GPR30 in the vasculature remain unclear. We examined whether putative agonists of GPR30 exert vasorelaxant and/or antioxidant effects similar to those reported for estrogen. Using wire myography, we assessed the role of the endothelium in relaxation responses to the GPR30 agonists, G-1 and 5408-0877 (1 nM-10 μM), in U-46619-precontracted common carotid arteries from Sprague-Dawley rats. Furthermore, using lucigenin (5 μM)-enhanced chemiluminescence, we tested the effect of G-1 (10 μM) on superoxide levels. Specific immunofluorescence was also used to confirm GPR30 expression in the arterial wall. We found that G-1 and 5408-0877 induced a concentration-dependent relaxation in carotid arteries from both male and female rats. Interestingly, G-1- and 5408-0877-induced relaxation was abolished by endothelium removal and abrogated in the presence of the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (100 μM). In addition, G-1 significantly decreased NADPH (100 μM)-stimulated superoxide production by carotid and intracranial (pooled basilar and middle cerebral) arteries but also attenuated the superoxide signal detected in a cell-free xanthine/xanthine oxidase assay. Furthermore, GPR30 immunoreactivity was observed in endothelial and vascular smooth muscle cells of carotid arteries from both genders. These findings indicate that GPR30 is expressed throughout the arterial wall and that GPR30 agonists elicit endothelial-derived nitric oxide-dependent relaxation of the carotid artery in male and female rats. Additionally, G-1 appears to directly scavenge superoxide anion.

G protein estrogen receptor; nitric oxide; G-1; 5408-0877; superoxide

UNTIL RECENTLY, IT WAS THOUGHT that the vascular actions of estrogens were solely mediated via activation of the classical nuclear receptors, estrogen receptor (ER) α and ERβ. Following activation, these receptors translocate to the nucleus to produce both genomic and rapid signaling events (14). However, neither inhibition nor removal of ERα and ERβ abolishes estrogen-dependent responses in the cardiovascular system (30, 36). Thus it was speculated that estrogen may elicit its cardiovascular actions via a different, nonconventional receptor. Indeed, over the last few years, emerging evidence has revealed the existence of a novel G protein-coupled receptor 30 (GPR30), also known as G protein estrogen receptor, which mediates some of the actions of estrogen, including rapid signaling events and rapid transcriptional activation (26).

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GPR30 was first discovered in the vasculature in 2007 by Haas and colleagues (9), who demonstrated GPR30 expression in the smooth muscle cells of human arteries and veins and confirmed that this novel receptor is regulated by estrogen. GPR30 expression has since been found in the mesenteric arteries of female mice (17) and aortas of mRen2. Lewis rats (13). Potential role(s) of GPR30 remained largely unknown until very recent studies demonstrated that stimulation of GPR30 with the selective agonist, G-1, acutely lowers blood pressure in normotensive and ovariectomized female mRen2. Lewis rats and also dilates rodent and human arteries (8, 13).

Isoflavones are a class of natural estrogenic compounds (phytoestrogens) found in soy products (27) and which appear to have cardiovascular protective actions (12). For example, isoflavones can lower blood pressure in spontaneously hypertensive (18, 19, 23, 25) and normotensive rats (16) as well as potently dilate systemic and cerebral arteries (4, 11, 31, 37). We have demonstrated that equol, the main active intestinal metabolite of the isoflavone daidzein, displays antioxidant activity in the basilar artery and has preserved vasorelaxant activity in carotid arteries of hypertensive rats (11). Although isoflavones are reported to have a similar affinity for the vascular ERβ to estrogen and less for ERα, it is not known whether isoflavones mediate their vascular effects via GPR30.

We hypothesized that GPR30 has both vasorelaxant and antioxidant properties that are similar to isoflavones. This hypothesis was tested by assessing the vasorelaxant properties of GPR30 agonists in carotid arteries from male and female rats using wire myography and their antioxidant properties using lucigenin-enhanced chemiluminescence. We also identified which vascular cell types expressed GPR30.

MATERIALS AND METHODS

All procedures were approved by the institutional animal ethics committee. In total, 31 male and 26 female Sprague-Dawley rats were studied. Rats were aged matched (8–12 wk; males 290 ± 6 g and females 274 ± 6 g) and killed by inhalation of an 80:20 mix of CO_2-O_2.

Localization of GPR30. Thoracic aortas and carotid and middle cerebral arteries were embedded in an optimum-cutting temperature Tissue-Tek (Bayer Diagnostics) mould and snap-frozen in liquid nitrogen. Arteries were sectioned (10 μm) on a cryostat (Leica) and thaw-mounted on 0.1% gelatinized slides. Sections were fixed in acetone for 15 min and washed in 0.01 M PBS (3 × 10 min). Arterial sections were then incubated with a GPR30 rabbit polyclonal antibody (1:200; AbCAM) overnight in a humid box. The following day, tissues were washed in 0.01 M PBS (3 × 10 min) to remove any excess antibody and incubated in a Texas Red-conjugated goat anti-rabbit IgG (1:200; Zymed Laboratories) for 3–4 h in a humid box. Sections were then washed in 0.01 M PBS (3 × 10 min) and mounted in buffered glycerol (0.5 M Na_2CO_3 added dropwise to 0.5 M $NaHCO_3$ to pH 8.6, combined 1:1 with glycerol). Tissue-mounted

slides were viewed and photographed on a Leica confocal scanning laser system.

