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GPER Mediates Functional Endothelial Aging in Renal Arteries

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Acetylcholine · Angiotensin · Endothelium · GPR30 · NADPH oxidase · Nitric oxide · Phenylephrine · Vascular

Abstract
Aging is associated with impaired renal artery function, which is partly characterized by arterial stiffening and a reduced vasodilatory capacity due to excessive generation of reactive oxygen species by NADPH oxidases (Nox). The abundance and activity of Nox depends on basal activity of the heptahelical transmembrane receptor GPER; however, whether GPER contributes to age-dependent functional changes in renal arteries is unknown. This study investigated the effect of aging and Nox activity on renal artery tone in wild-type and GPER-deficient (*Gper*$^{-/-}$) mice (4 and 24 months old). In wild-type mice, aging markedly impaired endothelium-dependent, nitric oxide (NO)-mediated relaxations to acetylcholine, which were largely preserved in renal arteries of aged *Gper*$^{-/-}$ mice. The Nox inhibitor gp91ds-tat abolished this difference by greatly enhancing relaxations in wild-type mice, while having no effect in *Gper*$^{-/-}$ mice. Contractions to angiotensin II and phenylephrine in wild-type mice were partly sensitive to gp91ds-tat but unaffected by aging. Again, deletion of GPER abolished effects of Nox inhibition on contractile responses. In conclusion, basal activity of GPER is required for the age-dependent impairment of endothelium-dependent, NO-mediated relaxation in the renal artery. Restoration of relaxation by a Nox inhibitor in aged wild-type but not *Gper*$^{-/-}$ mice strongly supports a role for Nox-derived reactive oxygen species as the underlying cause. Pharmacological blockers of GPER signaling may thus be suitable to inhibit functional endothelial aging of renal arteries by reducing Nox-derived oxidative stress and, possibly, the associated age-dependent deterioration of kidney function.

Introduction

Chronic kidney disease affects more than half of adults older than 70 years of age, and is strongly associated with an increased risk of cardiovascular disease and mortality [1]. Kidney function critically depends on adequate perfusion, which is provided by the renal artery. Advanced

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age is an independent risk factor for the development of both vascular and kidney disease [2], indicating a strong relationship between arterial and renal pathophysiology [3, 4]. In fact, age-dependent stiffening of renal arteries due to vascular calcification, fibrosis, inflammation, and a reduced vasodilatory capacity is centrally involved in detrimental renal changes, such as the development of glomerulosclerosis and tubulointerstitial fibrosis [3–5].

A common mediator of age-dependent structural and functional organ damage is excessive production of reactive oxygen species (ROS), commonly referred to as "oxidative stress" [6]. Among several cellular sources, ROS are mainly generated by NADPH oxidase (Nox) enzymes, of which 7 mammalian isoforms have been identified [7]. Nox-dependent regulation of redox-sensitive signaling pathways is particularly important in vascular and renal pathophysiology mainly involving Nox1, Nox2, and Nox4 [7, 8]. In arteries, ROS reduce the bioavailability of endothelium-derived nitric oxide (NO) with subsequent loss of its vasodilatory, anti-inflammatory, and antithrombotic properties [9]. At the same time, oxidative stress promotes arterial constriction through activators of G protein-coupled receptors (GPCRs), such as angiotensin and α-adrenergic receptors [2, 7]. Furthermore, ROS-induced alterations in cellular signaling pathways have been implicated in arterial remodeling and hypertrophy, as well as glomerulosclerosis, tubulointerstitial fibrosis, and proteinuria [7, 8]. Thus, therapeutic approaches interfering with increased ROS formation will likely reduce the progression both vascular and renal diseases associated with aging [7, 8, 10].

