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GPER is required for 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 myocardial 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, and 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 local 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.
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1. 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 can be 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 or 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 myocardium [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

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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 myocardial 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 male GPER-deficient ($Gper^{-/-}$) and wild-type mice.

2. Materials and methods

2.1. Materials

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

2.2. 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 12 h 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). Apical myocardium 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.

2.3. 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).

2.4. 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 $2^{-\Delta CT}$ method [35] with GAPDH serving as house-keeping control.

2.5. 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 ± s.e.m.; n equals the number of animals used. Statistical significance was accepted at a p value <0.05.

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′

3. Results

3.1. Aging upregulates myocardial ECE and ET receptor gene expression

To study whether aging affects the myocardial 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 $p < 0.001$, $n = 6–7$, Figs. 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 $n = 6–7$, $p < 0.001$, Fig. 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., Fig. 2).

3.2. GPER is required for the age-dependent upregulation of ECE-2 and ET_B receptor gene expression

To determine whether endogenous GPER affects the upregulation of the myocardial 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, Figs. 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, Fig. 1). Similarly, deletion of *Gper* inhibited the age-dependent upregulation of myocardial ET_B receptor gene expression (40% reduction, $n = 6$, $p < 0.01$ *vs.* wild-type control, Fig. 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., Fig. 3).

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

4. 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

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