Preparation of small vessel myograph. Carotid arteries were excised and placed in ice-cold Krebs-bicarbonate solution (composition in mmol/l: 118 NaCl, 4.5 KCl, 0.45 $MgSO_4$, 1.03 KH_2PO_4, 25 $NaHCO_3$, 11.1 glucose, and 2.5 $CaCl_2$). Connective tissue was removed, and individual rings (3–4 mm in length; internal diameter ~400 μm) were mounted in a Mulvany-Halpern myograph (Danish Myo Technology) for measurement of isometric force. Rings were bathed in 5 ml of Krebs-bicarbonate solution, which was maintained at 37°C and bubbled with carbogen (95% O_2 and 5% CO_2). A tension of 15 mN was applied to the blood vessels, and rings were allowed to equilibrate for 45 min.

Verification of endothelial integrity/disruption. For experiments in endothelium-intact vessels, endothelial integrity was assessed before experimentation by precontracting arteries with U-46619 (10 nM) and measuring the subsequent relaxation response to ACh (1 μM). In some rings, the endothelium was removed by gently abrading the luminal surface of the vessel with a stainless steel pin to determine the contribution of the endothelium to GPR30-mediated relaxation. The effectiveness of endothelial removal was verified by the lack of a relaxation response to ACh. Following application of ACh, vessels were rinsed in Krebs bicarbonate and allowed to equilibrate for 30 min.

Effect of GPR30 agonists on carotid arteries. To test whether GPR30 can regulate tone in carotid arteries, concentration-response curves to the selective and structurally similar GPR30 agonists (see Fig. 1), G-1 (Cayman) and 5408-0877 (ChemDiv) (1 nM-10 μM), or vehicle (DMSO), were constructed in U-46619-contracted arteries from both male and female rats. The response to each concentration was allowed to stabilize (~10–15 min) before the next concentration was applied. To verify that a stable contractile effect of U-46619 was maintained throughout each experiment, time control experiments were performed in contracted vessels. At the end of each experiment, sodium nitroprusside (SNP; 10 μM) was applied to the carotid arteries to establish maximum relaxation.

Effect of endothelial disruption and nitric oxide synthase inhibition on GPR30-mediated relaxation. To assess the contribution of the endothelium to GPR30-mediated relaxation, concentration-response curves to G-1, 5408-0877 (1 nM-10 μM), or vehicle [dimethyl sulfoxide (DMSO)] were performed in endothelium-denuded rings. Furthermore, indirect effects of G-1 (10 μM) on contractions to U-46619 (10 nM) were investigated in endothelium-intact and endothelium-denuded arteries after preincubation for 1 h with G-1 or vehicle (DMSO). Because studies have shown that estrogen mediates vasodilation via nitric oxide, similar protocols were performed in endothelium-intact arteries treated with the broad spectrum nitric oxide synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM; Sigma) or vehicle (Krebs bicarbonate). To verify the efficacy of L-NAME, we examined the subsequent effect of ACh (30 μM).

Effect of G-1 on superoxide production by vascular NADPH oxidase. NADPH (100 μM)-stimulated superoxide production by carotid (5 mm rings) and intracranial (pooled basilar and middle cerebral) arteries was measured by 5 μM lucigenin-enhanced chemiluminescence (24) in the presence of either G-1 (10 μM) or vehicle (0.1% DMSO). Background counts were subtracted and superoxide production normalized to dry tissue weight.

To assess whether G-1 might directly scavenge superoxide, we used a cell-free enzyme assay as previously described (3). In brief, superoxide was generated via the reaction of xanthine (100 μM) and xanthine oxidase (0.05 U/ml) and levels measured in the presence of either G-1 (10 μM), vehicle (0.1% DMSO), or superoxide dismutase (SOD; 250 U/ml) by 5 μM lucigenin-enhanced chemiluminescence.

Calculations and statistics. Relaxation responses (to ACh, G-1, and 5408-0877) were expressed as a percent of the relaxation to SNP. U-46619-mediated contractions were expressed as a percent of the U-46619-induced contraction performed before arteries being pretreated with G-1 or vehicle. All data are expressed as means ± SE, and n values refer to the number of animals in each group. A t-test or two-way ANOVA was used for comparisons, as appropriate. If differences were detected by ANOVA, individual groups were compared with the Student-Newman-Keuls test using SigmaStat 3.0. A probability of $P \leq 0.05$ was accepted as significant for all comparisons.

RESULTS

Localization of GPR30. A specific anti-GPR30 antibody was used to identify the cell types in the artery wall that express GPR30. GPR30 immunoreactivity was observed in both the endothelium and vascular smooth muscle of the carotid ($n = 4$; Fig. 2, *A* and *B*) and middle cerebral ($n = 4$; Fig. 2, *C* and *D*) arteries and the thoracic aorta (data not shown) of both male and female rats. The intensity of GPR30 immunofluorescence appeared to be increased in the endothelium compared with the vascular smooth muscle of all arteries examined. Furthermore, there was no apparent difference between genders in the intensity of staining. Immunoreactivity was absent in arterial sections that lacked the primary antibody.

GPR30 agonists induce relaxation of carotid arteries. The GPR30 agonist G-1 induced relaxation of carotid arteries from both males (e.g., 42 ± 8% at 10 μM; $n = 6$) and females (32 ± 7% at 10 μM; $n = 6$). Similarly, 5408-0877 induced a concentration-dependent relaxation in arteries from males (e.g., 35 ± 10% at 10 μM; $n = 6$) and females (34 ± 11% at 10 μM; $n = 6$). On some occasions, up to 100 μM agonist was applied, and this concentration caused no further relaxation than 10 μM (data not shown). The vasorelaxant effect of both agonists was concentration-dependent and significantly greater than the effect of vehicle (Fig. 3, *A* and *B*).

