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Obligatory role for GPER in cardiovascular aging and disease[^]

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Abstract

Pharmacological activation of the heptahelical G protein-coupled receptor GPER by selective ligands counteracts multiple aspects of cardiovascular disease. We thus expected that genetic deletion or pharmacological inhibition of GPER would further aggravate such disease states, particularly with age. To the contrary, we found that genetic ablation of *Gper* in mice prevented cardiovascular pathologies associated with aging by reducing superoxide ($\cdot\text{O}_2^-$) formation by NADPH oxidase (Nox) and reduced expression the Nox isoform *Nox1*. Blocking GPER activity pharmacologically with G36, a synthetic, small molecule, GPER-selective blocker (GRB), decreased Nox1 abundance and $\cdot\text{O}_2^-$ production to basal amounts in cells exposed to angiotensin II and in mice chronically infused with angiotensin II. Thus, this study revealed a role for GPER activity in regulating Nox1 abundance and associated $\cdot\text{O}_2^-$ -mediated structural and functional damage that contributes to disease pathology. Our results indicated that GRBs represent a new class of drugs that can indirectly reduce Nox activity and could be used for the treatment of chronic disease processes involving excessive $\cdot\text{O}_2^-$ formation, including arterial hypertension and diastolic heart failure.

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Competing interests: M.R.M., G.S., M.B., and E.R.P. are inventors on a U.S. patent application for the therapeutic use of compounds targeting GPER. E.R.P. and J.B.A. are inventors on U.S. patent Nos. 7,875,721 and 8,487,100 for GPER-selective ligands and imaging agents. The other authors declare that they have no competing interests.

Introduction

G protein-coupled receptors (GPCRs) exert both rapid and chronic effects (1). The G protein-coupled estrogen receptor GPER is a heptahelical receptor, originally designated GPR30, with amino acid homology to GPCRs for angiotensin II (Ang II) and chemokines, that is found in multiple cell types including vascular cells (2-4). GPER is localized predominantly to the endoplasmic reticulum and Golgi apparatus (5) and mediates cellular responses to estrogens, selective estrogen receptor modulators (SERMs), and xenoestrogens (6) through non-genomic as well as genomic mechanisms (5, 7-9). GPER activation results in the rapid mobilization of intracellular calcium (5, 10, 11) and activation of nitric oxide (NO) synthase (12), Akt (11, 13-16) and ERK (7, 11, 16), among other pathways (6). In addition, GPER, like many other GPCRs (1, 17), may also exhibit basal or intrinsic activities that contribute to the chronic regulation of genomic pathways. Such genomic effects have been suggested to be responsible for the increased vasoconstrictor tone observed in *Gper*-deficient mice (18). The beneficial cardiovascular effects of GPER-selective synthetic ligands in multiple disease models (4, 12, 19) have led to the currently prevailing concept that activation of GPER conveys organ protection (6, 11, 20-23).

Reactive oxygen species (ROS) are short-lived intermediates of oxidative metabolism that are essential for cardiovascular homeostasis (24). Excessive ROS production, however, occurs in many chronic disease processes and aggravates vasoconstriction and cell growth, thereby contributing to increased vascular tone, myocardial hypertrophy, fibrosis, heart failure, and aging (25, 26). The NADPH oxidase (Nox) family represents the principal physiological source of ROS in the cardiovascular system and is composed of 7 catalytic subunits termed Nox1-5 and Duox1-2 (27-29). Of these, Nox1, Nox2 and Nox4 have been implicated in both experimental and human hypertension and heart failure, yet their role(s) in cardiovascular aging are less understood (27, 28).

Given that studies utilizing the GPER-selective agonist G-1 have demonstrated that GPER activation conveys partial protection from vascular and myocardial disease (4, 13, 30) and given the central role of ROS in these chronic disease processes (24, 25, 27), we expected that pathologies characterized by increased bioavailability of ROS, particularly those associated with aging, would be exacerbated in the absence of functional GPER, resulting in even greater ROS production. We therefore set out to determine the functional and structural effects of genetic deletion (20) as well as pharmacologic inhibition (31, 32) of GPER on ROS-dependent pathologies affecting the cardiovascular system.

Results

GPER increases Nox-dependent vascular $\cdot\text{O}_2^-$ and vascular tone in aged arteries

We first examined the effects of *Gper* deletion on vascular oxidative stress by measuring the production of the unstable free radical superoxide ($\cdot\text{O}_2^-$) in the aorta of aged mice. To determine whether Nox enzymes are involved in the generation of $\cdot\text{O}_2^-$, we used a peptide termed gp91dstat (33), which is derived from a gp91^{phox} (now named Nox2) sequence in the region that interacts with the organizer protein p47^{phox}, thus disrupting p47^{phox} binding to and activation of associated catalytic Nox subunits, particularly Nox2 (34), but also Nox1 in

vascular smooth muscle cells (VSMCs) (29, 35-37). We found that in aged wild-type mice, ~50% of $\cdot\text{O}_2^-$ formation was Nox-dependent as it was blocked by gp91ds-tat (**Fig. 1A**, left panel). In contrast to our expectation of exacerbated $\cdot\text{O}_2^-$ production, $\cdot\text{O}_2^-$ formation in aged *Gper*^{-/-} mice was instead blunted by ~50-80% compared to wild-type mice (**Fig. 1, A and B**) and was unaffected by gp91ds-tat treatment (**Fig. 1A**), suggesting an inactive or absent Nox-mediated $\cdot\text{O}_2^-$ -producing pathway.

