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DELETION OF G PROTEIN-COUPLED ESTROGEN RECEPTOR INCREASES ENDOTHELIAL VASOCONSTRICTION

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Abstract

Endogenous estrogens mediate protective effects in the cardiovascular system, affecting both endothelium-dependent and -independent mechanisms. Previous studies have suggested that nonselective estrogen receptor agonists such as endogenous estrogens inhibit endothelium-dependent vasoconstriction; however, the role of estrogen receptors in this response has not yet been clarified. This study investigated whether the novel intracellular G protein-coupled estrogen receptor GPER regulates vascular reactivity in mice. Effects of chronic deficiency (using mice lacking the GPER gene) and acute inhibition (using the GPER-selective antagonist G15) on endothelium-dependent and -independent vascular reactivity, and the effects of GPER deficiency on vascular gene expression and structure were investigated. We found that chronic GPER deficiency is associated with increased endothelial prostanoid-mediated vasoconstriction, but had no effect on endothelial nitric oxide (NO) bioactivity, gene expression of endothelial NO synthase and thromboxane prostanoid (TP) receptor, or vascular structure. GPER deletion also increased TP receptor-mediated contraction. Acute GPER blockade enhanced endothelium-dependent contractions, and reduced endothelial NO bioactivity. Contractions to TP receptor activation were unaffected by G15. In conclusion, this study has identified GPER as the first estrogen receptor with inhibitory activity on endothelium-dependent contractility. These findings may be important for understanding and treating diseases associated with increased endothelial vasoconstrictor prostanoid activity such as hypertension and obesity.

Keywords

EDCF; Endothelium; Estrogen; GPER; GPR30; Nitric Oxide; Prostanoid

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Introduction

Vascular tone is maintained by a balanced release of endothelial relaxing factors including nitric oxide (NO), and endothelium-derived contracting factors (EDCF) such as prostanoids and endothelin.[1,2] NO is released under basal conditions, in response to shear stress and vasodilators such as acetylcholine.[3] Acetylcholine and other agonists also stimulate the production of EDCF, including cyclooxygenase (COX)-derived prostanoids that activate thromboxane prostanoid (TP) receptors on vascular smooth muscle cells (VSMC).[1,2,4] In arteries of patients with essential hypertension, endothelium-dependent NO generation is impaired, while activity of vasoconstrictor prostanoids is increased.[1,2] These abnormalities in endothelial cell function are associated with an increased cardiovascular risk.[5]

Endogenous estrogens beneficially modulate the activity of endothelial factors by increasing the transcription of the endothelial NO synthase (eNOS) gene,[6] and acutely stimulating NO production via eNOS.[7] Effects of estrogens are mediated by multiple receptors including the classical estrogen receptors ERα and ERβ,[8] with NO-dependent effects of estrogens requiring functional ERα.[7,9–11] Estrogens also bind to the novel, 7-transmembrane spanning intracellular G protein-coupled estrogen receptor GPER (previously termed GPR30)[8,12] cloned from human endothelial cells[13] and expressed throughout the cardiovascular system.[8] The development of the GPER-selective agonist G-1[14] has facilitated studies that demonstrate GPER activation induces acute vasodilation and lowers blood pressure in rodents.[15–19] Moreover, G-1-dependent relaxation is absent in GPER-knockout ($GPER^0$) mice, further corroborating the requirement of this receptor to mediate vascular responses.[15] We[18] and others[17,19] have shown that acute GPER-mediated vasodilator effects are at least partly endothelium- and NO-dependent.

The role of GPER in endothelium-dependent vasoconstriction is unknown. Natural estrogens such as 17β-estradiol, a non-selective agonist of ERα, ERβ, and GPER,[8] modulate vasoconstrictor prostanoid activity and the expression of TP receptors and prostanoid synthases.[20–26] Furthermore, inhibitory effects of 17β-estradiol on COX-dependent responses to vasoconstrictors have suggested a role of estrogen receptors,[27] although the specific estrogen receptor(s) involved in endothelium-dependent vasoconstriction have not been identified. Using $GPER^0$ mice and the GPER-selective antagonist G15,[28] the present study was therefore designed to test the hypothesis that GPER is involved in endothelium-dependent and -independent regulation of vasomotor tone, with a particular emphasis on endothelial vasoconstrictor prostanoids.