GPR30-mediated vasorelaxation is dependent on endothelium-derived nitric oxide. To test if the endothelium is required for GPR30-mediated relaxation, additional functional studies were performed in endothelium-intact vs. -denuded carotid

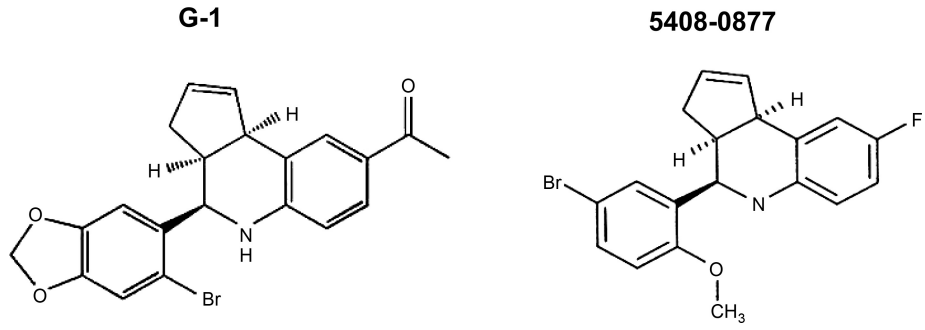

Fig. 1. Chemical structures of G protein-coupled receptor 30 (GPR30) agonists, G-1 and 5408-0877.

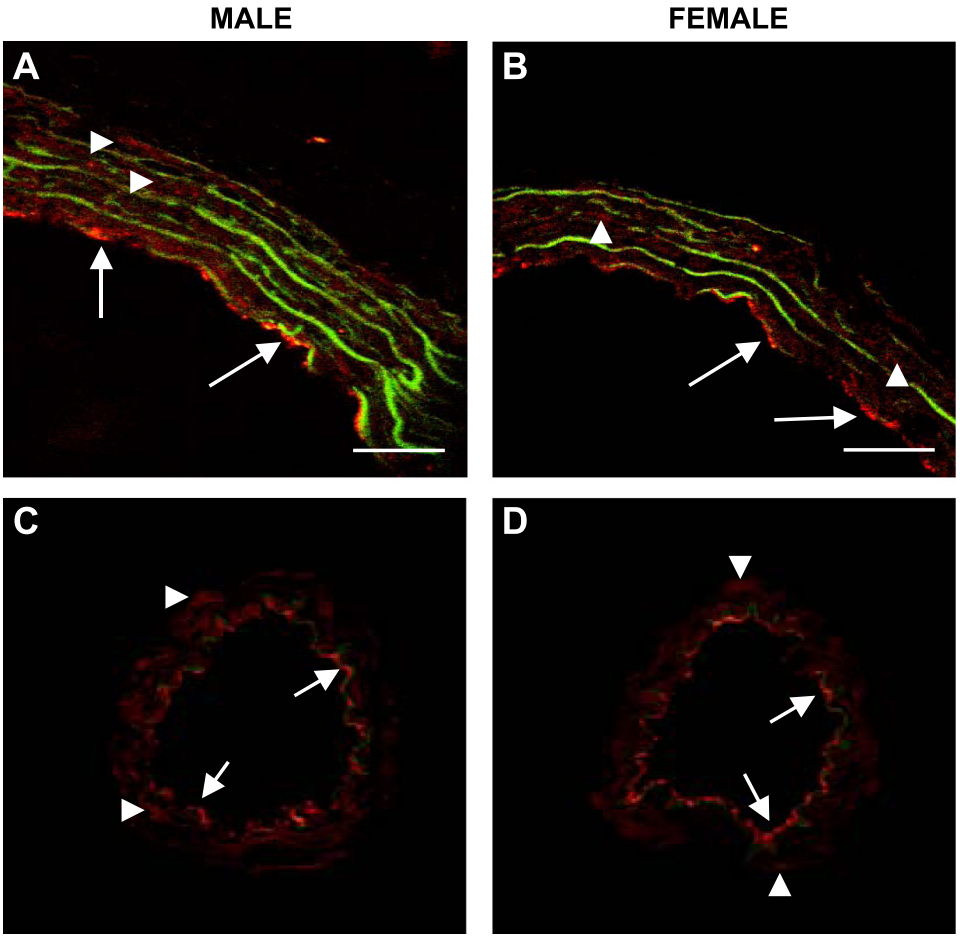

Fig. 2. Photomicrographs showing sections of carotid (*A* and *B*) and middle cerebral (*C* and *D*) arteries from male (*A* and *C*) and female (*B* and *D*) rats following GPR30 immunohistochemistry (red = GPR30; green = autofluorescence). GPR30 immunofluorescence was observed in both the endothelium (arrows) and smooth muscle (arrowheads). Scale bars = 40 μm.

artery rings. Relaxant responses to G-1 or 5408-0877 were abolished by removal of the endothelium in rings from both genders (Fig. 4, *A* and *B*), suggesting that activation of GPR30 elicits vasorelaxation via the release of an endothelium-derived relaxing factor. To further strengthen these observations, we assessed whether activation of GPR30 indirectly acts as a vasorelaxant by blocking the activity of U-46619-mediated contraction in arteries with and without the endothelium. Preincubation of G-1 significantly reduced U-46619-induced contraction in endothelium-intact carotid arteries but not in endothelium-denuded vessels (Fig. 5). These findings are consistent with the direct vasorelaxant actions mediated by GPR30 agonists. We thus examined the contribution of nitric oxide to GPR30-mediated vasorelaxation by performing further experiments in the absence or presence of the NOS inhibitor L-NAME (100 μM). Vascular relaxation by both G-1 and 5408-0877 was virtually abolished by L-NAME in male and female carotid arteries (Fig. 6, *A* and *B*). L-NAME also inhibited responses to ACh but not SNP, as expected (data not shown).