We next determined the effects of aging on vascular tone, which is characterized by increased $\cdot\text{O}_2^-$ formation that inactivates endothelium-derived vasodilatory NO (38). Impaired endothelium-dependent vasodilation represents an important predictor of mortality in patients with heart failure and hypertension (38-41). As expected, NO-mediated endothelium-dependent relaxation induced by acetylcholine (42) was reduced in aged (**Fig. 1C**) compared to young wild-type mice (**Fig. S1A**). This impairment was completely reversed by incubating arteries with gp91ds-tat, restoring vasodilation to an extent similar to that observed in young mice (**Fig. 1C** and **fig. S1A**). Further supporting the notion that impaired NO bioactivity was a result of oxidative stress in aged wild-type mice, the smooth muscle sensitivity to NO alone, generated by an exogenous NO donor, was not affected by aging (**Fig. S2**). In contrast, aged *Gper*^{-/-} mice were completely protected from the impairment in endothelium-dependent vasodilation observed in aged wild-type mice; in fact, the vasodilatory capacity was preserved and identical to that of young mice (**Fig. 1C**, **fig. S1A** and **fig. S2**).

In agreement with these observations, we found that in aged wild-type mice, vascular contractions in response to Ang II (a vasoactive peptide that stimulates Nox (43, 44)) were partially (~50%) blocked by gp91ds-tat (**Fig. 1D**), whereas gp91ds-tat had no effect on Ang II-mediated contractions of arteries from aged *Gper*^{-/-} mice. In line with the reduced $\cdot\text{O}_2^-$ formation in *Gper*^{-/-} mice (**Fig. 1, A and B**, and **fig. S1B**), contractions in response to Ang II were attenuated in aged (**Fig. 1D**) as well as in young *Gper*^{-/-} mice (**fig. S1C**). These findings, which contrast with the protective vascular role of *Gper* expression and/or GPER stimulation reported in previous studies (4, 12, 13, 30, 45), indicate instead that constitutive *Gper* expression is essential for increased vascular Nox bioactivity as well as Nox-mediated vasoconstriction and impaired endothelial cell function, particularly in the context of vascular aging.

GPER deletion prevents structural and functional cardiac aging and myocardial dysfunction

To determine whether *Gper*-dependent regulation of oxidative stress also plays a role in age-dependent structural and functional cardiac abnormalities, we next assessed $\cdot\text{O}_2^-$ production in the aging heart. Compared to wild-type mice, myocardial $\cdot\text{O}_2^-$ amounts were markedly lower in aged *Gper*^{-/-} mice (**Fig. 2A**). Given that oxidative stress is centrally involved in the structural changes that occur with cardiac aging (25, 26), we next examined myocardial histopathology. Whereas aging increased the left ventricular (LV) wall-to-lumen ratio by ~60% in wild-type mice, *Gper*^{-/-} mice were completely protected from age-dependent myocardial hypertrophy (**Fig. 2B** and **fig. S3A**). In addition, histological analyses of the myocardium of *Gper*^{-/-} mice revealed an absence of cardiomyocyte hypertrophy (**Fig. 2, C**

and **D**). Organ failure resulting from fibrosis accounts for at least one third of deaths worldwide (46), with myocardial fibrosis being a key feature of cardiac aging (25, 26). Aging in wild-type mice was associated with prominent and diffuse interstitial myocardial fibrosis and collagen IV accumulation, which again was generally absent in aged *Gper*^{-/-} mice (**Fig. 2, C, E and F**). The cardioprotective effects of *Gper* deletion on myocardial fibrosis and hypertrophy were already detectable at 12 months of age (although the differences were less prominent due to the reduced disease pathology in the wild-type mice), resulting in a lower LV wall-to-lumen ratio (**fig. S3A**), reduced cardiomyocyte hypertrophy (**fig. S3B**) and reduced myocardial fibrosis, as assessed by Sirius Red (**fig. S3C**) and collagen IV (**fig. S3D**) staining, although the reduction in the former did not reach significance at this age.

Given that *Gper* deletion prevented the structural cardiac abnormalities observed with aging, we next determined whether this translated into improved myocardial function in vivo. Echocardiography confirmed the marked increase in LV relative wall thickness and mass in wild-type mice compared to *Gper*^{-/-} mice (**Fig. 2G and 2H and table S1**). Consistent with the reduced ventricular fibrosis and stiffness, analysis of LV filling and diastolic mitral valve annulus velocities (47) revealed improved diastolic function and lower LV filling pressures in aged *Gper*^{-/-} mice (**Fig. 2I and table S1**). Together, the overall absence of myocardial fibrosis and hypertrophy in aged *Gper*^{-/-} mice translated into increased ventricular elasticity, as indicated by improved LV diastolic filling. These differences were independent of changes in systolic LV function or systemic hemodynamics (**Table S1**).

GPHER is essential for $\cdot\text{O}_2^-$ production in murine and human VSMCs through Nox1

Cardiac fibrosis involves an age-dependent localized activation of the renin-angiotensin system (RAS) (41, 46, 48), with its primary vasoactive peptide Ang II also promoting premature senescence through the induction of Nox (49, 50). Moreover, Ang II-induced ROS promote redox-sensitive cell functions such as intracellular calcium mobilization and contraction (51). Having established that *Gper* deficiency abrogates Nox-generated $\cdot\text{O}_2^-$ production in aged mice, we next examined the underlying mechanisms of the molecular regulation in vascular smooth muscle cells (VSMCs) isolated from wild-type and *Gper*^{-/-} mice (**fig. S4, A and B**). Consistent with the activation of Nox in intact arteries of wild-type mice (**Fig. 1**), Ang II-stimulated $\cdot\text{O}_2^-$ production in wild-type VSMCs was inhibited by gp91ds-tat (**Fig. 3A, left panel**). In cells lacking *Gper*, the Ang II-stimulating effect on $\cdot\text{O}_2^-$ generation was completely absent (**Fig. 3, A and B**), which was confirmed by electron paramagnetic resonance (EPR) spectroscopy using BMPO as a spin trap for $\cdot\text{O}_2^-$ (52-54) (**fig. S5**). Similarly, Ang II-induced, Nox-dependent mobilization of intracellular calcium (51) was absent in *Gper*-deficient VSMCs (**Fig. 3, C and D**). By contrast, intracellular calcium mobilization responses to the purinergic receptor agonist ATP (a Nox-independent stimulus (55)) were comparable in VSMCs from wild-type and *Gper*^{-/-} mice, thus excluding inherent defects in calcium signaling in VSMCs lacking *Gper* (**Fig. 3D**). In addition, absence of *Gper* did not affect the expression of the genes encoding the Ang II AT_{1A} and AT_{1B} receptors (**fig. S4C**).

