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GPER mediates the age-dependent upregulation of the myocardial endothelin system

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Abstract

Aims: Cardiac aging is associated with progressive structural changes and functional impairment, such as left ventricular hypertrophy, fibrosis and diastolic dysfunction. Aging also increases myocardial activity of endothelin-1 (ET-1), a multifunctional peptide with growth-promoting and pro-fibrotic activity. Because the G protein-coupled estrogen receptor (GPER) regulates vascular responsiveness to ET-1, we investigated whether GPER also plays a role in the regulation of the cardiac endothelin system with aging.

Main methods: Young (4 month-old) and aged (24 month-old) wild-type and *Gper*-deficient (*Gper*^{-/-}) mice were studied. Gene expression levels of prepro-ET-1, endothelin converting enzymes ECE-1 and ECE-2, and endothelin ET_A and ET_B receptors were determined by qPCR in left ventricular myocardium.

Key findings: Aging markedly increased steady-state mRNA expression levels of ECE-1, ECE-2, ET_A and ET_B receptors (each $p < 0.001$ vs. young mice). Deletion of *Gper* inhibited the age-dependent increase in ECE-2 and ET_B receptor mRNA levels (57% and 40% reduction, respectively, each $p < 0.01$ vs. wild-type mice), whereas gene expression of prepro-ET-1, ECE-1, or the ET_A receptor was unaffected in *Gper*^{-/-} mice.

Significance: We identified a novel regulatory mechanism through which the endogenous *Gper* facilitates the age-dependent increase in myocardial expression of ECE-2 and the ET_B receptor, which is compatible with an activating role of GPER for the cardiac endothelin system with aging. Targeting GPER signaling by selective antagonists may therefore be considered a new therapeutic approach to reduce age-dependent increased ET-1 activity and the associated development of left ventricular hypertrophy, fibrosis and heart failure.

Key words: Aging; Endothelin Converting Enzyme; Endothelin Receptor; Estrogen Receptor; ET-1; GPR30; Heart; Myocardium.

Introduction

With the anticipated aging of the world's population, current estimates predict a marked increase in the prevalence of heart failure and the associated cost of care, necessitating already more hospitalizations of older patients than any other medical condition in the Western civilization [1]. Indeed, the elderly account for more than 90% of patients with heart failure [2]. Cardiac aging in humans is associated with progressive structural changes and functional impairment, such as left ventricular hypertrophy, fibrosis and impaired diastolic function, changes that are recapitulated in experimental animals [3,4].

In rodents, age-dependent cardiac hypertrophy and fibrosis have been associated with increased myocardial expression of the multifunctional peptide endothelin-1 (ET-1) [5-7], which can induce cardiomyocyte growth and collagen synthesis in cardiac fibroblasts [8,9]. Similarly, aging is associated with increased cardiac expression of endothelin ET_A and ET_B receptors [6,10], whereas cardiomyocyte hypertrophy and myocardial fibrosis are attenuated in aged mice with cardiomyocyte-specific deletion of the ET_A receptor gene [6]. Furthermore, treatment with ET_A and dual ET_A/ET_B receptor antagonists improves cardiac function and survival in animal models of acute and chronic heart failure [11-13]. In symptomatic patients with advanced heart failure, myocardial ET-1 peptide levels are also increased, which may be related to elevated expression of endothelin converting enzyme-1 (ECE-1) that catalyzes proteolytic cleavage of the precursor peptide big-ET-1 to form ET-1 [14]. Together, these findings suggest that cardiac aging activates the local endothelin system, yet the underlying mechanisms are still unclear.

Although myocardial function in adulthood and during aging critically depends on the function of G protein-coupled receptors (GPCRs) such as ET_A and ET_B receptors [15], much less is known about the orphan GPCR GPR30 that shows strong expression in the heart [16-18]. GPR30 was later identified to bind and induce rapid signaling in response to estrogen [19,20], which led to its designation as G protein-coupled estrogen receptor (GPER) [21]. However, chronic *Gper*-dependent effects in the absence of circulating ovarian estrogens have also been reported [22-24]. Activation of GPER using its selective agonist G-1 ameliorates cardiac function, hypertrophy, and fibrosis in animals with hypertensive cardiomyopathy or congestive heart failure [25-29], and reduces infarct size and improves cardiac remodeling after experimentally induced myocardial ischemia and reperfusion injury [30-33]. Whether GPER function plays a role in cardiac aging is unknown.