G-1 directly scavenges superoxide. In the presence of the GPR30 agonist G-1, NADPH-stimulated superoxide carotid and intracranial cerebral arteries was ~30–40% lower than levels generated by arteries treated with vehicle (Fig. 7, *A* and *B*). However, levels of superoxide generated in the xanthine/

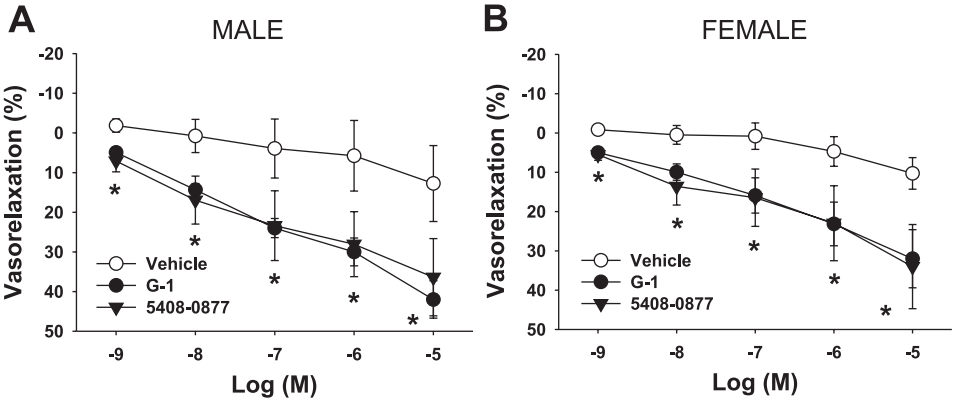

Fig. 3. Vasorelaxant responses to increasing concentrations of the GPR30 agonists, G-1 and 5408-0877, in carotid arteries from male (*A*) and female (*B*) rats (n = 6/group). *$P <$ 0.05 vs. vehicle.

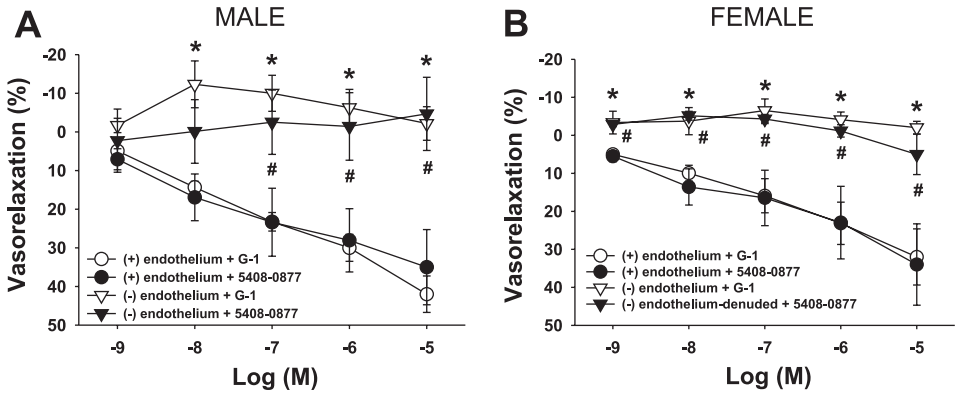

Fig. 4. Vasorelaxant responses to increasing concentrations of GPR30 agonists G-1 and 5408-0877 in endothelium-intact and endothelium-denuded arteries from male (*A*) and female (*B*) rats ($n = 5$–6/group). $P < 0.05$ vs. G-1 (*) and vs. 5408-0877 (#).

xanthine oxidase cell-free system were similarly attenuated in the presence of G-1 (Fig. 7*C*). SOD (250 U/ml) abolished the superoxide signal in this assay, confirming that the signal was exclusively due to reaction of superoxide with lucigenin.

DISCUSSION

The major findings of the present study are: *1*) GPR30 immunoreactivity is apparent in endothelial and vascular smooth muscle cells of thoracic aorta and middle cerebral and carotid arteries from both genders; *2*) GPR30 agonists, G-1 and 5408-0877, induce similar concentration-dependent relaxations of rat isolated carotid arteries from male and female rats; *3*) there was no gender difference in these relaxation responses; *4*) G-1- and 5408-0877-induced relaxation is abolished by both endothelium removal and NOS inhibition; and *5*) G-1 reduces levels of vascular superoxide, probably by direct inactivation rather than GPR30-mediated enzyme inhibition. Collectively, these results suggest that activation of GPR30 elicits relaxation of the carotid artery in both male and female rats via release of endothelium-derived nitric oxide.

It is well documented that classical estrogen receptors contribute to many signaling events in healthy and diseased blood vessels (7). Conversely, very little is understood about the role of the novel estrogen receptor, GPR30, in regulating vascular tone. This study has found that activation of GPR30 by two agonists, G1 or 5408-0877, elicits concentration-dependent relaxation of male and female rat carotid arteries. It was very recently reported that the selective GPR30 agonist, G-1, acutely lowers arterial pressure in normotensive rats and induces relaxation of various rodent and human blood vessels (8). Similar blood pressure-lowering effects were reported to occur in ovariectomized female hypertensive mRen2. Lewis rats by Lindsey et al. (13). These authors also found that G-1 induces relaxation of the thoracic aorta of intact and ovariectomized female rats. Furthermore, Haas et al. (8) showed that G-1-mediated vasorelaxation was absent in mice deficient of GPR30, consistent with G-1 being a specific agonist for GPR30. We observed a small but significant vasorelaxation at 10 nM G-1, whereas Lindsey et al. (13) only found significant vasorelaxation of the aorta to occur at >1 μM. This could reflect a more efficient signaling by G protein-coupled receptor or a greater binding affinity for G-1 in carotid artery vs. thoracic aorta. To our knowledge, this is the first study to demonstrate the vasorelaxant actions of the potentially selective GPR30 agonist, 5408-0877. It remains to be elucidated, however, as to whether 5408-0877 selectively stimulates GPR30 and not ERα or ERβ.