Methods

Vascular Function Experiments

Male C57Bl6 and GPER-deficient ($GPER^0$) mice (3 months of age) were anesthetized by intraperitoneal injection of sodium pentobarbital and exsanguinated by cardiac puncture. All procedures were approved by and carried out in accordance with institutional policies and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The aorta was excised and prepared for measurements of isometric force in organ baths as described.[15,18,29] To study acute effects of GPER inhibition on vascular function, selected rings were pretreated with the GPER-selective antagonist G15 (3 μmol/L)[28] or vehicle (ethanol 0.27%, DMSO 0.03% vol/vol) for 20 min. Some rings were also pretreated with the non-selective COX inhibitor indomethacin (10 μmol/L), the TP receptor antagonist SQ 29,548 (1 μmol/L), or the NO synthase inhibitor L-NAME (300 μmol/L) for 30 min. Concentration-response curves to acetylcholine (0.1 nmol/L – 100 μmol/L), sodium nitroprusside (SNP, 0.01 nmol/L – 10 μmol/L), and U46619 (0.1 nmol/L – 10 μmol/L) were obtained. Basal NO bioactivity was determined as described.[30] To exclude interference with

NO-dependent effects of GPER,[17–19] responses to SNP and U46619 were recorded in the presence of L-NAME.

Histological Analyses and Immunohistochemistry

Paraffin-embedded cross sections (2 μm) of WT and GPER^0 aortas were stained with hematoxylin and eosin using a standard protocol. For immunohistochemical analyses, sections were stained as described[31] using the following antibodies: α-smooth muscle actin (1:200), proliferating cell nuclear antigen (PCNA, 1:500), and Ki-67 (1:100).

Quantitative Real-time Polymerase Chain Reaction (qPCR)

RNA from thoracic aorta of WT and GPER^0 mice was extracted and reverse transcribed. PCR was performed using TaqMan gene expression assays. Gene expression was calculated using the $2^{-\Delta\Delta C(T)}$ method.[32]

Statistical Analyses

Area under the curve (AUC) and EC_{50} values (as negative logarithm: pD_2) of concentration-response curves were calculated by non-linear regression analysis.[33] Data were analyzed using two-way repeated-measures ANOVA followed by Bonferroni post hoc analysis, the unpaired Student's *t*-test or the Mann-Whitney *U* test where appropriate. Values are shown as means±SEM. A $P<0.05$ value was considered statistically significant.

For expanded Materials and Methods please see the online supplement at http://hyper.ahajournals.org.

Results

Effect of GPER Deficiency on Endothelium-Dependent Vascular Reactivity

In order to assess the effect of endogenous GPER expression on endothelial vasoreactivity, we examined acetylcholine-induced endothelium-dependent relaxation and contraction[34,35] in the aorta of wild type (WT) and GPER^0 mice. Endothelium-dependent, receptor-stimulated NO release by acetylcholine induced similar extents of relaxation in phenylephrine-preconstricted rings from WT and GPER^0 mice (Figure 1A). However, at acetylcholine concentrations ≥0.1 μmol/L, which stimulate the release of EDCF,[21–23,34–36] a 2.3-fold increased contraction in GPER^0 aorta was observed (44.1±2.3% *vs*. 19.6±6.4%, $n=5$, $P<0.01$ *vs*. WT, Figure 1A). Consistent with an EDCF-mediated response,[1,2] the non-selective COX-inhibitor indomethacin and the TP receptor antagonist SQ 29,548 completely blocked contractions to acetylcholine ($n=4–5$, $P<0.05$ *vs*. untreated vessel rings, Figure 1A). To assess the role of GPER in EDCF-mediated activation of TP receptor signaling in VSMC,[1,2] we measured concentration-dependent contraction to the selective TP receptor agonist U46619. Contractions to U46619 were increased in GPER^0 aorta (maximal effect 197±8% *vs*. 156±10%; AUC 586±22 *vs*. 435±27 AU; pD_2 8.01±0.03 *vs*. 7.82±0.12; $n=5–6$, $P<0.01$ *vs*. WT), consistent with an inhibitory effect on EDCF-mediated VSMC contractility by endogenous GPER in WT mice. TP receptor gene expression was unaffected by GPER deficiency (Table 1), which also had no effect on gene expression levels of prostanoid generating enzymes, including COX-1, prostacyclin synthase, and thromboxane A_2 synthase (Table 1).