We next sought to determine whether the effects of GPER on $\cdot\text{O}_2^-$ production observed in murine VSMCs extended to human VSMCs. Knockdown of GPER with siRNA abolished the ability of primary human VSMCs to generate $\cdot\text{O}_2^-$ in response to Ang II (**Fig. 4A**). To determine whether the effects of GPER were mediated through rapid non-genomic signaling alone or involved long-term genomic effects, we treated human VSMCs with the GPER-selective antagonist G36, a synthetic, small molecule GPER blocker (GRB) (32). Acute treatment (30 min) with gp91ds-tat, but not G36, abolished Ang II-stimulated $\cdot\text{O}_2^-$ production (**Fig. 4B**). In contrast, prolonged treatment with G36 (for 72 h) completely abrogated Ang II-induced $\cdot\text{O}_2^-$ formation (**Fig. 4B**), suggestive of mechanisms regulating gene transcription. Consistent with the lack of acute effects, G36 did not display direct antioxidant activity (**fig. S6**).

Given that Nox inhibition by gp91ds-tat reduced vascular $\cdot\text{O}_2^-$ production in mice as well as in murine and human VSMCs only in the presence of GPER, we next determined whether the vascular abundance of Nox1, Nox2 or Nox4 catalytic subunits, which have been implicated in both experimental and human hypertension (27-29), was affected by intrinsic GPER activity. Although gp91ds-tat is traditionally thought only to disrupt Nox2 activity (33), studies have demonstrated that gp91ds-tat also blocks $\cdot\text{O}_2^-$ -mediated effects mediated by Nox1 in VSMCs, likely through its interaction with p47^{phox} (56, 57). In fact, p47^{phox} in VSMCs facilitates activation of Nox1 (58), the closest homologue of Nox2, but not that of Nox4 (59), and also mediates Ang II-induced, redox-dependent signaling (37, 60). Feed-forward mechanisms have also been observed in which ROS production by one Nox subtype or other sources results in the activation of additional Nox subtype(s), suggesting that inhibition of any intermediate could block downstream events (61, 62). We found that in human VSMCs treated with G36 for 72 h, the protein abundance of Nox1 was reduced by ~70% compared to solvent, whereas that of Nox2 and Nox4 was unaffected (**Fig. 4C**). Similarly, only the protein abundance of Nox1, but not that of Nox2 or Nox4, was substantially lower in murine VSMCs from *Gper*^{-/-} mice as compared to wild-type mice (**Fig. 4D**). The reduced Nox1 protein abundance was commensurate with a similar reduction in the mRNA abundance in VSMCs isolated from *Gper*^{-/-} mice, with gene expression of *Nox2* and *Nox4* again being unaffected (**Fig. 4E**). *Gper* deficiency also reduced Nox1 mRNA abundance in the aorta and myocardium of aged *Gper*^{-/-} mice (**Fig. 4F**), both of which displayed markedly reduced $\cdot\text{O}_2^-$ bioactivity compared to wild-type mice (**Fig. 1** and **2**). To verify that the decreased Nox1 abundance indeed accounted for the inability of *Gper*-deficient VSMCs to generate $\cdot\text{O}_2^-$ in response to Ang II, we restored Nox1 abundance in these cells using a Nox1-expressing adenovirus. Reintroduction of Nox1 into *Gper*-deficient VSMC restored their capacity to generate $\cdot\text{O}_2^-$ in response to Ang II (**Fig. 4G**), further suggesting an obligatory role for GPER in the increase in Nox1 abundance and associated ROS-dependent cellular functions.

Genetic ablation or pharmacologic inhibition of GPER prevents arterial hypertension

To explore whether the protective effects of *Gper* deletion extended to cardiovascular disease conditions other than those associated with aging, we increased Nox1 abundance and activity in vivo by infusing mice with Ang II, a critical mediator of Nox1-dependent $\cdot\text{O}_2^-$ production, vascular dysfunction and increased vascular tone (44). Animals lacking *Gper*

were resistant to the Ang II-induced increase in blood pressure observed in wild-type mice (**Fig. 5A**). Furthermore, vascular $\cdot\text{O}_2^-$ generation and the increase in vascular Nox1 abundance in mice in response to Ang II infusion required the presence of *Gper* (**Fig. 5, B to D**). As previously reported in wild-type mice (44), $\cdot\text{O}_2^-$ generated in response to Ang II impaired endothelial cell function as evident from the blunted NO-dependent vasodilation in response to acetylcholine. By contrast, the attenuation of the vasodilator response was completely absent in *Gper*^{-/-} mice infused with Ang II (**Fig. 5E**). In line with $\cdot\text{O}_2^-$ -mediated impairment of NO bioactivity, the inherent vascular smooth muscle sensitivity to NO, as determined with an exogenous NO donor, was not affected by either Ang II infusion or by GPER deficiency or inhibition (**fig. S7**). These data further confirm that *Gper* is required to increase Nox1 abundance and the resulting $\cdot\text{O}_2^-$ production, vascular dysfunction and increases in vascular tone.

The Nox pathway has been recognized as a therapeutic target for ROS-dependent pathologies in humans (46, 63, 64). To determine in vivo whether decreasing Nox1 protein by pharmacological GPER inhibition can be achieved, we again utilized the GRB G36 (32). Not only did G36 treatment prevent the Ang II-mediated increase in Nox1 protein abundance (**Fig. 5D**), it also markedly reduced vascular $\cdot\text{O}_2^-$ production (**Fig. 5, B and C**) and restored the vasodilatory response (**Fig. 5E**). These effects of G36 resulted in a substantial inhibition of the Ang II-mediated increase in blood pressure (**Fig. 5A**). Given that GPER mediated increases in Nox1 protein abundance, these results identify GRBs as a member of a new class of drugs that act as Nox down-regulators.