Given our previous observation that ET-1-mediated vasoconstriction is attenuated by G-1 [34] and potentiated in *Gper*-deficient male mice (e.g. in the absence of ovarian estrogen production) [23], we hypothesized that GPER may play a regulatory role in the cardiac endothelin system with age. We therefore set out to study steady-state gene expression of

ET-1, as well as ECE-1, ECE-2, ET_A and ET_B receptors in left ventricular myocardium of young and aged GPER-deficient (*Gper*^{-/-}) and wild-type male mice.

Materials and Methods

Materials

All materials were from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Animals

Male *Gper*^{-/-} mice (Proctor & Gamble, Cincinnati, OH, USA, provided by Jan S. Rosenbaum) were generated and backcrossed onto the C57BL/6 background as described [24]. *Gper*^{-/-} and wild-type littermates (Harlan Laboratories, Indianapolis, IN, USA) were housed at the Animal Resource Facility of the University of New Mexico Health Sciences Center with unlimited access to water and a rodent diet devoid of alfalfa or soybean meal to minimize the occurrence of natural phytoestrogens (Teklad 2020SX, Harlan Laboratories, Madison, WI, USA). Animals were maintained under controlled temperature of 22-23 °C on a 12h light-dark cycle. At 4 or 24 months of age, mice were sacrificed by intraperitoneal injection of sodium pentobarbital (2.2 mg/g body weight). The apex of the left ventricle was collected, immediately snap-frozen in liquid nitrogen and stored at -80 °C until further analysis. 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.

Isolation and reverse transcription of myocardial mRNA

Frozen left ventricular myocardium (20 mg) was disrupted and homogenized using a rotor-stator homogenizer, and total RNA was extracted using the silica-based RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA, USA). RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA).

Quantitative real-time polymerase chain reaction (qPCR)

SYBR Green-based detection of amplified gene-specific cDNA fragments was performed on a 7500 FAST real-time PCR system (Applied Biosystems). The sets of primers used are given in Table 1. Relative gene expression was determined using the 2^{-ΔCT} method [35] with GAPDH serving as house-keeping control.

Statistical analyses

Data was analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test for multiple comparisons (Prism version 5.0 for Macintosh, GraphPad Software, San Diego, CA, USA). Values are expressed as mean \pm s.e.m.; n equals the number of animals used. Statistical significance was accepted at a p value <0.05 .

Results

Aging upregulates myocardial ECE and ET receptor gene expression

To study whether aging affects the cardiac endothelin system, gene expression levels of its individual components were quantified in left ventricular myocardium of young (4 month-old) and aged (24 month-old) wild-type mice. Aging was associated with a marked up-regulation of the ECEs and ET receptors in left ventricular myocardium: mRNA levels of ECE-1, ECE-2, ET_A and ET_B receptors were 4-fold to 12-fold higher compared to young mice (all $p<0.001$, $n=6-7$, Figures 1 and 2), whereas prepro-ET-1 expression was unaffected by aging. Furthermore, gene expression levels of ECE-1 were 19-fold and 6-fold higher than ECE-2 in left ventricular myocardium of young and aged mice, respectively (each $n=6-7$, $p<0.001$, Figure 1). In contrast, mRNA levels of ET_A and ET_B receptors were similar within each age group (each $n=6-7$, $p=n.s.$, Figure 2).

GPER mediates the age-dependent upregulation of ECE-2 and ET_B receptor gene expression

To determine whether endogenous GPER affects the upregulation of the cardiac endothelin system with aging, left ventricular myocardium of *Gper*^{-/-} mice was analyzed. In young mice, myocardial gene expression of prepro-ET-1, ECE-1, ECE-2, ET_A or ET_B receptors was unaffected by deletion of *Gper* (each $n=5-7$, $p=n.s.$ vs. wild-type control, Figures 1 and 2). In contrast, the increase in ECE-2 mRNA level with aging was markedly reduced in myocardium lacking *Gper* (57% reduction, $n=6$, $p<0.001$ vs. wild-type control, Figure 1). Similarly, deletion of *Gper* significantly inhibited the age-dependent upregulation of myocardial ET_B receptor gene expression (40% reduction, $n=6$, $p<0.01$ vs. wild-type control, Figure 2).