We have recently identified an unexpected role of a basally active GPCR termed GPER, a heptahelical transmembrane receptor that also signals via estrogen-dependent mechanisms [11], which is required for Nox1 expression and activity in the aorta and hearts of aged mice, as well as in human aortic smooth muscle cells [12]. Ligand-independent GPER activity was also found to mediate Nox1-dependent impaired vascular function in arterial hypertension [12]; however, whether GPER contributes to age-dependent functional changes of renal arteries is unknown.

We hypothesized that basal GPER activity contributes to the deterioration of renal artery function with age involving Nox-dependent mechanisms. To this end, we studied the effects of GPER deletion and Nox activity on endothelium-dependent, NO-mediated vasodilation and vasoconstrictor responses mediated by angiotensin AT_1 and α-adrenergic receptors in the renal artery from young and aged male mice.

Methods

Transgenic Mice and Aging Model

Gper$^{-/-}$ mice were generated and backcrossed as described [12]. Male wild-type C57BL/6 and *Gper*$^{-/-}$ mice were housed at the Animal Resource Facility of the University of New Mexico Health Sciences Center under controlled temperature (22°C) on a 12-h light-dark cycle with unrestricted access to water and a rodent diet devoid of alfalfa or soybean meal to minimize the presence of natural phytoestrogens (Teklad 2020SX, Harlan Laboratories, Madison, WI, USA). Animals were killed by intraperitoneal injection of sodium pentobarbital (2.2 mg/g body weight) at 4 and 24 months of age, because age-dependent functional and structural changes in 24-month-old mice closely resemble human vascular aging [13]. All procedures were approved by and carried out in accordance with institutional policies and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Preparation of Renal Arteries

Immediately after sacrifice, renal arteries were carefully excised and dissected free from adherent connective tissue and fat in cold (4°C) physiological saline solution (PSS; composition in mmol/L: 129.8 NaCl, 5.4 KCl, 0.83 $MgSO_4$, 0.43 NaH_2PO_4, 19 $NaHCO_3$, 1.8 $CaCl_2$, and 5.5 glucose; pH 7.4). Using two 25 μm tungsten wires, vessels were mounted in organ chambers of a Mulvany-Halpern myograph (620 M Multi-Channel Myograph System, Danish Myo Technology, Aarhus, Denmark) containing PSS (37°C, pH 7.4, bubbled with 21% O_2, 5% CO_2, and balanced N_2).

Vascular Reactivity Studies

Recordings of isometric tension were performed as described [14] using a PowerLab 8/35 data acquisition system and LabChart Pro software (AD Instruments, Colorado Springs, CO, USA). Briefly, arteries were stretched stepwise to the optimal level of passive tension for force generation, and the functional integrity of vascular smooth muscle was confirmed by repeated exposure to KCl (PSS with substitution of 60 mmol/L potassium for sodium). In subsequent experiments, the role of NADPH oxidase was studied by randomly treating the left or right renal artery with the Nox1/2-selective inhibitor gp91ds-tat (3 μmol/L for 30 min) [15]. This peptide is derived from a $gp91^{phox}$ (now termed Nox2) sequence in the region that interacts with the organizer protein $p47^{phox}$, thus disrupting $p47^{phox}$ binding to and activation of the homologous Nox2 and Nox1 catalytic subunits [7, 12]. To study endothelium-dependent, NO-mediated relaxations, arteries were precontracted with phenylephrine to ~70% of KCl-induced contractions, and responses to acetylcholine (0.1 nmol/L–10 μmol/L) were recorded. Precontraction did not differ between groups. Similarly, responses to the NO donor sodium nitroprusside (10 μmol/L) were determined. Contractions to agonists of angiotensin AT_1 and α-adrenergic receptors, angiotensin II (100 nmol/L), and phenylephrine (0.3 μmol/L), respectively, were studied in separate experiments.

