Activation of GPR30 attenuates diastolic dysfunction and left ventricle remodelling in oophorectomized mRen2.Lewis rats

Hao Wang1, Jewell A. Jessup1, Marina S. Lin1, Clarissa Chagas1, Sarah H. Lindsey2, and Leanne Groban1,3*

1Department of Anesthesiology, Wake Forest School of Medicine, Winston-Salem, Medical Center Boulevard, Winston-Salem, NC 27157-1009, USA; 2Hypertension and Vascular Research Center, Wake Forest School of Medicine, Winston-Salem, NC, USA; and 3Department of Physiology and Pharmacology, Wake Forest School of Medicine, Winston-Salem, NC, USA

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Aims

GPR30 is a novel oestrogen receptor expressed in various tissues, including the heart. We determined the role of GPR30 in the maintenance of left ventricular (LV) structure and diastolic function after the surgical loss of ovarian hormones in the female mRen2.Lewis rat, a model emulating the cardiac phenotype of the post-menopausal woman.

Methods and results

Bilateral oophorectomy (OVX) or sham surgery was performed in study rats; the selective GPR30 agonist, G-1 (50 μg/kg/day), or vehicle was given subcutaneously to OVX rats from 13–15 weeks of age. Similar to the cardiac phenotype of sham rats, G-1 preserved diastolic function and structure relative to vehicle-treated OVX littermates independent of changes in blood pressure. G-1 limited the OVX-induced increase in LV filling pressure, LV mass, wall thickness, interstitial collagen deposition, atrial natriuretic factor and brain natriuretic peptide mRNA levels, and cardiac NAD(P)H oxidase 4 (NOX4) expression. In vitro studies showed that G-1 inhibited angiotensin II-induced hypertrophy in H9c2 cardiomyocytes, evidenced by reductions in cell size, protein content per cell, and atrial natriuretic factor mRNA levels. The GPR30 antagonist, G15, inhibited the protective effects of oestradiol and G-1 on this hypertrophy.

Conclusion

These data show that the GPR30 agonist G-1 mitigates the adverse effects of oestrogen loss on LV remodelling and the development of diastolic dysfunction in the study rats. This expands our knowledge of the sex-specific mechanisms underlying diastolic dysfunction and provides a potential therapeutic target for reducing the progression of this cardiovascular disease process in post-menopausal women.

Keywords

Cardiac hypertrophy • Diastolic dysfunction • GPR30 • mRen2.Lewis rat • Post-menopausal woman

1. Introduction

The prevalence of diastolic dysfunction dramatically increases in post-menopausal women.1 Unlike men of equivalent age, post-menopausal women predominantly exhibit impairments in diastolic function with concentric left ventricular (LV) remodelling and eventual diastolic heart failure rather than systolic dysfunction, eccentric remodelling, and systolic heart failure.2 Diastolic heart failure, also known as heart failure with preserved ejection fraction, accounts for 40–60% of chronic heart failure cases in industrialized nations,3 and over 75% of these cases are in older women.4 Hospital admissions for diastolic heart failure have increased steadily over the past two decades, with female patients having the highest annual percentage increase in hospitalization rates when compared with males with heart failure.3 This may be due in part to delayed diagnoses of heart failure in women compared with men.5,6 In addition, the prevalence and prognostic impact of its precursor, diastolic dysfunction, has only recently been recognized and the mechanisms are not yet clear.7 Evidence suggests that the loss of oestrogens contributes to the development of hypertension and cardiac hypertrophy in post-menopausal women,8–10 and these diseases are risk factors for diastolic dysfunction.11,12 While the prevalence of hypertension rises with age for both genders, the near doubling in the prevalence of hypertension in women aged 70–79 compared with those aged 50–
59 may be attributed, in part, to the decline in oestrogen during meno-
pause.\textsuperscript{13,14} Moreover, the evolution of LV hypertrophy differs between pre- and post-menopausal women, with a greater induction of hyper-
trophy when circulating oestrogen levels are reduced.\textsuperscript{15} Experimental
studies in adult female rats show that ovariectomy eliminates female-
specific protection against volume-induced cardiac hypertrophy and re-
modelling,\textsuperscript{16} while oestradiol administration attenuates hypertrophy
associated with pressure overload\textsuperscript{17} and ageing.\textsuperscript{18} Although activation of the
classical oestrogen receptors (ER-\textalpha and ER-\textbeta) located on myo-
cytes, fibroblasts, and the extracellular matrix might be involved in
oestrogen-mediated cardioprotection,\textsuperscript{19} the exact mechanisms for
the beneficial effects of oestrogen on cardiac hypertrophy, fibrosis,
and diastolic dysfunction remain unclear.