Chronic and Acute Effects of GPER Inhibition on Endothelial NO and EDCF Activity

Based on previous findings that the GPER-selective agonist G-1 induces endothelium- and NO-dependent relaxation,[17–19] we next investigated the role of GPER in basal and stimulated bioactivity of NO. GPER deficiency had no effect on receptor-stimulated, NO-

mediated relaxation (Figure 1A), on basal NO bioactivity (41.9±9.3% *vs*. 46.0±9.2%, *n*=6–9, *P*=n.s. *vs*. WT, Figure 2A), or on eNOS gene expression (Table 1). In contrast, acute inhibition of GPER with the selective antagonist G15 reduced basal NO bioactivity in WT mice (38.3±5.7% *vs*. 57.7±5.2%, *n*=4–6, *P*<0.05 *vs*. vehicle, Figure 2A). Moreover, receptor-stimulated endothelial NO release in response to acetylcholine in WT mice was attenuated by G15 (maximal effect - 60.7±3.5% *vs.* −74.4±2.5%; AUC 90±11 *vs.* 133±9 AU; *n*=5–6, *P*<0.05 *vs.* vehicle, Figure 2B). At higher concentrations of acetylcholine, blockade of GPER by G15 yielded an increase in contraction to acetylcholine[21–23,34–36] (9-fold at 0.1 mmol/L, *n*=5–6, *P*<0.05 *vs.* vehicle, Figure 3A). In contrast, contraction to TP receptor activation by U46619 remained unaffected by G15 (Figure 3B) compatible with the notion that acute GPER inhibition augments endothelial EDCF activity and/or release but not VSMC TP receptor signaling. In addition, G15 alone had no effect on vascular tone in quiescent aortic rings (Meyer MR and Prossnitz ER, unpublished data, 2011).

Acute and Chronic Effects of GPER Inhibition on Endothelium-Independent Relaxation

Endothelium-independent, NO-mediated relaxation in response to SNP was slightly but significantly enhanced in $GPER^0$ mice (maximal effect −111±1% *vs.* −106±1%, *P*<0.01 *vs.* WT; AUC 421±7 *vs.* 402±7 AU, *P*=n.s.; pD_2 8.84±0.05 *vs.* 8.82±0.08, *P*=n.s.; *n*=5–6, Figure 4A). GPER deficiency had no effect on gene expression levels of the α- or β-subunits of soluble guanylate cyclase, the downstream target of NO in VSMC[3] (Table 1). Moreover, G15 did not acutely affect endothelium-independent relaxation (Figure 4B), suggesting that only the chronic lack of GPER to a very small extent affects smooth muscle sensitivity to NO.

Vascular Histology and Immunohistochemistry

To determine whether the increased vasoreactivity in $GPER^0$ animals might be associated with structural changes in the vasculature, histological analyses of the aorta were performed. Hematoxylin and eosin staining of aorta cross sections revealed structurally normal vascular walls in WT and $GPER^0$ animals, in particular no changes in thickness of the smooth muscle layer (Figure 5). There was also no evidence of changes in VSMC differentiation and proliferation as assessed by staining for α-smooth muscle actin (Figure 5), PCNA, and Ki-67 (Amann K and Prossnitz ER, unpublished data, 2011).

Discussion

The present study provides evidence that endogenous GPER regulates vascular activity of prostanoid-dependent endothelial vasoconstriction. Chronic deficiency of the GPER gene has no effect on NO bioactivity but is associated with enhanced VSMC contraction to endothelium-dependent, COX-derived vasoconstrictor prostanoids and responses to TP receptor activation. In addition, histological and immunohistochemical analyses revealed structurally normal arteries in $GPER^0$ mice. By contrast, acute blockade of GPER decreases both basal and stimulated endothelial NO bioactivity and increases EDCF-mediated responses while contractions to TP receptor activation remain unchanged. These data identify GPER as a novel, inhibitory regulator of endothelial vasoconstrictor prostanoids, and as the first estrogen receptor specifically associated with the regulation of endothelial vasoconstriction.