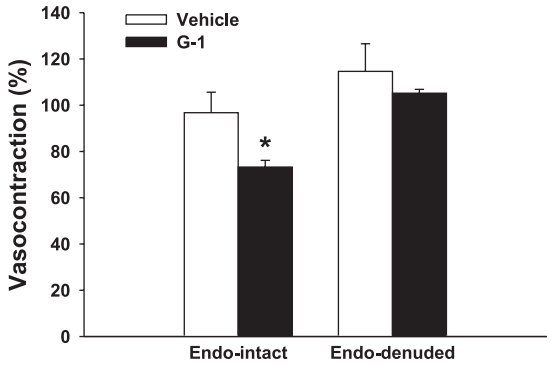

Fig. 5. Vasocontraction responses to U-46619 in both endothelium-intact and endothelium-denuded carotid arteries from male rats preincubated with G-1 or vehicle [dimethyl sulfoxide (DMSO)] for 1 h ($n = 4$). *$P < 0.05$ vs. vehicle.

To establish in which vascular cell type GPR30 is expressed in, we performed immunohistochemistry on various arterial sections using a specific GPR30 antibody. Positive GPR30 immunofluorescence was observed in both endothelial and vascular smooth muscle cells of thoracic aortas and carotid and cerebral arteries of male and female rats. Similar anti-GPR30 immunoreactivity was identified in the thoracic aorta of ovariectomized mRen2. Lewis female rats (13). In addition, Isensee et al. (10) reported that GPR30 is located in endothelial cells of small systemic arteries, but, interestingly, it is only located in the vascular smooth muscle of cerebral vessels. Consistent with this latter point, Haas et al. (9) demonstrated GPR30 is located in the vascular smooth muscle of human arteries and veins. Although the findings of these previous studies, in addition to those of the present study, have provided strong evidence that GPR30 is located in vascular endothelial and smooth muscle cells, understanding the role of GPR30 in each cell type remained to be elucidated.

Studies of estrogen-mediated activation of ERα and/or ERβ have demonstrated that the resulting vasodilatation is endothelium dependent in various vascular beds (21, 29, 34, 35). To determine if GPR30 also mediates vasorelaxation via a similar mechanism, we investigated GPR30 agonist-induced responses in endothelium-denuded arteries. The experiments revealed that G-1- and 5408-0877-induced relaxation of carotid arteries from male and female rats is endothelium-dependent, suggesting that activation of GPR30 located in the intima results in the release of an endothelium-derived relaxing factor. These find-

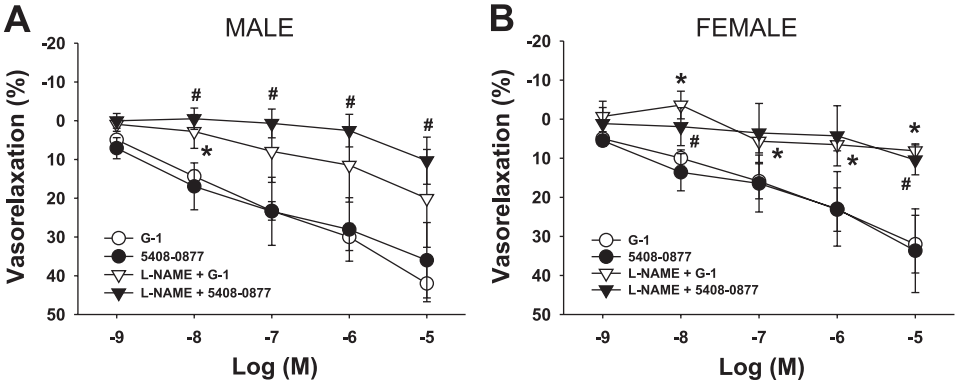

Fig. 6. Vasorelaxant responses to increasing concentrations of the GPR30 agonists, G-1 and 5408-0877, in arteries from male (*A*) and female (*B*) rats with or without N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM; n = 5–6/group). $P < 0.05$ vs. G-1 (*) and vs. 5408-0877 (#).

ings were supported by additional studies that showed pretreating vessels with G-1 significantly reduced U-46619-mediated contraction in endothelium-intact, but not in endothelium-denuded, arteries. Our findings corroborate those reported by Lindsey et al. (13) who demonstrated G-1-mediated relaxation is abrogated in endothelium-denuded arteries from both intact and ovariectomized female rats. Interestingly, it appears that GPR30 in vascular smooth muscle cells may not contribute to the regulation of vascular tone because, in endothelium-denuded arteries, GPR30 agonists did not elicit relaxation and G-1 preincubation had no significant effect on U-46619-induced contraction. It is possible that GPR30 in these cells may instead play a role in cell growth, since GPR30 activation is reported to inhibit cell proliferation in human vascular smooth muscle cells that express neither ERα nor ERβ (8). In the same study, however, it was reported that stimulation of GPR30 by G-1 blocks serotonin-induced changes in intracellular calcium in vascular smooth muscle cells, which indicates that GPR30-mediated vasorelaxation involves calcium antagonistic or desensitizing effects. Moreover, G-1 induced a robust increase in extracellular signal-regulated kinase (ERK) 1/2 phosphorylation and thus the authors speculated that GPR30 might antagonize changes in intracellular calcium caused by vasoconstrictor agonists, possibly via ERK1/2. It is possible that the differences between our findings and those reported by Haas et al. are due to variations between the preparations employed. Our isolated carotid artery preparation allows study of vascular function in a more physiological setting compared with cultured vascular smooth muscle cells. Indeed, relatively short-term culture is known to elicit changes in vascular smooth muscle transient receptor potential canonical protein, voltage-gated potassium channel, and calcium-activated potassium channel expression/function (1, 32). These channels are important regulators of calcium channels, and it is thought that such changes likely occur because of enzymatic digestion, exposure to artificial substrata and culture media, and a lack of normal intercellular communication, cell contacts, and tangential stress. Additional explanations for the varying results between our study and Haas et al. (8) is that different arteries from different species were examined.