Discussion

The results presented in the current study demonstrate that inhibiting GPER activity conveys protection from myocardial and vascular diseases associated with increased Nox1-derived oxidative stress, including cardiovascular aging and arterial hypertension. These data may seem counterintuitive at first when compared to the current body of evidence suggesting the protective role of GPER-selective agonists (GRAs) in the cardiovascular system (4). GRAs, such as G-1 (10), rapidly activate Nox-independent pathways thought to mediate salutary vascular effects, such as Akt and ERK (4). In particular, G-1, unlike the GRB G36, induces eNOS phosphorylation and the subsequent generation of NO, which mediates indirect antioxidant effects through inactivation of $\cdot\text{O}_2^-$ (12). Thus, both GRBs and GRAs improve disease outcome by reducing ROS activity through the same receptor, albeit through entirely distinct mechanisms. These findings place GPER at the center of the balance between the beneficial L-arginine-NO synthase pathway and harmful excessive ROS generation. Such dichotomous effects also exist for other hormones (such as insulin) with the ultimate (patho)physiological effect dependent upon the stage of the disease process (65).

Our results have identified new and important chronic functions of intrinsic GPER activity that determine Nox1 abundance. Indeed, regulation of gene expression through constitutive signaling is prevalent among G protein-coupled receptors (17). The GPER-dependent increase in Nox1 abundance and Nox1-dependent ROS formation was likely a key factor in the pathogenesis of increased vasoconstriction associated with hypertension or aging, as well as age-dependent cardiac remodeling. Indeed, arterial hypertension, left-ventricular

hypertrophy and the associated diastolic dysfunction in humans are important predictors of the development of heart failure (26), the prevalence of which increases with age and has reached epidemic proportions (66).

The present study has certain limitations. The results were obtained in experimental models of vascular and myocardial aging, heart failure and arterial hypertension. Investigating the association between oxidative excess and cardiovascular injury in humans is complicated because most of these patients are on ROS-inhibiting treatments (such as angiotensin converting enzyme inhibitors and statins), which makes it difficult to unmask mechanisms as described in the present study (67). Moreover, whether human Nox enzymes such as Nox5, which is not encoded in the rodent genome (27, 63), are involved in these disease processes requires further study. Given the results from the in vitro studies with primary vascular human cells, it remains to be shown whether G36 inhibits Nox1 activity in humans, which would also require monitoring the safety of GRB treatment. Because ROS are implicated in the progression of many chronic non-communicable diseases, GRBs may find application in a broader array of indications.

In summary, this study has identified an obligatory role for the intrinsic activity of GPER as an activator of *Nox1* expression that mediates structural and functional injury in vascular and myocardial diseases. Although the role of ROS in the aging process and chronic diseases is well recognized, simple scavenging of ROS with antioxidants has been largely unsuccessful therapeutically (68), likely due to the localized production and ensuing effects of ROS. Inhibition of Nox activity through the use of small molecules, some with limited selectivity (69), is currently being evaluated in clinical trials. The present study however introduces a new class of drugs, Nox down-regulators, which, as shown for G36, reduce Nox protein abundance, thus directly limiting $\cdot\text{O}_2^-$ production by one of its main sources. Therapeutically reducing the expression of *Nox* could provide an effective approach to target chronic disease conditions involving excessive Nox-mediated $\cdot\text{O}_2^-$ formation. The therapeutic application of GRBs may include prevention of organ injury due to Nox-dependent chronic diseases such as heart failure and arterial hypertension, while also treating or delaying pathologies associated with aging and rare diseases such as progeria syndromes (40).

Materials and Methods

Study Design

The aim of this study was to explore whether and through which mechanisms GPER promotes cardiovascular oxidative stress induced by aging or Ang II. For this purpose, we used wild-type and *Gper*-deficient mice as a model of aging, as well as mice chronically infused with Ang II. In addition, the GRB G36 was employed to test whether pharmacological targeting of GPER reduced oxidative stress. Detailed mechanistic studies were carried out in primary VSMCs isolated from wild-type and *Gper*^{-/-} mice, as well as in human VSMCs. Mice were randomly and equally assigned to different treatment groups. Animal studies were conducted in a controlled and non-blinded manner. Study designs included: (i) two-way factorial designs comparing *Gper*^{+/+} and *Gper*^{-/-} mice, in the presence or absence of inhibitors; (ii) similar two-way factorial designs with repeated measures

(concentrations, time); (iii) one-way factorial repeated measure designs within *Gper*^{+/+} cohort, controlling for baseline differences, over time and (iv) two-way factorial designs with repeated measures designs between the *Gper*^{+/+} and *Gper*^{-/-} groups, controlling for baseline differences, over time.

Transgenic mice and aging model

Male *Gper*^{-/-} mice (provided by Jan S. Rosenbaum, Proctor & Gamble Co.) were generated and backcrossed 10 generations onto the C57BL/6 background (Harlan Laboratories) as described (12). Wild-type C57BL/6 and *Gper*^{-/-} littermates were housed at the Animal Resource Facility of the University of New Mexico Health Sciences Center under controlled temperature of 22–23 °C on a 12 h light-dark cycle with unrestricted access to standard chow and water. Mice aged 24 months, which show functional and structural changes resembling human cardiovascular aging (25), were used as a model of aging. Animals were euthanized at the age of 4, 12 or 24 months by intraperitoneal injection of sodium pentobarbital (2.2 mg g⁻¹ body weight). 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.

Chronic Ang II infusion model

Micro-osmotic pumps (Alzet model 1002, Durect) were implanted subcutaneously in the midscapular region of wild-type and *Gper*^{-/-} mice under isoflurane (3%) anesthesia. Pumps continuously delivered PBS or Ang II (MP Biomedicals) at a rate of 0.7 mg kg⁻¹ per day for 14 days (43, 44). Three days prior to pump implantation, pellets continuously releasing the GRB G36 (32) (33 µg per day, Innovative Research of America) or placebo were implanted subcutaneously into the right hindlimb of a subset of wild-type mice.