Acetylcholine induces the release of two counter-acting endothelial mediators in the mouse aorta: NO-mediated relaxation is induced at low concentrations of acetylcholine whereas a moderate EDCF-mediated contraction is observed at higher concentrations of acetylcholine.[34] In the present study, we demonstrate that GPER deficiency augments acetylcholine-mediated contraction, which is completely abolished by either inhibition of

COX or TP receptors, consistent with an EDCF-based mechanism.[1,2] Moreover, direct activation of the EDCF-responsive element in VSMC, the TP receptor, reproduced the enhanced constrictor response in the absence of GPER with TP receptor gene expression levels being unaffected. Similar to the present findings, sensitivity to the prostanoid PGH_2 is increased in spontaneously hypertensive rats (SHR) compared to normotensive animals, with no change in TP receptor expression levels in SHR.[36,37] This suggests that a hyper-responsiveness of VMSC may contribute to endothelium-dependent contractions in both $GPER^0$ mice and the SHR,[2,36] possibly mediated by either increased calcium influx following TP receptor activation or enhanced sensitivity of contractile proteins.[38] Consistent with this possibility, we observed that the calcium sensitivity of VSMC to endothelin-1-mediated constrictions is enhanced in $GPER^0$ arteries.[39] The present study also shows that endothelium-independent NO-mediated relaxation was slightly increased in $GPER^0$ vessels with no change in guanylate cyclase gene expression, suggesting the possibility that chronic enhanced sensitivity to endothelium-derived constrictors might be compensated for by enhanced smooth muscle sensitivity to NO.

The enhanced EDCF response in $GPER^0$ mice is likely unrelated to reduced NO release,[1,2] since GPER deficiency had no effect on basal or stimulated NO bioactivity, or eNOS gene expression. Moreover, GPER deficiency had no effect on the gene expression levels of enzymes involved in prostanoid synthesis, including COX-1, which mediates endothelium-dependent contractions in large murine arteries,[35] as well as synthases for thromboxane A_2 and prostacyclin, molecules that function as EDCF under certain conditions.[1,2] It is possible that an increased prostanoid release alone or in conjunction with an exaggerated production of reactive oxygen species (ROS), which stimulate formation of vasoconstrictor prostanoids,[1,2] contributes to the enhanced EDCF response in $GPER^0$ animals.

Acute GPER inhibition by G15 reduced endothelium-dependent, receptor-stimulated and basal NO bioactivity. This is consistent with previous studies demonstrating that stimulation of GPER using the selective agonist G-1 causes acute relaxation that is endothelium- and NO-dependent,[17–19] an effect blocked by G15.[19] In the present study, we also report that at higher concentrations capable of EDCF stimulation,[21–23,34–36] acetylcholine causes a pronounced endothelium-dependent contraction in the presence of G15. This may be in part due to the observed G15-dependent reduction of NO bioactivity, which potentiates EDCF-mediated contractions.[1,2] Moreover, the response to VSMC TP receptor activation was unaffected by G15, suggesting no increase in VSMC sensitivity to prostanoids in contrast to the observation in $GPER^0$ mice.

The activity of endothelium-derived vasoconstrictor prostanoids increases with aging and diseases such as hypertension and obesity, conditions associated with structural changes in the vascular wall.[1,2,40,41] In the present study, we used healthy adult mice and found no effect of GPER deficiency on media thickness, differentiation, or proliferation in the smooth muscle layer of the aorta consistent with normal vascular structure. Our results confirm previous findings in adult female $GPER^0$ mice showing normal structure of the aortic media.[42] In these animals, media thickness of non-pressure-fixed mesenteric arteries was also unaffected by GPER deficiency, whereas the vessel diameter was slightly decreased in nine months old $GPER^0$ compared to WT mice.[42] However, mean arterial blood pressure (MAP) in anesthetized $GPER^0$ animals did not increase with age, although the MAP of $GPER^0$ mice at nine months was higher than WT mice of the same age.[42] Whether and to what extent the increased endothelium-dependent vasoreactivity observed in $GPER^0$ in the present study contributes to blood pressure or structural vascular changes associated with the physiological aging process[40] remains to be determined.