Endothelium-dependent effects of estrogen in the vasculature commonly involve endothelial factors, including nitric oxide (21, 29, 34, 35). Having found that GPR30 activation induces endothelium-dependent vasorelaxation, we considered

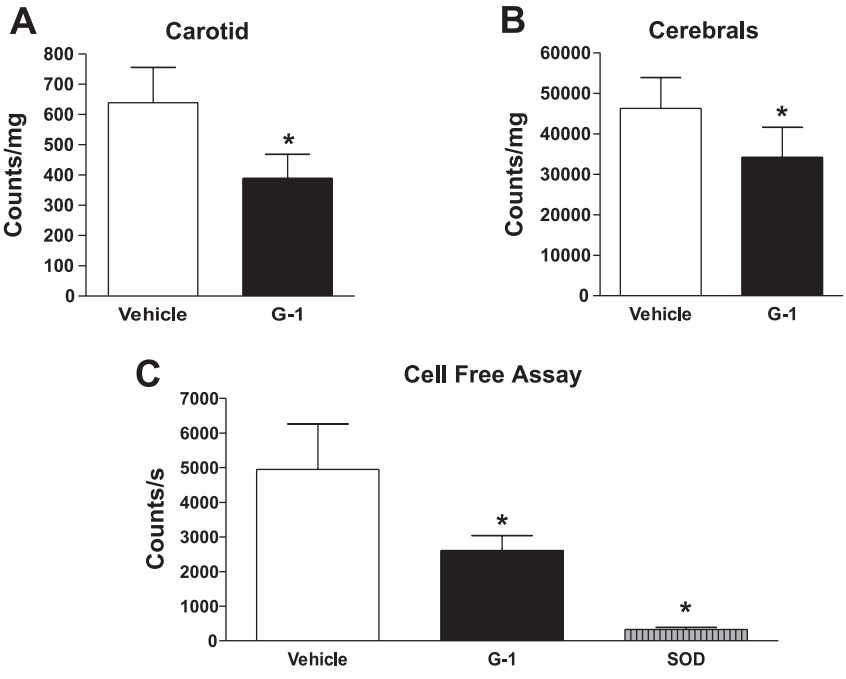

Fig. 7. Superoxide production was measured by lucigenin (5 mM)-enhanced chemiluminescence. *A* and *B*: effect of NADPH (100 μM) on superoxide production by carotid (*A*) and cerebral (*B*) arteries (n = 5) in the presence or absence of G-1 (10 μM). *C*: a xanthine/xanthine oxidase (X-XO)-stimulated cell free activity assay (n = 5) with or without G-1 (10 μM) or superoxide dismutase (SOD; 200 U/ml). *$P < 0.05$ vs. control.

the possibility that, like classical estrogen receptors, GPR30 mediates relaxation via nitric oxide signaling. We therefore tested this hypothesis by assessing GPR30-mediated relaxation in arteries pretreated with the NOS inhibitor L-NAME. The results confirmed that the relaxant responses to GPR30 agonists were not only endothelium dependent but also nitric oxide mediated. This finding indicates that the mechanism underlying GPR30-mediated relaxation of the normal carotid artery differs from that which exists in the aorta of hypertensive rats (13), which instead occurs through regulation of the renin-angiotensin system. Thus different signaling mechanisms may contribute to GPR30-mediated relaxation in different vascular beds, and in health vs. disease; hence, further studies are required to fully clarify this. However, the mechanisms by which activated GPR30 triggers the release of nitric oxide in vascular endothelial cells remains to be determined. A possible mechanism is that stimulation of GPR30 leads to phospholipase C activation and subsequent inositol 1,4,5-trisphosphate production (28), resulting in intracellular calcium release from the endoplasmic reticulum. It is well established that increased calcium release in vascular endothelial cells is involved in the generation of nitric oxide from NOS (2). It is important to note that the G protein-coupled receptor-mediated phospholipase C/calcium signal transduction cascade, resulting in generation of nitric oxide, has been widely reported in the vasculature (6).

Isoflavones display significant binding and activity toward GPR30 (15, 33). Hence, it has been suggested that GPR30 may be an important receptor for mediating vascular responses to isoflavones (27). However, our observation that GPR30-mediated relaxation occurs via activation of the endothelial nitric oxide pathway may indirectly suggest that some isoflavones, such as equol, daidzein, trans-tetrahydrodaidzein, and dehydroequol, do not mediate vasorelaxation via GPR30. Several groups, including ours, have shown that these isoflavones mediate vasorelaxation independent of endothelial nitric oxide signaling, including in the rat carotid artery (5, 11, 22). Conversely, isoflavones have been reported to elicit endothelium-dependent relaxation of some vessels (4, 5, 31); therefore, future studies are necessary to ascertain whether isoflavones that mediate endothelium-dependent relaxation indeed activate GPR30.