Calculations and Statistical Analyses

Relaxation is expressed as the percentage of precontraction, and contraction is given as the percentage of contraction compared to KCl. EC_{50} values (given as negative logarithm: pD_2), area under the curve (given as arbitrary units), and maximal responses (E_{max}) were calculated by non-linear regression analysis [16]. Data

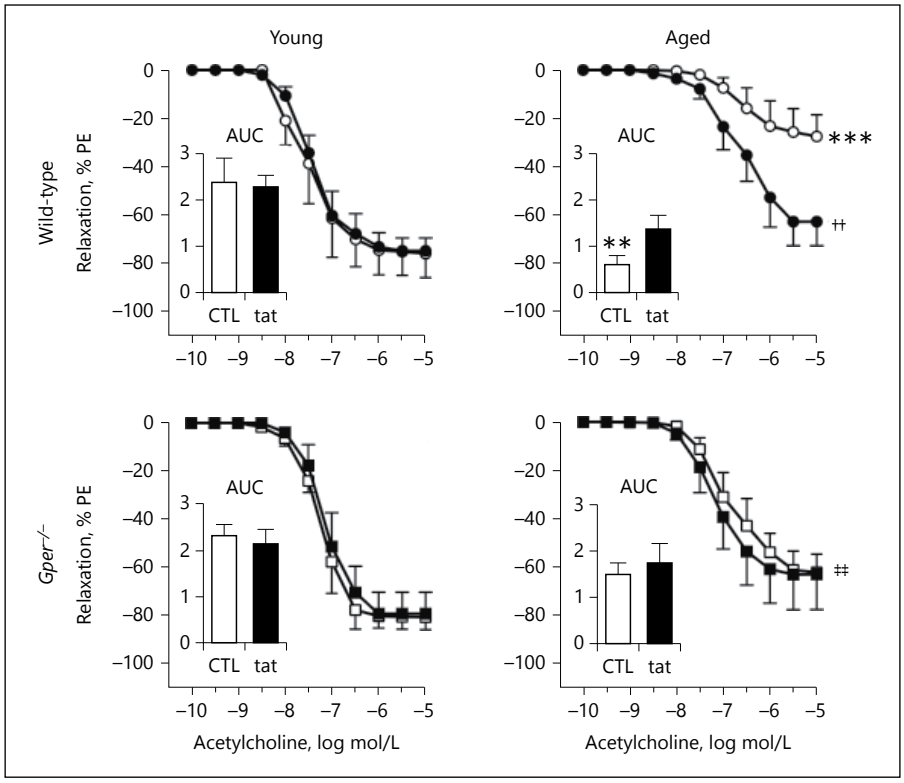

Fig. 1. Effect of aging and GPER on endothelium-dependent relaxation to acetylcholine in renal arteries. The role of Nox-derived oxidative stress was assessed using the Nox1/2-inhibitor gp91ds-tat (tat, 3 μmol/L) in wild-type and *Gper*$^{-/-}$ mice at 4 (young) or 24 (aged) months of age. Responses are expressed as percent of precontraction to phenylephrine (PE). Insets: area under the curve (AUC) of the concentration-response curves given as arbitrary units. Data (n = 4–5 per group) are means ± SEM. ** $p < 0.01$, *** $p < 0.001$ vs. young; †† $p < 0.01$ vs. untreated control (CTL); ‡‡ $p < 0.01$ vs. wild-type.

were analyzed by 1-way or 2-way analysis of variance, as appropriate, followed by the Bonferroni post hoc test to correct for multiple comparisons (GraphPad Prism version 5.0 for Macintosh, GraphPad Software, San Diego, CA, USA). Values are expressed as means ± SEM; n equals the number of animals per group. Statistical significance was accepted at a p value <0.05.

Drugs

Gp91ds-tat was from AnaSpec (Fremont, CA, USA), and sodium nitroprusside was from MP Biomedicals (Solon, OH, USA). All other drugs were from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions were prepared according to the manufacturer's instructions, and diluted in PSS to the required concentrations before use.