GPER is expressed and functional in the cardiovascular system of both males and females.[15,17,42,43] Similar to the findings of the present study in male GPER^0 mice, the absence of ERα or ERβ adversely affects endothelial-dependent vasoreactivity and blood pressure in male animals as well as in men.[9,44–46] Thus, estrogen receptors are involved in the regulation of endothelium-derived factors independent of sex.[9] Interestingly, many G protein-coupled receptors display a basal intrinsic activity even in the absence of their ligand that might be sufficient to mediate GPER-dependent vascular effects.[47] Furthermore, the activity of the enzyme aromatase within the vascular wall (where it is localized intracellularly, like GPER, to the endoplasmic reticulum[12,48]), mediates local conversion of androgen precursors into the non-selective estrogen receptor agonist 17β-estradiol, which could yield local concentrations high enough to activate estrogen receptors.[49] In fact, male mice lacking the enzyme aromatase display impaired endothelium-dependent relaxation.[50] These findings are supported by human studies in men treated with an aromatase inhibitor, which impairs endothelium-dependent, flow-mediated dilation.[51] Furthermore, there is evidence that 17β-estradiol inhibits endothelium-dependent contractions in salt-sensitive hypertension: in the renal artery, ovary-intact females exhibit much lower EDCF-mediated contractions than males, and only females are sensitive to inhibition of these responses by 17β-estradiol.[23] As it is unclear to what degree GPER may have mediated these estrogen-dependent effects, further studies are required to clarify any sex-dependent roles of GPER or other ERs for EDCF or NO bioactivity under healthy conditions and in the presence of disease.

Perspectives

Conditions such as aging, hypertension, and obesity accelerate vascular disease development and are associated with exacerbated endothelium-dependent contractions and impaired NO bioactivity.[1,2,41] In this study, we have identified GPER as the first estrogen receptor to specifically regulate endothelial protanoid vasoconstrictor activity. The ability of endogenous GPER to mitigate endothelium-dependent vasoconstriction and to enhance basal and stimulated endothelial NO bioactivity suggests the therapeutic potential of GPER-selective agonists such as G-1[14] to improve endothelium-dependent vascular dysfunction in conditions associated with increased cardiovascular risk.

Supplementary Material

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[1]E.R.P. holds a US patent on G-1 and G15.

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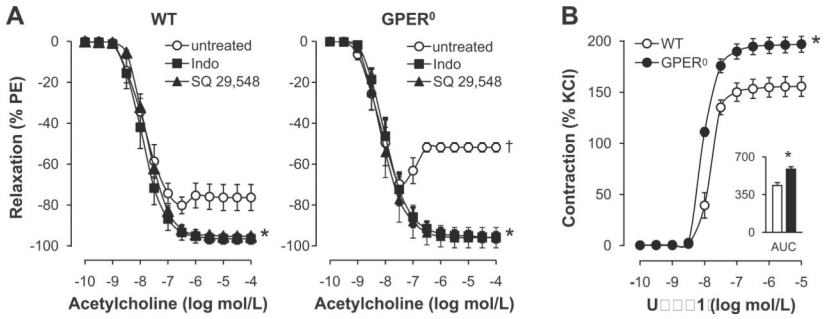

Figure 1.

A, Endothelium-dependent responses to acetylcholine in the aorta of WT and GPER^0 mice. Responses in the absence and presence of the COX-inhibitor indomethacin (Indo) or the TP receptor antagonist SQ 29,548 are shown. Values are means±SEM; n=4–5. *P<0.05 *vs.* untreated rings; †P<0.01 *vs.* WT. **B,** Concentration-dependent contraction and area under the curve (AUC) in response to the TP receptor agonist U46619 in WT and GPER^0 aorta in the presence of L-NAME. Values are means±SEM; n=4–6. *P<0.01 *vs.* WT.