Because isoflavones possess antioxidant activity in cerebral arteries (11), and NADPH oxidases are a major source of superoxide in these vessels (20), we tested whether activation of GPR30 might modulate the activity of vascular NADPH oxidases. Using the chemiluminescence probe lucigenin, we found that, in the presence of G-1, levels of superoxide generated by carotid and cerebral arteries in response to NADPH were significantly lower than in untreated arteries. However, subsequent experiments showed that G-1 also reduced levels of superoxide generated in the cell-free xanthine/xanthine oxidase assay. Taken together, these findings suggest that activation of GPR30 does not suppress vascular NADPH oxidase but, interestingly, that G-1 directly scavenges superoxide.

In conclusion, this study demonstrated that the novel GPR30 receptor is expressed in the endothelium and vascular smooth muscle of rat aorta and cerebral and carotid arteries. Moreover, agonists of GPR30 elicit endothelium-dependent, nitric oxide-mediated vascular relaxation in carotid arteries of male and female rats. Furthermore, the GPR30 agonist G-1 also acts as an antioxidant but unlikely inhibits NADPH oxidase-derived superoxide. As a provasodilator receptor, GPR30 has a potentially important physiological role in mediating estrogen signaling, and it could represent a potential therapeutic target in the cerebral circulation during vascular diseases such as stroke.

GRANTS

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES

1. **Bergdahl A, Gomez MF, Wihlborg AK, Erlinge D, Eyjolfson A, Xu SZ, Beech DJ, Dreja K, Hellstrand P.** Plasticity of TRPC expression in arterial smooth muscle: correlation with store-operated Ca^{2+} entry. *Am J Physiol Cell Physiol* 288: C872–C880, 2005.
2. **Bredt DS, Snyder SH.** Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc Natl Acad Sci USA* 87: 682–685, 1990.
3. **Chan EC, Drummond GR, Woodman OL.** 3′,4′-Dihydroxyflavonol enhances nitric oxide bioavailability and improves vascular function after ischemia and reperfusion injury in the rat. *J Cardiovasc Pharmacol* 42: 727–735, 2003.
4. **Chin-Dusting JP, Boak L, Husband A, Nestel PJ.** The isoflavone metabolite dehydroequol produces vasodilatation in human resistance arteries via a nitric oxide-dependent mechanism. *Atherosclerosis* 176: 45–48, 2004.
5. **Chin-Dusting JP, Fisher LJ, Lewis TV, Piekarska A, Nestel PJ, Husband A.** The vascular activity of some isoflavone metabolites: implications for a cardioprotective role. *Br J Pharmacol* 133: 595–605, 2001.
6. **Christopoulos A, El Fakahany EE.** The generation of nitric oxide by G protein-coupled receptors. *Life Sci* 64: 1–15, 1998.
7. **Deroo BJ, Korach KS.** Estrogen receptors and human disease. *J Clin Invest* 116: 561–570, 2006.
8. **Haas E, Bhattacharya I, Brailoiu E, Damjanovic M, Brailoiu GC, Gao X, Mueller-Guerre L, Marjon NA, Gut A, Minotti R, Meyer MR, Amann K, Ammann E, Perez-Dominguez A, Genoni M, Clegg DJ, Dun NJ, Resta TC, Prossnitz ER, Barton M.** Regulatory role of G protein-coupled estrogen receptor for vascular function and obesity. *Circ Res* 104: 288–291, 2009.
9. **Haas E, Meyer MR, Schurr U, Bhattacharya I, Minotti R, Nguyen HH, Heigl A, Lachat M, Genoni M, Barton M.** Differential effects of 17beta-estradiol on function and expression of estrogen receptor alpha, estrogen receptor beta, and GPR30 in arteries and veins of patients with atherosclerosis. *Hypertension* 49: 1358–1363, 2007.
10. **Isensee J, Meoli L, Zazzu V, Nabzdyk C, Witt H, Soewarto D, Effertz K, Fuchs H, Gailus-Durner V, Busch D, Adler T, de Angelis MH, Irgang M, Otto C, Noppinger PR.** Expression pattern of G protein-coupled receptor 30 in LacZ reporter mice. *Endocrinology* 150: 1722–1730, 2009.
11. **Jackman KA, Woodman OL, Chrissobolis S, Sobey CG.** Vasorelaxant and antioxidant activity of the isoflavone metabolite equol in carotid and cerebral arteries. *Brain Res* 1141: 99–107, 2007.
12. **Jackman KA, Woodman OL, Sobey CG.** Isoflavones, equol and cardiovascular disease: pharmacological and therapeutic insights. *Curr Med Chem* 14: 2824–2830, 2007.
13. **Lindsey SH, Cohen JA, Brosnihan KB, Gallagher PE, Chappell MC.** Chronic treatment with the G protein-coupled receptor 30 agonist G-1 decreases blood pressure in ovariectomized mRen2. *Lewis rats Endocrinology* 150: 3753–3758, 2009.
14. **Luksha L, Kublickiene K.** The role of estrogen receptor subtypes for vascular maintenance. *Gynecol Endocrinol* 25: 82–95, 2009.
15. **Maggiolini M, Vivacqua A, Fasanella G, Recchia AG, Sisci D, Pezzi V, Montanaro D, Musti AM, Picard D, Ando S.** The G protein-coupled receptor GPR30 mediates c-fos up-regulation by 17beta-estradiol and phytoestrogens in breast cancer cells. *J Biol Chem* 279: 27008–27016, 2004.