Given that activation of GPER induces production of nitric oxide (NO) by endothelial NO synthase (eNOS) [22,24,34], and since the cardiac eNOS and endothelin systems closely interact in the pathogenesis of cardiac dysfunction, hypertrophy and fibrosis [36,37], we next determined myocardial eNOS gene expression in wild-type and *Gper*^{-/-} mice. Surprisingly, eNOS mRNA levels were neither affected by aging nor by *Gper* deletion ($n=5-7$, $p=n.s.$, Figure 3).

Taken together, the presence of *Gper* in aged mice is required to facilitate the upregulation of specific components of the cardiac endothelin system with age, including ECE-2 and the ET_B receptor.

Discussion

This study identifies endogenous GPER as an age-dependent stimulatory regulator of myocardial ECE-2 and ET_B receptor gene expression in male mice. In the presence of GPER, increased ECE-2 expression is likely to contribute to augmented local synthesis of ET-1. Thus, GPER may facilitate the activation of the cardiac endothelin system with aging [5-7,10]. Furthermore, GPER-dependent regulation of ET_B receptor expression suggests functional cross-talk between the two GPCRs. Since increased activity of the cardiac endothelin system has been implicated in the progression of heart failure with aging [5-7,10], inhibiting GPER may provide a new approach to reduce myocardial ECE-2 and ET_B receptor expression and thus increased ET-1 activity.

Elevated circulating levels and myocardial expression of ET-1 have been associated with age-dependent cardiac hypertrophy and fibrosis [5-7], and can be observed in animals and humans with heart failure [11-14]. In the present study, we found an age-dependent increase in myocardial ECE-1, ECE-2, as well as ET_A and ET_B receptor gene expression, while prepro-ET-1 mRNA levels were unaffected by aging. However, given that ET-1 is formed locally through a 39-amino acid intermediate, big-ET-1, which undergoes subsequent proteolytic cleavage by ECE-1 and ECE-2 [14,38], enhanced conversion of precursor peptides by ECE may be relevant for elevated ET-1 levels in cardiac aging or age-related cardiovascular disease. Among the ECE identified, the membrane bound metalloproteinase ECE-1 was found to display increased expression levels in the myocardium of patients with advanced heart failure [14], and inhibition of ECE-1 activity or expression reduces *in vitro* hypertrophy of cardiomyocytes stimulated by the adrenergic agonist phenylephrine [39]. ECE-2, which similar to GPER [19] is expressed intracellularly in the Golgi network [38], plays a role in the embryonic development of cardiac outflow structures, and may be functionally involved in cardiac ET-1 formation similar to ECE-1 [40]. Although the role of ECE-2 in cardiac aging is unclear and its expression level is substantially lower compared to ECE-1, we observed a substantial age-dependent increase in ECE-2 mRNA levels that largely depended on the presence of *Gper*. Thus, GPER-driven ECE-2 expression may potentiate age-dependent increases in cardiac ET-1 production. In addition, it is conceivable that local ET-1 activity would be further enhanced by increased expression of ET_A and/or ET_B receptors with aging [6,10], which have been implicated in age-dependent pathologies such as podocyte injury and glomerulosclerosis. Indeed, ET_A receptor blockade for several weeks

in part reverses structural and functional renal aging [41,42]. Given that ET_B receptor mRNA expression is also stimulated by the presence of GPER as shown in the present study, this may further underscore a role of the endogenous GPER for increased activity of the cardiac endothelin system with aging.

The role of GPER in the physiological aging process of the heart is unknown. Previous studies have shown impaired vascular function related to GPER in blood vessels from aged female rats [43], while metabolic effects on glucose tolerance and inflammation become evident in male *Gper*^{-/-} mice with age [44]. In line with these findings, the present study now also provides evidence of a novel role for GPER in cardiac aging. This may not be surprising given the high cardiac expression of GPER [16-18] and its emerging protective role in heart failure models upon activation by the selective ligand G-1 [25-32]. To the contrary, the observation that GPER expression is required to increase certain components of the myocardial endothelin system was unexpected, even more so since we have previously observed that deletion of *Gper* increases vasoconstrictor responses to ET-1 [23], whereas G-1 inhibits ET-1-dependent contractions of coronary arteries [34]. However, these GPER-mediated effects on vascular reactivity to ET-1 have been observed in blood vessels from healthy young animals as opposed to the present findings regarding the cardiac endothelin system in mice at advanced age, which has recently been considered a “disease-like state” [3]. It may therefore be possible that GPER – contrary to its effects under healthy conditions – contributes in part to the increased activity of myocardial ET-1 with cardiac aging, as recently also observed for ET-1-stimulated migration of certain cancer cells [45]. Furthermore, activation of GPER potently induces eNOS-derived NO formation [22,24,34], an effect that may explain the inhibition of ET-1-dependent contractions in blood vessels. On the other hand, despite the strong interaction between the cardiac eNOS and endothelin systems [36,37], we found that neither *Gper* deletion nor aging affected myocardial eNOS expression, indicating that GPER – at least on the gene expression level – regulates the cardiac endothelin but not the eNOS system with aging.