Vascular smooth muscle cells

Primary aortic VSMCs from wild-type and *Gper*^{-/-} mice (*n* = 13 per genotype) were isolated and cultured as described (12). Human aortic VSMCs (Lonza) were cultured according to the provider's recommendations. Experiments were performed with cells derived from passages 2 to 5 for murine and 2 to 8 for human VSMCs. For functional assays, cells at sub-confluence were rendered quiescent by overnight serum starvation.

Measurement of superoxide (·O₂⁻) by lucigenin-enhanced chemiluminescence

Following euthanization, the aorta was immediately excised, carefully cleaned from perivascular adipose and connective tissue, opened longitudinally, and cut into segments of identical size (3 mm) in cold (4 °C) physiological saline solution (PSS, composition in mmol L⁻¹: 129.8 NaCl, 5.4 KCl, 0.83 MgSO₄, 0.43 NaH₂PO₄, 19 NaHCO₃, 1.8 CaCl₂, and 5.5 glucose; pH 7.4). Tissues were transferred and equilibrated in HEPES-buffered PSS (composition in mmol L⁻¹: 134 NaCl, 6 KCl, 1 MgCl, 10 HEPES, 2 CaCl₂, 0.026 EDTA, and 10 glucose; pH 7.4) in a humidified incubator at 37 °C for 60 min. In addition to intact isolated arteries, vascular smooth muscle cells (VSMCs) and a cell free ·O₂⁻ generating system by adding the substrate xanthine (100 µmol L⁻¹, Calbiochem) to xanthine oxidase (0.05 mU, Calbiochem) were employed. Chemiluminescence was measured in dark-adapted

HEPES-PSS containing 5 $\mu\text{mol L}^{-1}$ lucigenin (Enzo Life Sciences) at 37 °C (70). After equilibrating for 15 min, $\cdot\text{O}_2^-$ production was induced by Ang II (100 nmol L^{-1}) (70). Where indicated, tissues or cells were pretreated with the Nox-selective inhibitor gp91ds-tat (Anaspec, 3 $\mu\text{mol L}^{-1}$) (28, 33), the GRB G36 (32) (10 nmol L^{-1} , 100 nmol L^{-1} , or 1 $\mu\text{mol L}^{-1}$), the $\cdot\text{O}_2^-$ dismutase mimetic tempol (100 $\mu\text{mol L}^{-1}$, Tocris Bioscience) (71), or vehicle (DMSO 0.01%). Luminescence was measured 10-times in 20 sec intervals using a Synergy H1 multi-mode microplate reader (BioTek), and readings were averaged to reduce variability (70). A background reading was subtracted, and $\cdot\text{O}_2^-$ production normalized to surface area of vascular segments (72) or to VSMC number (70), respectively.

In-situ detection of $\cdot\text{O}_2^-$ by dihydroethidium (DHE)

The thoracic aorta was equilibrated in HEPES-PSS in a humidified incubator at 37 °C for 60 min, and treated with the Nox-selective inhibitor gp91ds-tat (3 $\mu\text{mol L}^{-1}$) (28, 33) for 30 min when indicated. Tissues were frozen in optimum cutting temperature (O.C.T.) compound (Sakura Finetek), cut on a cryostat into 10 μm thick sections, and stored on glass slides at -80 °C. For staining, sections were incubated with DHE (5 $\mu\text{mol L}^{-1}$, Invitrogen) in HEPES-PSS for 15 min at room temperature in the dark (73). In separate experiments, VSMCs were grown on poly-L lysine coated coverslips, which were incubated with DHE (5 $\mu\text{mol L}^{-1}$) in HEPES-PSS for 30 min at 37 °C in the dark (73). Where indicated, VSMCs were pretreated with Nox-selective inhibitor gp91ds-tat (3 $\mu\text{mol L}^{-1}$) (28, 33) for 30 min, and $\cdot\text{O}_2^-$ production was stimulated by Ang II (100 nmol L^{-1}) for 20 min prior to imaging. Slides with VSMCs or aortic sections were carefully washed, mounted in HEPES-PSS with coverslips, and immediately imaged by epifluorescence microscopy (Axiovert 200M, Zeiss) using a rhodamine filter with exposure intensity adjusted to background fluorescence (73). Signal intensity was quantified using ImageJTM software (National Institutes of Health).

$\cdot\text{O}_2^-$ detection by spin trapping combined with electron paramagnetic resonance (EPR) spectroscopy

The nitron 5-*tert*-butoxycarbonyl 5-methyl-1-pyrroline *N*-oxide (BMPO, Enzo Life Sciences) was used as the spin trap for $\cdot\text{O}_2^-$ generated from VSMC, which was monitored using EPR spectroscopy as described (52-54). Briefly, serum-starved VSMCs were suspended in serum-free medium supplemented with BMPO (50 mmol L^{-1}) and diethylenetriaminepentaacetic acid (100 $\mu\text{mol L}^{-1}$), and $\cdot\text{O}_2^-$ production was stimulated by Ang II (100 nmol L^{-1} , 30 min at 37°C). Supernatant containing spin-trapped $\cdot\text{O}_2^-$ was snap-frozen in liquid nitrogen and stored at -80 °C for less than one week. After thawing, supernatant was immediately transferred to custom-made gas-permeable Teflon tubing (Zeus Industries), folded four times, and inserted into a quartz EPR tube open at each end. The quartz EPR tube was inserted into the cavity of an EPR spectrometer (EleXsys 540 X-band, Bruker) operating at 9.8 GHz and 100-kHz field modulation, and the spectra of BMPO-OOH, spin-trapped $\cdot\text{O}_2^-$, was recorded after spectrometer tuning at room temperature. The EPR spectrum was acquired with a scan time of 40 s, and 20 scans were obtained and averaged to produce significant signal-to-noise ratio. Instrument settings were as follows: magnetic field, 3,509 G; scan range, 70 G; microwave power, 21 mW; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; time constant, 20 ms. The EPR spectra were collected, stored, and manipulated using Xepr software (Bruker).