Results

GPER Deletion Preserves Renal Artery NO Bioactivity with Aging

In renal arteries of young wild-type and *Gper*$^{-/-}$ mice, endothelium-dependent, NO-mediated relaxation induced by acetylcholine was similar and unaffected by the Nox1/2 inhibitor gp91ds-tat (Fig. 1). By contrast, in wild-type mice, aging markedly reduced endothelium-dependent relaxation (E_{max}, –27 ± 9 vs. –75 ± 10%, n = 4–5, $p <$ 0.001 vs. young; Fig. 1), also indicated by reduced sensitivity (pD_2, 6.4 ± 0.2 vs. 7.4 ± 0.2, n = 4–5, $p < 0.01$ vs. young) and area under the curve values (0.4 ± 0.2 vs. 1.9 ± 0.4, n = 4–5, $p < 0.01$ vs. young). This age-dependent deterioration in NO bioactivity was largely absent in mice lacking *Gper* (E_{max}, –61 ± 8 vs. –27 ± 9%, n = 4–5, $p < 0.01$ vs. wild-type; Fig. 1). As a result, vasodilatory responses were 2.3-fold greater in aged *Gper*$^{-/-}$ mice. The Nox1/2 inhibitor gp91ds-tat abolished this difference by greatly enhancing relaxation in aged wild-type mice (2.3-fold, from –27 ± 9 to –63 ± 11%, n = 5, $p < 0.01$), to the level observed in *Gper*$^{-/-}$ mice (–61 ± 8%). Furthermore, gp91ds-tat had no effect in *Gper*$^{-/-}$ mice (Fig. 1). Vasodilation in response to the exogenous NO donor sodium nitroprusside was unaffected by aging or *Gper* deletion, indicating that the differences observed in acetylcholine-mediated relaxation are not due to alterations in the sensitivity to NO (not shown). Thus, aging impairs endothelium-dependent, NO-mediated relaxation in renal arteries largely through increased Nox activity, an effect that is absent in mice lacking *Gper*.

GPER Mediates Nox-Dependent Signaling to GPCR Agonists

Given that *Gper* mediates age-dependent, Nox-mediated changes in endothelium-dependent relaxation of