Interestingly, despite low endogenous estrogen production, studies in mice have previously established that endogenous GPER is also active in the cardiovascular system of males [22,23] and ovariectomized females [24], e.g. in the absence of ovarian estrogen production. The present study extends these previous findings demonstrating that deletion of the *Gper* gene in male mice is sufficient to regulate the expression of components of the cardiac endothelin system with aging. Such effects may be explained by a certain level of intrinsic activity generally attributed to GPCRs [46], or by localized myocardial conversion of androgen precursors into estrogens catalyzed by the enzyme aromatase, which facilitates sufficient localized estrogen biosynthesis that might activate signaling of GPER and/or the “classical” estrogen receptors, ER α and ER β [47].

Conclusion

The present study presents evidence for a novel regulatory mechanism that is required to stimulate the age-dependent increase in myocardial expression of ECE-2 and ET_B receptors through the presence of the *Gper* gene. This endogenous effect of GPER will likely increase the overall activity of the cardiac endothelin system with aging. Aged mice closely mimic changes of cardiac aging in humans, including left-ventricular hypertrophy, fibrosis, and diastolic dysfunction, which has been associated with increased activity of ET-1 [3-7,10], yet the extent to which GPER-mediated, age-dependent upregulation of ECE-2 and ET_B expression translates into structural or functional cardiac changes remains to be clarified. Given these GPER-mediated effects, targeting GPER signaling by selective antagonists may be considered a new therapeutic approach to reduce increased activity of the cardiac endothelin system with aging and the development of heart failure associated with it [3-7,10].

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Financial Disclosure and Competing Interests Statement

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

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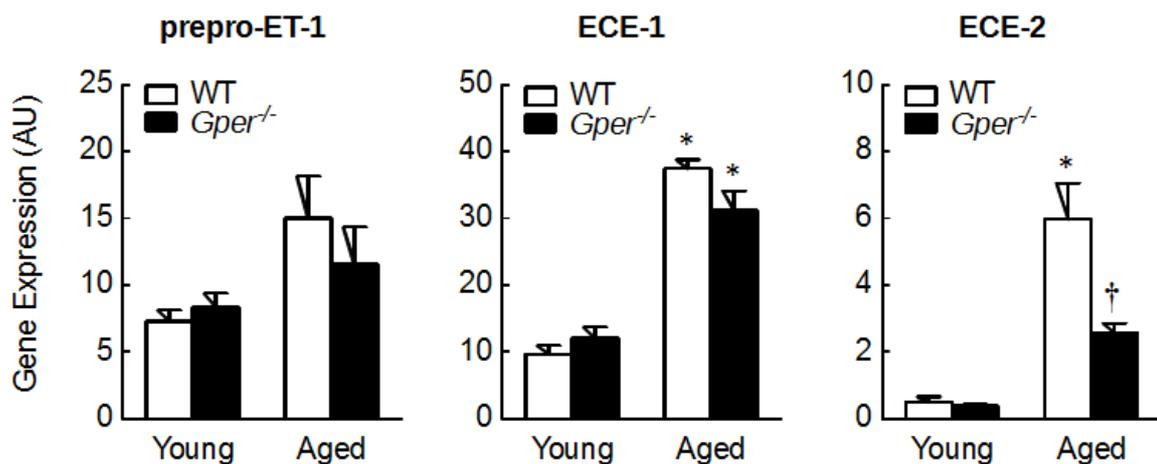
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Table 1. Primer sets used for qPCR.