Vascular reactivity studies

The aorta was immediately excised after euthanization, carefully cleaned from perivascular adipose and connective tissue and cut into 2 mm long rings in cold (4 °C) PSS. Aortic rings were mounted in myograph chambers (multi-channel myograph system 620M, Danish Myo Technology) onto 200 µm pins (18, 74). A PowerLab 8/35 data acquisition system and LabChart Pro software (AD Instruments) were used for recording of isometric tension. Experiments to determine vascular reactivity of aortic rings were performed as described (18, 74). Briefly, rings were equilibrated in PSS (37 °C; pH 7.4; oxygenated with 21% O₂, 5% CO₂, and balanced N₂) for 30 min and stretched step-wise to the optimal amount of passive tension for force generation. Functional integrity of vascular smooth muscle was confirmed by repeated exposure to KCl (PSS with substitution of 60 mmol/L potassium for sodium), with resulting contractions demonstrating no differences between groups. Selected arteries were pretreated with the Nox-selective inhibitor gp91ds-tat (3 µmol L⁻¹) (28, 33) for 30 min. Contractions to Ang II (100 nmol L⁻¹) were studied in the abdominal aorta in the presence of the NO synthase inhibitor L-N^G-nitroarginine methyl ester (L-NAME, 300 µmol L⁻¹, incubation for 30 min, Cayman Chemical) (75) to exclude Ang II-mediated release of NO (76). Ang II-induced contractions exhibit rapid desensitization in the mouse vasculature with a nearly complete loss of tension after about 2 min, thus preventing the recording of responses to increasing concentrations (75, 77). To study endothelium-dependent, NO-mediated relaxations, rings from the thoracic aorta were precontracted with phenylephrine (Sigma-Aldrich) to 80% of KCl-induced contractions, and responses to acetylcholine (0.1 nmol L⁻¹ – 10 µmol L⁻¹, Sigma-Aldrich) were recorded. Similarly, endothelium-independent, NO-mediated relaxations to sodium nitroprusside (SNP, 1 – 10 µmol L⁻¹, MP Biomedicals) were determined. Precontraction did not differ between groups. To exclude any GPER-dependent effects on vasoconstrictor prostanoids (18), responses were obtained in the presence of the cyclooxygenase-inhibitor meclofenamate (1 µmol L⁻¹, incubation for 30 min, Cayman Chemical). Contractions were calculated as the percentage of contraction to KCl, and relaxation was expressed as the percentage of phenylephrine-induced precontraction.

Cardiac histopathology

After sacrifice, hearts were excised, fixed in 4% paraformaldehyde and embedded into paraffin blocks. Histological sections (2 µm) were stained with hematoxylin-eosin, Sirius Red or by immunohistochemistry using a polyclonal goat antibody recognizing collagen IV (Southern Biotech) as described (78). Morphometric analysis of free left ventricle wall thickness and ventricular lumen area was performed using light microscopy at 400-fold magnification and cellSensTM software (Olympus), with left ventricular wall thickness based on analysis of 10 randomly selected measure points. Cardiomyocyte cross-sectional area was determined by analysis of 15 anterolaterally located cardiomyocytes using cellSens software. Myocardial fibrosis on Sirius Red or collagen type IV stained paraffin sections was graded using a semi-quantitative fibrosis score (0 = no staining; 1 = less than 25%; 2 = 26–50 %; 3 = 51–75%; 4 = more than 75% of cardiac tissue with positive staining). For each heart, the mean score evaluated on 10 power fields at 200-fold magnification was calculated.

High-resolution, high-frequency echocardiography

Mice were lightly sedated using inhaled isoflurane anesthesia, placed on a heat-pad to maintain body temperature, and echocardiography was performed using a Vevo® LAZR photoacoustic imaging system (VisualSonics) using high-resolution, high-frequency ultrasound at 40 MHz (47, 79). Conventional B-mode, M-mode, pulsed wave- and tissue-doppler images were acquired by an experienced, blinded operator to ensure a standardized, consistent technique, and LV dimensions were quantified as described (47, 79). LV ejection fraction was determined by speckle-tracking based wall motion analysis (47, 79, 80) using VevoStrain software (VisualSonics). Analysis of diastolic function included transmitral flow velocity waveforms obtained from pulsed-wave Doppler to calculate the ratio of early (E)-to-late (atrial, A) LV filling velocities (E/A ratio), and the mitral annulus diastolic velocity (e' waves) obtained from pulsed-wave tissue Doppler imaging as well as the calculated E/e' ratio as measures of diastolic function and LV filling pressures.

Measurement of arterial blood pressure

Systolic blood pressure was measured in conscious mice using a volume-pressure recording noninvasive monitoring system (CODA-6, Kent Scientific) as described (12), which produces blood pressure readings with similar sensitivity and specificity as invasive measurements (12). This blood pressure measurement technique has successfully been applied in the chronic Ang II infusion model (43, 44).

Intracellular calcium mobilization

VSMCs were loaded with 5 $\mu\text{mol L}^{-1}$ indo1-AM (Invitrogen) and 0.05% pluronic F-127 (Invitrogen) in Hanks' buffered salt solution (HBSS) supplemented with NaCl (150 mmol L^{-1}), CaCl_2 (2 mmol L^{-1}), and HEPES (20 mmol L^{-1} ; pH 7.4) for 30 min at room temperature in the dark. Cells were washed and resuspended in HBSS (10^6 cells per mL), and calcium mobilization in response to Ang II (100 nmol L^{-1}) and adenosine triphosphate (ATP, 1 $\mu\text{mol L}^{-1}$, Sigma-Aldrich) was determined ratiometrically using λ_{ex} 340 nm and λ_{em} of 405 and 490 nm at 37 °C in a QM-2000-2 spectrofluorometer (Photon Technology International).

VSMC transduction with Nox1 adenovirus or transfection with GPER-targeted siRNA

VSMCs from *Gper*^{-/-} mice were plated at ~600,000 cells per T25 flask, washed in PBS, and infected with Nox1GFP and GFP control adenovirus constructs (59) at 400 MOI overnight in low serum (1% FBS) DMEM. Cells were allowed to recover for 48 h prior to experiments. Transduction efficiency was determined by GFP expression. For siRNA, human aortic VSMCs were transfected with siGPER (Dharmacon ON-Targetplus J-005563-08) or control (siCTL) siRNA (Dharmacon ON-Targetplus D-001810-02) using Lipofectamine 2000 (Invitrogen) for 6–8 h in serum-free medium, washed, and returned to normal medium as described by the manufacturer. Subsequent experiments were performed 72 h after transfection.