With the recent discovery of the G protein-coupled oestrogen
receptor (GPR30) in the heart,\textsuperscript{12,20} the potential mechanisms for
oestrogen-mediated cardioprotection are expanded. GPR30, also
known as GPER, is located on the cell membrane and endoplasmic reti-
culum,\textsuperscript{21,22} and is widely distributed, independent of species and sex,
among a variety of mammalian tissues including the heart, lung, liver,
adrenal gland, intestine, ovary, and brain.\textsuperscript{23,24} GPR30 activation by its
specific agonist, G-1, improves contractile function and reduces infarct
size in isolated rat and mouse hearts subjected to ischaemia/reperfu-
sion injury.\textsuperscript{20,25–27} We found increased cardiac GPR30 mRNA levels
in the female mRen2.Lewis rat following chronic consumption of a
high-salt diet\textsuperscript{12} or surgically induced oestrogen loss (see Supplementary
material online). Further studies from our laboratory show that in
vivo activation of GPR30, with G-1, attenuates the adverse effects of
high salt on diastolic function and cardiac remodelling.\textsuperscript{12} Indeed, en-
dogenous oestrogens could have contributed to the anti-remodelling
and lusitropic benefits following high salt consumption,\textsuperscript{28} since oes-
tradiol binds GPR30 at an affinity similar to G-1.\textsuperscript{29} Nonetheless, these
data support the notion that cardiac GPR30 is increased during periods of physiological and pathophysiological stress, and that its ac-
tivation could provide valuable cardioprotection.

Therefore, using the oophorectomized (OVX) mRen2.Lewis rat, an
established rodent model that mimics the cardiac phenotype of
women following surgical or natural cessation of ovarian hormone
production,\textsuperscript{28,30–34} we hypothesized that GPR30 activation by G-1
preserves diastolic function and mitigates cardiac remodelling follow-
ing the loss of oestrogens.

2. Methods

2.1 Animals

Female mRen2.Lewis rats were obtained from the Hypertension and Vascu-
lar Research Center Congenic Colony at Wake Forest School of Medi-
cine and all studies were approved by the institution’s Animal Care and
Use Committee. All animal procedures conformed to the Guide to the
Care and Use of Laboratory Animals published by the US National Institutes
of Health. Rats were individually housed in an Association for Assessment
and Accreditation of Laboratory Animal Care-approved, temperature
controlled facility with ad libitum access food and water.

2.2 Experimental protocol

At 4 weeks of age, rats were randomly assigned to undergo either OVX
(n = 16) or sham operation (Sham; n = 10) performed under 2% isoflu-
ane anaesthesia, as previously described.\textsuperscript{30,31} The adequacy of anaesthesia
was monitored by observation of slow breathing, loss of muscular tone,
and no response to surgical manipulation. The success of OVX and the
subsequent depletion of circulating oestrogens were confirmed using a
serum oestradiol assay (5 pg/mL detection limit; Polymedco, Cortlandt
Manor, NY, USA) at the end of the experiment (data not shown). Once the rats reached 13 weeks of age, the OVX group was further ran-
domly divided to receive either the GPR30 agonist, G-1, (OVX-G1; n = 7;
Cayman Chemical Company, Ann Arbor, MI, USA) diluted in a DMSO/
EtOH mixture for a targeted dose of 50 mcg/kg/day or the DMSO/
EtOH vehicle (OVX-V; n = 9) and were administered subcutaneously
via osmotic mini-pumps (DURECT Corporation, Cupertino, CA, USA).
This dose of G-1 was determined by an initial pilot study conducted in
our laboratory that demonstrated lusitropic effectiveness without signifi-
cant blood pressure reductions (Figure 1). Weekly body weight and systol-
ic blood pressures by tail-cuff plethysmography (NIBP-LE5001, Panlab,
Barcelona, Spain) were monitored throughout the study. Rats were eutha-
nized via exsanguination by cardiac puncture while under ketamine/xyla-
zeine anaesthesia (ketamine HCL 60 mg/kg and xylazine HCL 5 mg/kg)
at 15 weeks of age, following echocardiographic evaluation. Whole hearts
were isolated and further dissected to isolate the left ventricle, right vent-
tricle, and atria. Tissue weights and tibial lengths were measured with an
analytical scale and a micrometer; respectively. The left ventricle was cut
into pieces for RNA, western blot, and histological analyses.