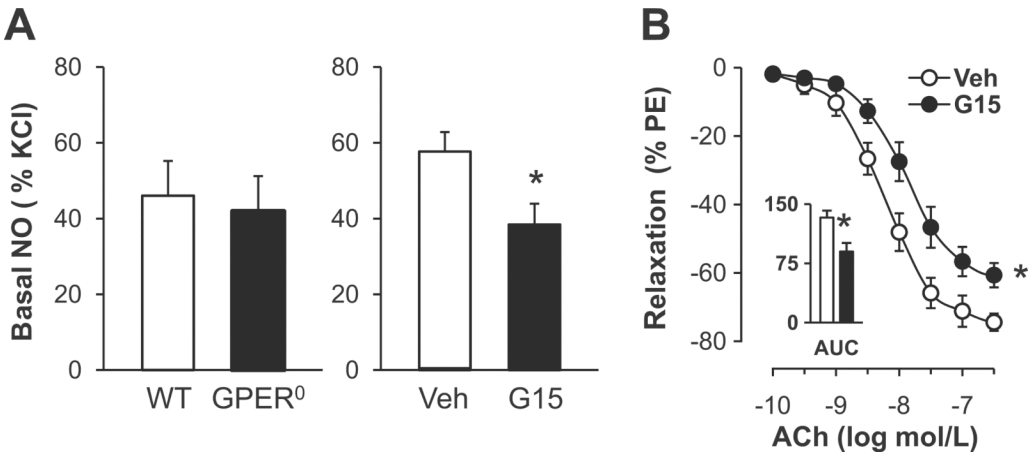

Figure 2.

A, Basal NO release in aorta from WT and GPER^0 mice (left panel), and acute effects of the GPER-selective antagonist G15 (right panel). Basal NO release was calculated as the increase in contraction to phenylephrine (300 nmol/L) in rings treated with L-NAME compared to untreated rings. Values are means±SEM; n=4–9. *P<0.05 *vs.* vehicle. **B,** Acute effect of G15 on vascular reactivity to receptor-stimulated NO release by acetylcholine (ACh). AUC, Area under the curve. Values are means±SEM; n=5–6. *P<0.05 *vs.* vehicle (Veh).

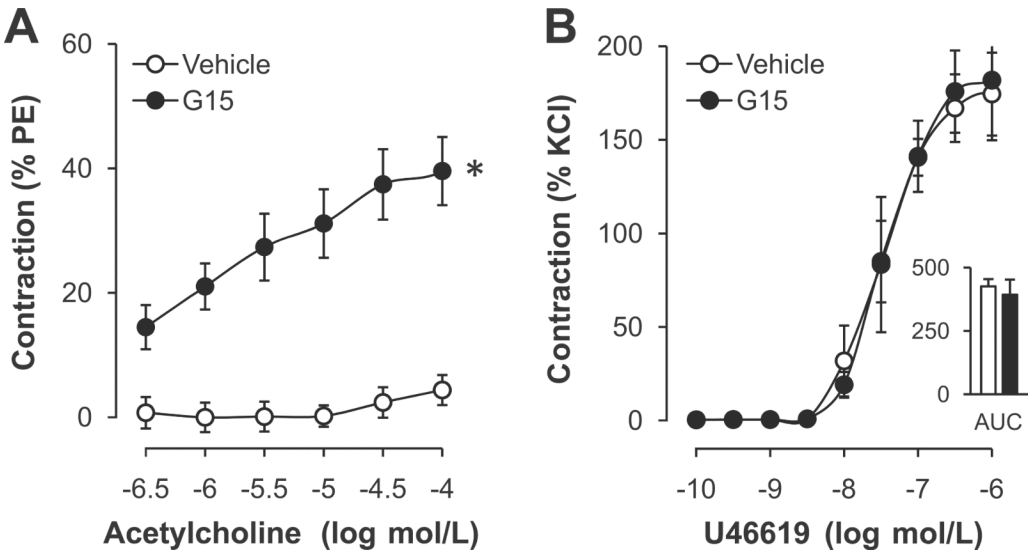

Figure 3.

A, Acute effects of the GPER-selective antagonist G15 on endothelium-dependent contraction to acetylcholine. Values are means±SEM; n=5–6. *P<0.05 *vs.* vehicle. **B,** Concentration-dependent contraction and area under the curve (AUC) to U46619 after acute G15 incubation in the presence of L-NAME. Values are means±SEM; n=4–5.