Quantification of gene and protein expression

RNA was extracted, reverse-transcribed and analyzed using SYBR Green-based detection of amplified gene-specific cDNA fragments by qPCR performed in triplicate as described (18). The following primer pairs have been used: 5'-CAT CCA GTC TCC AAA CAT GAC A-3' (forward) and 5'-GCT ACA GTG GCA ATC ACT CCA G-3' (reverse) for amplification of a specific cDNA fragment encoding mouse Nox1 (GenBank ID: NM_172203.1); 5'-ACT CCT TGG GTC AGC ACT GG-3' (forward) and 5'-GTT CCT GTC CAG TTG TCT TCG-3' (reverse) for amplification of a specific cDNA fragment encoding mouse Nox2 (GenBank ID: NM_007807.4); 5'-TGA ACT ACA GTG AAG ATT TCC TTG AAC-3' (forward) and 5'-GAC ACC CGT CAG ACC AGG AA-3' (reverse) for amplification of a specific cDNA fragment encoding mouse Nox4 (GenBank ID: NM_015760.4); 5'-GCG GTC TCC TTT TGA TTT CC-3' (forward) and 5'-CAA AGG GCT CCT GAA ACT TG-3' (reverse) for amplification of a specific cDNA fragment encoding mouse AT_{1A} receptor (GenBank ID: NM_177322.3); 5'-TAT TTT CCC CAG AGC AAA GC-3' (forward) and 5'-TGT TGC TTC CTT GTC CCT TG-3' (reverse) for amplification of a specific cDNA fragment encoding mouse AT_{1B} receptor (GenBank ID: NM_175086.3); and 5'-TTC ACC ACC ATG GAG AAG GC-3' (forward) and 5'-GGC ATG GAC TGT GGT CAT GA-3' (reverse) for amplification of a specific cDNA fragment encoding mouse GAPDH (GenBank ID: NM_008084.2), which served as the housekeeping control.

For determination of protein abundance by Western blot, VSMCs were lysed in NP-40 buffer supplemented with protease inhibitor (1 µg/mL), 10% SDS, 0.5% sodium fluoride, and 0.5% sodium orthovanadate. 20 or 40 µg of lysate were loaded on 10% SDS-PAGE gel (Thermo Scientific), blotted onto polyvinylidene fluoride membrane (Millipore), and blocked with 3% newborn calf serum in Tris-buffered saline with Tween-20 (0.1%). Blots were incubated overnight at 4 °C with primary antibodies recognizing Nox1 (Sigma-Aldrich), Nox2 (Boster), or Nox4 (Boster), washed, incubated with secondary HRP-conjugated antibodies (1:5000) for 1 h at room temperature, and developed with Super Signal West Pico Chemiluminescent substrate (Thermo Scientific). Blots performed in duplicate were imaged and quantified using ImageJ densitometry analysis software.

Immunofluorescence of Nox1 and GPER

Aortic sections frozen in O.C.T. compound were fixed in 4% paraformaldehyde, blocked and permeabilized in PBS containing normal goat serum (3%) and TritonX-100 (0.01%, EM Science). Sections were incubated with rabbit antibody recognizing murine Nox1 (1:100, Sigma-Aldrich) or negative control IgG (1:100, Sigma-Aldrich) overnight at 4 °C, washed, incubated with goat antibody recognizing rabbit IgG conjugated to Alexa Fluor 488 (Invitrogen) for one hour, washed, mounted in Vectashield (Vector Laboratories), and imaged utilizing a Leica SP5 confocal microscope. Signal intensity was quantified using ImageJ software. VSMCs were stained with a rabbit antiserum recognizing murine GPER as described (12).

Statistical analysis

Statistical analysis for in vitro and in vivo experiments was performed using GraphPad Prism™ version 5.0 for Macintosh (GraphPad Software). When comparing two groups, the

two-tailed, unpaired Student's *t*-test was performed. When comparing multiple groups, data were analyzed by two-way analysis of variance (ANOVA), with repeated measures as appropriate, followed by Bonferroni's post-hoc test to correct for multiple comparisons. Values are expressed as mean±sem; *n* equals the number of independent animals or cell preparations used. Statistical significance was accepted at a *P* value < 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Genetic deletion of *Gper* abrogates Nox activity and prevents enhanced vasoconstriction in vascular aging. Intact arteries of aged (24 month-old) wild-type (*Gper*^{+/+}) and *Gper*^{-/-} mice were analyzed. (A), (B) Nox activity was determined by measuring vascular superoxide ($\cdot\text{O}_2^-$) production using chemiluminescence (A) or DHE fluorescence (B, scale bar, 200 μm). To quantify the amount of $\cdot\text{O}_2^-$ generated by Nox, subsets of arteries were treated with the Nox inhibitor gp91ds-tat (tat). (C), (D) Endothelium-dependent, NO-mediated vasodilation in response to acetylcholine (C) and contractions to angiotensin II (Ang II, D) in intact arteries in the presence or absence of gp91ds-tat (tat). Data are mean $\pm$ sem; $n = 3$ –4 mice per group in (A), $n = 5$ –10 mice per group in (B), $n = 4$ –5 mice per group in (C), (D). * $P < 0.05$, ** $P < 0.01$ compared to control (CTL); † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ compared to wild-type mice (ANOVA with Bonferroni post-hoc tests in (A), (D); repeated measures ANOVA with Bonferroni post-hoc tests in (C); Student's *t*-test in (B)).

Fig. 2.