2.3 Echocardiographic evaluation

LV function and dimensions were assessed prior to the protocol termina-
tion in anaesthetized (ketamine/xylazine: 80/12 mg/kg) animals using a
Philips 5500 echocardiograph (Philips Medical Systems, Andover, MA,
USA) and a 12 MHz phased array probe as previously described.\textsuperscript{12,28,30,31}

2.4 Determination of collagen deposition in the heart

LV specimens were fixed in 4% paraformaldehyde and embedded into
collagen blocks; 4 \mu m sections were stained with picrosirius red, as previ-
ously described.\textsuperscript{30,31} Images were captured using an Axiosvert 200 micro-
scope (Thorwood, NY, USA). The ratio of collagen-stained pixels to
unstained pixels was quantified using NIH ImageJ software (http://
rsweb.nih.gov/ij/).

2.5 Cardiomyocyte size measurement

Cardiomyocyte cross-sectional areas were measured in paraffin-
embedded LV sections. Alexa Flour 488-conjugated wheat germ agglutinin
(WGA, 10 \mu g/mL, Invitrogen, Carlsbad, CA, USA) was employed to delin-
eate cellular membranes. TO-PRO-3 (1/1000 dilution, Invitrogen, Carls-
bad, CA, USA) was used for nuclear staining. Images were captured

<table>
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<th>Days after treatment</th>
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<tr>
<td>3</td>
<td>160</td>
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<td>7</td>
<td>120</td>
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Figure 1 Tail-cuff systolic blood pressure in conscious, sham-operated and ovariectomized female mRen2.Lewis rats treated with G-1 or vehicle for 2 weeks. Values are means \pm SEM; OVX, ovariectomized; V, vehicle. \*P < 0.05 vs. sham. n = 7–10/group.
using a Zeiss LSM 510 confocal microscope (Thornwood, NY, USA) and analysed with Zeiss LSM Image Browser (version 3.2.0.70). For each section, at least 300 round cells with centrally located nuclei were measured, and the average value was used as the final cardiomyocyte size.

2.6 Immunofluorescence staining and immunocytochemistry analysis

Immunofluorescent staining of heart sections and immunocytochemistry in cultured cells were performed using standard procedures. Images were captured using a Zeiss LSM 510 confocal microscope. For detailed methodology, please refer to the Supplementary material online.

2.7 Cell culture and treatment

The H9c2 myoblast cell line, derived from embryonic rat heart, was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) with 10% heat-inactivated foetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 mg/mL), at 37 °C in 5% CO2 and 95% air, at a relative humidity of 95% and split 1:4 at confluence (80%). Before each experiment, cells were seeded in six-well plates or chamber slides (Lab-TekIII Chamber Slides: Thermo Scientific NUNC 177437) at the density of 5 × 10^4 cells/cm² and starved for 18 h in DMEM containing 0.1% FBS. Cells were subsequently treated with angiotensin II (Ang II, 10−7 M, Bachem), oestradiol (E2) (10−7 M, Sigma), G-1 (10−7 M, Cayman Chemical Company), G15 (10−8 M, Tocris Bioscience).

After an additional 24-h incubation, cells were fixed with cold ethanol for immunocytochemical staining, lysed with TRIzol (Invitrogen, Carlsbad, CA, USA) for real-time (RT)–PCR analysis, or trypsinized for protein analysis. Protein content was determined using a Biorad protein assay kit (Hercules, CA, USA).

2.8 Analysis of gene expression by quantitative real-time PCR

RT–PCR was used to detect gene mRNA levels in cardiac tissue or H9c2 cells. For detailed methodology, please refer to the Supplementary material online.

2.9 Western blot analysis

LV tissue homogenates were separated by SDS–PAGE and transferred onto membranes as described previously.12,31 ImmunobLOTS were probed using antibodies for NAD(P)H 4 (NOX4) (2 μg/mL; Abcam, Cambridge, MA, USA) collagen I (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), collagen III (1:200; Santa Cruz Biotechnology), transforming growth factor-beta-1 (TGF-β1) (1:500; Santa Cruz Biotechnology), sarco-plasmic endoplasmic reticulum ATPase (SERCA2:1:1000; Abcam), and phosphoholamin (PLB) (1:5000; Abcam). The PLB-to-SERCA2 ratio was used as a measure of SERCA2 inhibition. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:5,000; Cell Signaling, Danvers, MA, USA) was used as a loading control. The bands were digitized using MCID image analysis software (Imaging Research, Inc., Ontario, Canada). Each band was expressed in arbitrary units and normalized to its own GAPDH.

2.10 Statistical analyses

All results were expressed as mean ± SEM. For all endpoints, one-way ANOVA evaluated significant effects among the groups. Significant interactions between the groups were further characterized using Student–Newman–Keuls post hoc analyses. Analyses were performed using GraphPad Prism version 5 (GraphPad, San Diego, CA, USA). Differences were considered significant at \( P < 0.05 \).