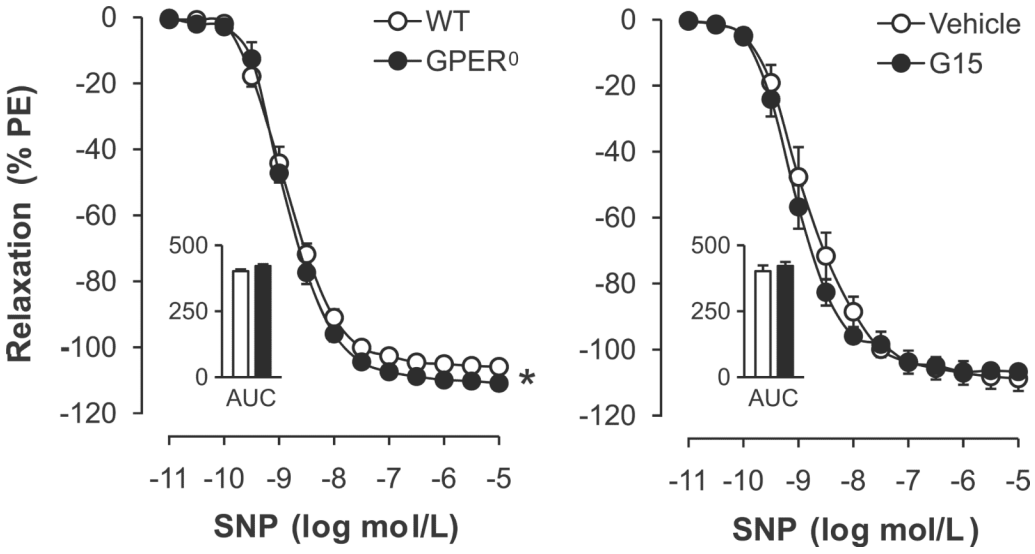

Figure 4.

Endothelium-independent relaxation to the NO donor sodium nitroprusside (SNP) in aorta from WT and GPER^0 mice (left panel), and role of acute inhibition with the GPER-selective antagonist G15 (right panel). Responses were recorded in the presence of L-NAME. AUC, Area under the curve. Values are means±SEM; n=5–6. *P<0.01 *vs.* WT.

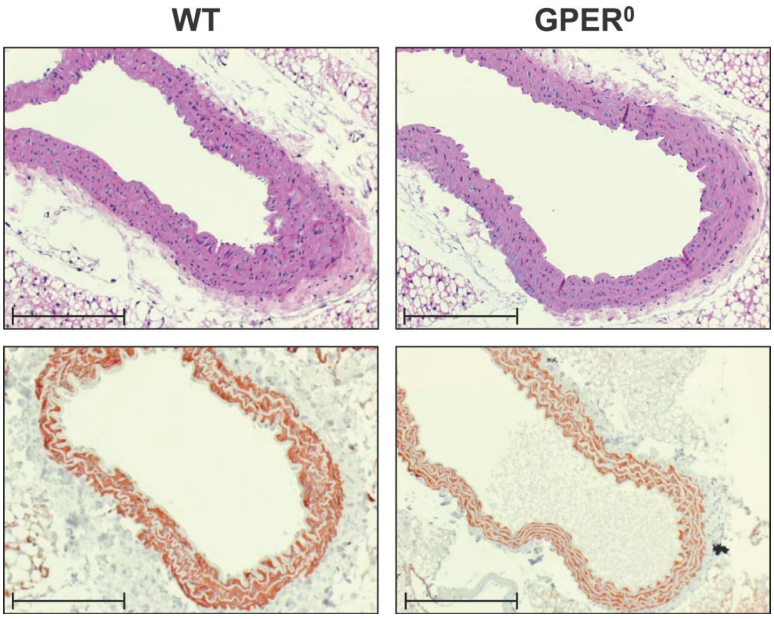

Figure 5.
Histological sections of thoracic aorta of WT and GPER^0 mice. Staining with hematoxylin and eosin (top) and immunohistochemical staining for α-smooth muscle actin (bottom) are shown revealing normal vascular structure. Scale bar, 100 μm.

Table 1

Vascular gene expression levels (calculated using the $2^{-\Delta\Delta C(T)}$ method[32] and expressed as arbitrary units) in WT and GPER^0 mice.

Gene	WT	GPER^0	*P*-Value
Cyclooxygenase 1	28.98±3.07	21.82±2.70	0.13
Prostacyclin Synthase	2.427±0.313	1.830±0.213	0.15
Thromboxane A_2 Synthase	517.4±71.0	428.8±10.7	0.25
TP Receptor	158.5±15.2	168.9±10.7	0.59
Endothelial NO Synthase	120.9±33.6	121.9±30.9	0.98
sGC (α-subunit)	187.4±42.1	171.7±19.6	0.75
sGC (β-subunit)	170.1±32.5	140.6±7.2	0.41

Values are means±SEM; *n*=4–5. TP receptor, thromboxane prostanoid receptor; sGC, soluble guanylate cyclase; NO, nitric oxide.