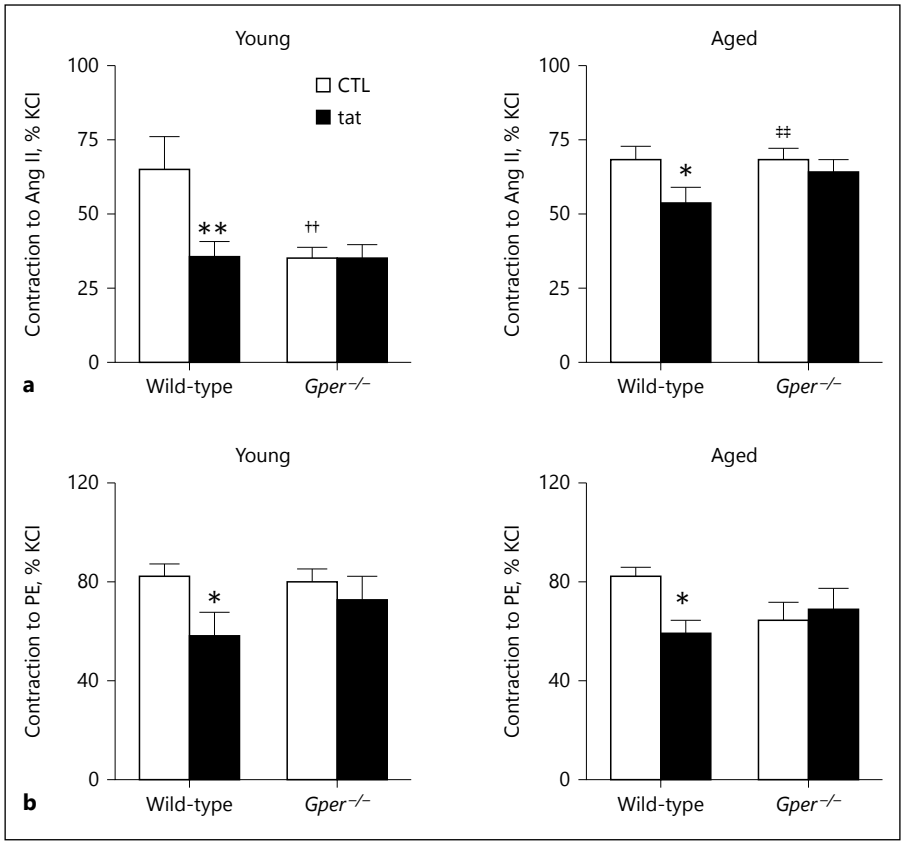

Fig. 2. Effect of aging and GPER on Nox-mediated contractions to GPCR agonists in renal arteries. Responses were recorded in the presence and absence of the Nox1/2-inhibitor gp91ds-tat (3 μmol/L) in young (4 months old) and aged (24 months old) wild-type and *Gper*$^{-/-}$ mice. **a** Contractions to the angiotensin AT_1 receptor agonist angiotensin II (Ang II, 100 nmol/L). **b** Contractions to the α-adrenergic agonist phenylephrine (PE, 0.3 μmol/L). Responses are expressed as percent of contraction to KCl (60 mmol/L). Data (n = 5–7 per group) are means ± SEM. * $p < 0.05$, ** $p < 0.01$ vs. untreated control (CTL); †† $p < 0.01$ vs. wild-type; ‡‡ $p < 0.01$ vs. young.

renal arteries, we next studied whether similar alterations also exist in contractile responses induced by agonists of other GPCRs. In wild-type mice, contractions to angiotensin II were unaffected by aging (Fig. 2a); the Nox1/2 inhibitor gp91ds-tat reduced contractions to angiotensin II in young (1.9-fold) and aged (1.3-fold) animals (both n = 5–7, $p < 0.05$; Fig. 2a). Deletion of *Gper* reduced contractions to Ang II in young mice only (~50% reduction, n = 5–7, $p < 0.01$; Fig. 2a). Notably, in the absence of *Gper*, Nox1/2 inhibition had no effect on angiotensin II-induced contractions regardless of age (Fig. 2a).

Aging did not affect contractions to the α-adrenergic agonist phenylephrine independent of genotype. However, responses were partially blocked by gp91ds-tat in young and aged wild-type (both ~30%, n = 4–7, $p < 0.05$), but not in *Gper*$^{-/-}$ mice (Fig. 2b). Together, given that the Nox1/2 inhibitor gp91ds-tat is ineffective in mice lacking the *Gper* gene, these findings indicate that basal *Gper* activity is required for Nox-mediated contractions to GPCR agonists in addition to the age-dependent impaired NO bioactivity in renal arteries.

Discussion

In the present study, we have sought to characterize functional changes in the renal artery during the physiological aging process and to assess whether and how GPER, a GPCR recently found to regulate Nox1 protein abundance and function [12], contributes to ROS-dependent modulation of endothelium-dependent and -independent vascular tone. The results show that endothelium-dependent, NO-mediated vasodilation in the renal artery is markedly reduced with aging, a functional change that largely depends on the activity of both *Gper* and Nox. These results are compatible with our previous report showing that Nox-derived ROS formation is largely absent in the heart and aorta of aged *Gper*$^{-/-}$ mice [12]. In both studies, we used the Nox1/2-selective inhibitor gp91ds-tat [15], a peptide derived from a $gp91^{phox}$ (now termed Nox2) sequence in the region that interacts with the organizer protein $p47^{phox}$, thus disrupting $p47^{phox}$ binding to and activation of the homologous Nox2 and Nox1 catalytic subunits [7, 12]. Detailed mechanistic studies in murine and human vascular smooth muscle cells revealed that GPER in fact selectively regulates Nox1

protein abundance and function, while having no effect on Nox2 [12]. It is thus highly likely that Nox1, facilitated by basal GPER activity, is centrally involved in the age-dependent regulation of renal artery tone.