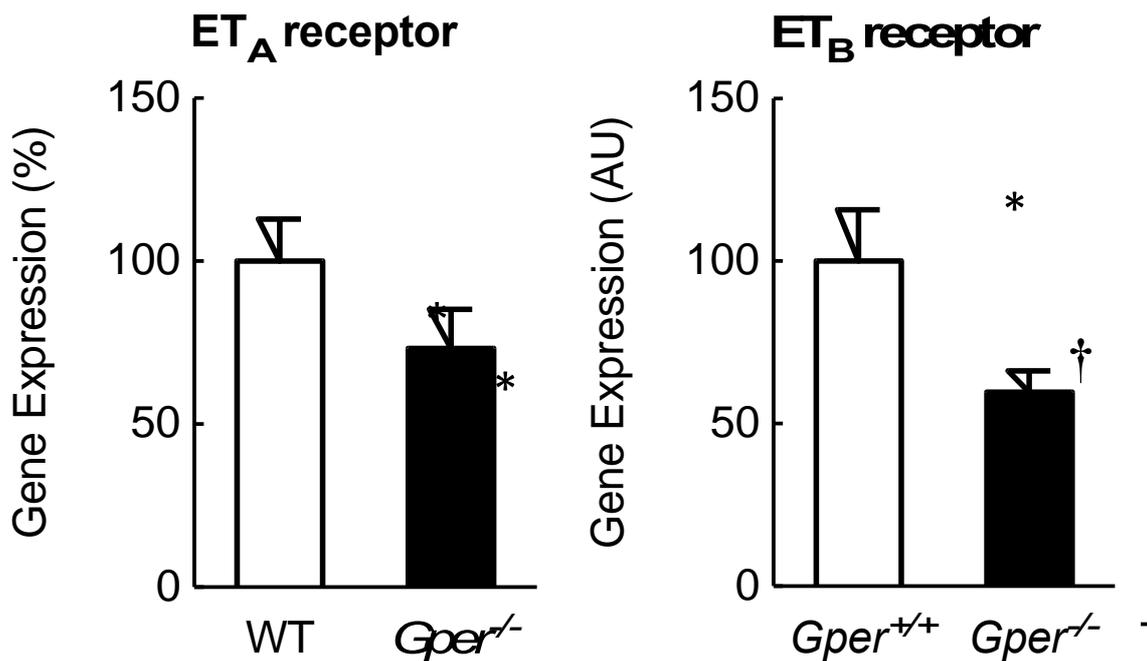
Gene (GenBank ID)	Forward Primer	Reverse Primer
prepro-ET-1 (U35233.1)	5'-AAC TCA GGG CCC AAA GTA CC-3'	5'-TTT GCA ACA CGA AAA GAT GC-3'
ECE-1 (NM_199307.2)	5'-GCC TAC CGG GCG TAC CAG AAC-3'	5'-GGT GTG CGG ACA GAG CAC CAG-3'
ECE-2 (AF396699)	5'-CCC GTG AAC GCT TAC TAC CTT-3'	5'-GGT CAT CAA AGG CAT GTG TCA-3'
ET _A receptor (BC008277)	5'-GAA GGA CTG GTG GCT CTT TG-3'	5'-CTT CTC GAC GCT GTT TGA GG-3'
ET _B receptor (BC026553)	5'-CGG TAT GCA GAT TGC TTT GA-3'	5'-CAC CTG TGT GGA TTG CTC TG-3'
eNOS (NM_008713)	5'-AGA GCC TGC AAT TAC TAC CA-3'	5'-GTG GAT TTG CTG CTC TGT AG-3'
GAPDH (NM_008084)	5'-TTC ACC ACC ATG GAG AAG GC-3'	5'-GGC ATG GAC TGT GGT CAT GA-3'

Figure 1. Gene expression levels of prepro-ET-1 and endothelin converting enzymes ECE-1 and ECE-2 in left ventricular myocardium of young (4 month-old) and aged (24 month-old) wild-type (WT) and *Gper*-deficient (*Gper*^{-/-}) mice. All data ($n=5-7$) are mean \pm s.e.m.; * $P<0.001$ vs. young mice, † $p<0.001$ vs. wild-type control. AU, arbitrary units.



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Figure 2. Gene expression levels of endothelin ET_A and ET_B receptors in left ventricular myocardium of young (4 month-old) and aged (24 month-old) wild-type (WT) and *Gper*-deficient (*Gper*^{-/-}) mice. All data (*n*=5-7) are mean±s.e.m.; **P*<0.01 vs. young mice, †*p*<0.01 vs. wild-type control. AU, arbitrary units.



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Figure 3. Gene expression levels of endothelial NO synthase (eNOS) in left ventricular myocardium of young (4 month-old) and aged (24 month-old) wild-type (WT) and *Gper*-deficient (*Gper*^{-/-}) mice. All data ($n=5-7$) are mean \pm s.e.m. AU, arbitrary units.