Obligatory role for GPER in aging cardiomyopathy and diastolic dysfunction. Hearts of aged (24 month-old) wild-type and *Gper*^{-/-} mice were analyzed. **(A)** Myocardial superoxide ($\cdot\text{O}_2^-$) production determined by DHE fluorescence. Scale bar, 500 μm . **(B)** Myocardial hypertrophy as measured by left ventricular wall-to-lumen ratio in cardiac cross sections. Scale bar, 2 mm. **(C)** Representative histologic myocardial sections stained with hematoxylin-eosin (left, scale bar, 100 μm) and Sirius Red (right, scale bar, 200 μm). Cardiomyocyte hypertrophy analyzed by cross-sectional area **(D)**, quantitation of interstitial fibrosis **(E)** and representative histologic sections stained for type IV collagen **(F)**, scale bar, 100 μm . **(G-I)** Dimensions and function of the left ventricle (LV) determined by echocardiography. Representative parasternal M-mode images **(G)**, calculated LV mass **(H)** and measures of LV filling pressures and diastolic dysfunction (E/e' ratio, **I**) are shown. Data are mean $\pm$ sem; $n = 4-5$ mice per group in **(A)**, $n = 6-7$ mice per group in **(B)**, $n = 6-8$ mice per group in **(C-F)**, $n = 3$ mice per group in **(G-I)**. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to wild-type mice (Student's *t*-test).

Fig. 3.

GPER is required for Ang II-induced superoxide (O_2^-) production and mobilization of intracellular calcium in VSMCs. Nox activity stimulated by Ang II was inhibited using gp91dstat (tat). (A), (B) Ang II-induced O_2^- production detected by DHE fluorescence (A) and chemiluminescence (B). (C), (D) Mobilization of intracellular calcium ($[\text{Ca}^{2+}]_i$) determined utilizing the Ca^{2+} sensor dye indo1-AM in response to Ang II (representative tracing in C, cumulative data in D) and ATP (D). F_{405}/F_{490} , ratiometric detection of emitted light at 405 nm and 490 nm. $**P < 0.01$, $***P < 0.001$ compared to control (CTL); $\dagger P < 0.05$, $\dagger\dagger P < 0.01$, $\dagger\dagger\dagger P < 0.001$ compared to VSMCs isolated from wild-type (*Gper*^{+/+}) mice. Data are mean $\pm$ sem; $n = 5-7$ independent experiments per group in (A), $n = 5$ independent experiment per group in (B), 3-5 independent experiments per group in (D), all with VSMCs from 2 independent isolations. ANOVA with Bonferroni post-hoc tests in (A), (D); Student's *t*-test in (B).

Fig. 4.

GPER increases Nox1 abundance and activity. **(A)** Ang II-induced superoxide ($\cdot O_2^-$) production detected by chemiluminescence in human VSMCs with GPER-targeted gene silencing (siGPER). * $P < 0.05$ compared to control siRNA (siCTL). siGPER treatment decreased GPER protein abundance by 89 $\pm$ 5% compared to siCTL treatment (representative data shown in inset). **(B), (C)** Ang II-induced $\cdot O_2^-$ production (detected by chemiluminescence, **B**) and protein abundance of Nox1, Nox2 and Nox4 (**C**) in human VSMCs treated with the GPER-selective antagonist G36 (1 μ mol L⁻¹) for 30 min (acute, **B**) or 72 h (chronic, **B, C**). Nox activity was inhibited using gp91ds-tat (tat). * $P < 0.05$, ** $P < 0.01$ compared to control (CTL, DMSO 0.01%); † $P < 0.05$ compared to acute treatment. **(D), (E)** Protein (**D**) and mRNA (**E**) abundance of *Nox1*, *Nox2* and *Nox4* in VSMCs isolated from wild-type (*Gper*^{+/+}) and *Gper*^{-/-} mice. ** $P < 0.01$ compared to wild-type mice. **(F)** *Nox1* mRNA abundance in aorta and myocardium of aged (24 month-old) *Gper*^{+/+} and *Gper*^{-/-} mice. * $P < 0.05$ compared to *Gper*^{+/+}. **(G)** Ang II-induced $\cdot O_2^-$ production detected by chemiluminescence in VSMCs isolated from *Gper*^{-/-} mice and infected with *Nox1*-expressing adenovirus (AdNox1). * $P < 0.05$ compared to vector control (AdGFP). Data are mean $\pm$ sem; $n = 3$ independent transfections per group in **(A)**, $n = 4$ –8 independent experiments per group in **(B)**, $n = 3$ –4 independent experiments and Western blots per group in **(C)** and **(D)**, $n = 3$ –4 VSMC preparations in **(E)**, $n = 4$ –10 mice per group in **(F)**, $n = 3$ independent infections per group in **(G)**. ANOVA with Bonferroni post-hoc tests in **(B)**; Student's *t*-test in **(A)**, **(C)**–**(G)**.

Fig. 5.

Genetic or pharmacologic ablation of GPER prevents Ang II-induced hypertension, oxidative stress, and increases in Nox1 abundance. Wild-type (*Gper*^{+/+}) and *Gper*^{-/-} mice were infused with Ang II (0.7 mg kg⁻¹ per day) or vehicle (control, CTL) for 14 days. A subset of wild-type mice was also treated with the GRB G36. **(A)** Systolic arterial blood pressure in conscious animals. **(B)**, **(C)** Vascular $\cdot\text{O}_2^-$ generation as measured by chemiluminescence **(B)** and DHE staining **(C)**, scale bars, 300 μm . **(D)** Vascular Nox1 protein abundance detected by immunofluorescence. **(E)** Endothelium-dependent, NO-mediated vasodilation in response to acetylcholine. Data are mean $\pm$ sem; $n = 4-5$ mice in **(A)**, $n = 3-5$ mice in **(B)**, $n = 3-4$ mice in **(C)**, $n = 3$ mice in **(D)**, $n = 5-9$ mice in **(E)**. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to genotype-matched CTL; † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ compared to Ang II-treated wild-type mice (repeated measures ANOVA with Bonferroni post-hoc tests in **(A)**, **(E)**; ANOVA with Bonferroni post-hoc tests in **(B)**, **(D)**). PE, phenylephrine.