In contrast to the aorta or carotid arteries from young mice [12, 17], the endothelium-dependent, NO-mediated relaxant response induced by acetylcholine was about 20% less potent in renal arteries, and impaired to an even greater extent with aging. Although this age-dependent reduction was largely reversed by inhibiting Nox, several mechanisms may also play a role. Acetylcholine-dependent generation of cyclooxygenase-derived vasoconstrictor prostanoids counteracts the vasodilatory activity of NO [18], and their formation and activity increases with aging [19], an effect that may become insensitive to Nox1/2 inhibition by gp91ds-tat [17]. Of note, compared to other arterial beds, the renal artery produces higher levels of endothelium-derived vasoconstrictor prostanoids [20], which are co-stimulated with NO in response to acetylcholine. Second, the renal artery may be more sensitive to uncoupling of endothelial NO synthase compared to other vascular beds, an effect that can be induced by Nox1-derived ROS [21]. As a result, uncoupled endothelial NO synthase increasingly produces ROS instead of vasodilatory NO, which is further reduced by direct interaction with superoxide and hydrogen peroxide [22].

We also studied whether and how aging and GPER deficiency affect renal artery contractility to GPCR agonists, including angiotensin II and phenylephrine. Previously, angiotensin II was used to stimulate Nox activity in vascular smooth muscle cells [23] and subsequent cloning of Nox1 [24]. Indeed, angiotensin II has emerged as the prototypic inducer of Nox1 both at the molecular and functional level [7]. We recently found that, in the murine aorta of wild-type mice, contraction to angiotensin II (and the ROS formation associated with it) is partially sensitive to inhibition by gp91ds-tat; of note, mice lacking GPER showed an equal reduction in contraction to angiotensin II, with gp91ds-tat having no additional effect, indicating that Nox-dependent contractions to angiotensin II require functional GPER [12]. The present study extends these findings, showing a similar regulation of angiotensin II-dependent contractions by GPER in the renal artery, suggesting a common mechanism in different vascular beds that persists independent of age. Furthermore, contractions to the α-adrenergic agonist phenylephrine were partly mediated by GPER-dependent Nox activity, indicating that the ROS-dependent regulation of vascular tone by GPER is not limited to a specific agonist, but may be involved in a broader number of signaling pathways governed by Nox1.

The present study was conducted in male animals to exclude any effects of endogenous (ovarian) estrogen, the prototypical physiological GPER agonist [11]. The data reinforce that ligand-independent or basal activity of GPER affects regulation of vascular tone, consistent with our previous studies [12, 25, 26]. Indeed, many GPCRs exhibit basal or intrinsic activity sufficient to regulate cell signaling [27]. Furthermore, effects reported in the present study are independent of blood pressure, as both wild-type and GPER-deficient mice at 3 and 24 months of age, respectively, remain normotensive [12, 17].

In conclusion, this study shows that aging substantially attenuates endothelium-dependent, NO-mediated relaxation in the renal artery, which is largely preserved in GPER-deficient mice due to a lack of Nox activity. These findings indicate a novel role for GPER in ROS-dependent functional aging of the renal artery. With the availability of small molecule GPER blockers such as G36 [28], which represent a new drug class capable of selectively reducing the increased expression and activity of Nox1 [12], future therapeutic applications in disease conditions characterized by oxidative stress may emerge [29]. Inhibition of GPER signaling may thus provide a novel approach to inhibit the age-dependent impairment of renal artery function and, possibly, the associated detrimental structural and functional renal changes.

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Disclosure Statement

M.R.M., M.B., and E.R.P. are inventors on a US patent application for the therapeutic use of compounds targeting GPER. E.R.P. is an inventor on US patent Nos. 7,875,721 and 8,487,100 for GPER-selective ligands and imaging agents.

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