16. **Mahn K, Borras C, Knock GA, Taylor P, Khan IY, Sugden D, Poston L, Ward JP, Sharpe RM, Vina J, Aaronson PI, Mann GE.** Dietary soy isoflavone induced increases in antioxidant and eNOS gene expression lead to improved endothelial function and reduced blood pressure in vivo. *FASEB J* 19: 1755–1757, 2005.
17. **Martensson UE, Salehi SA, Windahl S, Gomez MF, Sward K, Daszkiewicz-Nilsson J, Wendt A, Andersson N, Hellstrand P, Grande PO, Owman C, Rosen CJ, Adamo ML, Lundquist I, Rorsman P, Nilsson BO, Ohlsson C, Olde B, Leeb-Lundberg LM.** Deletion of the G protein-coupled receptor 30 impairs glucose tolerance, reduces bone growth, increases blood pressure, and eliminates estradiol-stimulated insulin release in female mice. *Endocrinology* 150: 687–698, 2009.
18. **Martin DS, Breitkopf NP, Eyster KM, Williams JL.** Dietary soy exerts an antihypertensive effect in spontaneously hypertensive female rats. *Am J Physiol Regul Integr Comp Physiol* 281: R553–R560, 2001.
19. **Martin DS, Williams JL, Breitkopf NP, Eyster KM.** Pressor responsiveness to angiotensin in soy-fed spontaneously hypertensive rats. *Can J Physiol Pharmacol* 80: 1180–1186, 2002.
20. **Miller AA, Drummond GR, Schmidt HH, Sobey CG.** NADPH oxidase activity and function are profoundly greater in cerebral versus systemic arteries. *Circ Res* 97: 1055–1062, 2005.
21. **Muller-Delp JM, Lubahn DB, Nichol KE, Philips BJ, Price EM, Curran EM, Laughlin MH.** Regulation of nitric oxide-dependent vasodilation in coronary arteries of estrogen receptor-α-deficient mice. *Am J Physiol Heart Circ Physiol* 285: H2150–H2157, 2003.
22. **Nevala R, Korpela R, Vapaatalo H.** Plant derived estrogens relax rat mesenteric artery in vitro. *Life Sci* 63: L-100, 1998.
23. **Nevala R, Vaskonen T, Vehniainen J, Korpela R, Vapaatalo H.** Soy based diet attenuates the development of hypertension when compared to casein based diet in spontaneously hypertensive rat. *Life Sci* 66: 115–124, 2000.
24. **Paravicini TM, Gulluyan LM, Dusting GJ, Drummond GR.** Increased NADPH oxidase activity, gp91phox expression, and endothelium-dependent vasorelaxation during neointima formation in rabbits. *Circ Res* 91: 54–61, 2002.
25. **Park E, Shin JI, Park OJ, Kang MH.** Soy isoflavone supplementation alleviates oxidative stress and improves systolic blood pressure in male spontaneously hypertensive rats. *J Nutr Sci Vitaminol (Tokyo)* 51: 254–259, 2005.
26. **Prossnitz ER, Arterburn JB, Smith HO, Oprea TI, Sklar LA, Hathaway HJ.** Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. *Annu Rev Physiol* 70: 165–190, 2008.
27. **Prossnitz ER, Barton M.** Signaling, physiological functions and clinical relevance of the G protein-coupled estrogen receptor GPER. *Prostaglandins Other Lipid Mediat* 89: 89–97, 2009.
28. **Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER.** A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* 307: 1625–1630, 2005.
29. **Rosenfeld CR, Cox BE, Roy T, Magness RR.** Nitric oxide contributes to estrogen-induced vasodilation of the ovine uterine circulation. *J Clin Invest* 98: 2158–2166, 1996.
30. **Rubanyi GM, Freay AD, Kauser K, Sukovich D, Burton G, Lubahn DB, Couse JF, Curtis SW, Korach KS.** Vascular estrogen receptors and endothelium-derived nitric oxide production in the mouse aorta. Gender difference and effect of estrogen receptor gene disruption. *J Clin Invest* 99: 2429–2437, 1997.
31. **Sobey CG, Weiler JM, Boujaoude M, Woodman OL.** Effect of short-term phytoestrogen treatment in male rats on nitric oxide-mediated responses of carotid and cerebral arteries: comparison with 17beta-estradiol. *J Pharmacol Exp Ther* 310: 135–140, 2004.
32. **Tang G, Wang R.** Differential expression of KV and KCa channels in vascular smooth muscle cells during 1-day culture. *Pflugers Arch* 442: 124–135, 2001.
33. **Thomas P, Dong J.** Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption. *J Steroid Biochem Mol Biol* 102: 175–179, 2006.
34. **Thompson LP, Pinkas G, Weiner CP.** Chronic 17beta-estradiol replacement increases nitric oxide-mediated vasodilation of guinea pig coronary microcirculation. *Circulation* 102: 445–451, 2000.
35. **Traupe T, Stettler CD, Li H, Haas E, Bhattacharya I, Minotti R, Barton M.** Distinct roles of estrogen receptors alpha and beta mediating acute vasodilation of epicardial coronary arteries. *Hypertension* 49: 1364–1370, 2007.
36. **Ullrich ND, Krust A, Collins P, MacLeod KT.** Genomic deletion of estrogen receptors ERα and ERβ does not alter estrogen-mediated inhibition of Ca^{2+} influx and contraction in murine cardiomyocytes. *Am J Physiol Heart Circ Physiol* 294: H2421–H2427, 2008.
37. **Walker HA, Dean TS, Sanders TA, Jackson G, Ritter JM, Chowienczyk PJ.** The phytoestrogen genistein produces acute nitric oxide-dependent dilation of human forearm vasculature with similar potency to 17beta-estradiol. *Circulation* 103: 258–262, 2001.