3. Results

3.1 Blood pressure changes during the experiment

Consistent with our previous studies,28,30,31 loss of ovarian hormones exacerbated hypertension in the female mRen2.Lewis rat. At 13 weeks of age, systolic blood pressure was 173 ± 6 mmHg in OVX rats vs. 147 ± 3 mmHg in intact littermates \((P < 0.001)\). Since chronic G-1 treatment, at the dose of 400 μg/kg/day, has significant blood pressure lowering effects in this strain,34 and since afterload is an important factor that affects diastolic function and myocardial relaxation,35 we conducted a pilot study to determine the ideal lusitropic dose of G-1, as determined by tissue Doppler mitral annular descent (e′) that did not alter blood pressure (Figure 1). Accordingly, we chose to chronically administer G-1 at 50 μg/kg/day, as <100 μg/kg/day of G-1 had no effect on blood pressure (data not shown).

Serial systolic blood pressures during the 2-week treatment period were not different between OVX-vehicle and OVX-G-1 treated rats, but both OVX-groups had significantly higher blood pressures compared with their oestrogen-intact littermates at each time-point (Figure 1).

3.2 G-1 attenuated the adverse effects of oestrogen loss on diastolic function

In the present study, we found that cardiac GPR30 mRNA levels were increased by 60% in OVX rats when compared with sham-operated littermates (see Supplementary material online, Figure S1). We evaluated the cardioprotective potential of GPR30 activation by its agonist, G-1, in OVX-mRen2.Lewis rats using both conventional and tissue Doppler echocardiography. While overt differences in the early-to-late (E/A) transmitral Doppler filling ratios were not observed among groups \((P = 0.08;\) see Supplementary material online, Table S1), the 2-week subcutaneous infusion of G-1 attenuated the decrease in myocardial relaxation (e′), increase in Doppler-derived left ventricle filling pressures \((E/e'\)) and increase in cardiac gene expression of brain natriuretic peptide (BNP) and atrial natriuretic factor (ANF) that occurred following the surgical loss of oestrogens in this strain (Figure 2). Systolic function, as determined by per cent fractional shortening and velocity of circumferential fibre shortening (Vcf), was not affected by the loss of oestrogens or the administration of G-1 (Figure 2B and Table 1).

3.3 G-1 attenuated the effect of oestrogen loss on hypertrophic and extracellular LV remodelling

The early surgical loss of oestrogen (at 4 weeks of age) led to significant increases in heart weight and left ventricle weight by 15 weeks of age (Table 1 and Figure 3). The whole heart weight increased by 20% and the LV weight increased by 14% in the OVX- compared with sham-operated rats \((P < 0.05)\). Interestingly, 2 weeks of G-1 attenuated the effect of ovarian hormone loss on heart weight. (Table 1, Figure 3C). These findings are consistent with echocardiographic-derived morphometric observations. In the hearts of OVX rats, the posterior and anterior wall thicknesses, relative wall thickness, and LV mass were significantly increased when compared with measures from the sham-operated group.
These increases were also mitigated by 2 weeks of G-1 treatment (Table 1, Figure 3A and B). Moreover, cardiomyocyte size was significantly increased in OVX- vs. oestrogen-intact rats, and this effect was inhibited by G-1 (Figure 3D and E).

Consistent with our previous findings, collagen deposition was significantly enhanced in LV sections from OVX-mRen2.Lewis rats when compared with sham-operated littermates (OVX: 5.7 ± 0.4% vs. Sham: 4.3 ± 0.4%, P < 0.05). This increase in interstitial fibrosis was attenuated by G-1 (OVX-G1: 5.0 ± 0.4% vs. OVX-V: 5.7 ± 0.4%, P < 0.05) (Figure 4A). In hearts from OVX rats, collagen III protein expression was increased and collagen I was decreased when compared with hearts from sham-operated rats. These changes were attenuated in hearts from G-1 treated rats (Figure 4B). In addition, cardiac TGF-β1 mRNA was significantly increased by OVX and attenuated by G-1 (see Supplementary material online, Figure S2B). Likewise, TGF-β1 protein expression was increased in hearts from OVX rats and attenuated by G-1 (see Supplementary material online, Figure S2A).

Table 1 Heart weight, heart rate, and echocardiography parameters

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<tr>
<th></th>
<th>Sham</th>
<th>OVX-V</th>
<th>OVX-G1</th>
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<tr>
<td>N</td>
<td>10</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Heart rate (b.p.m.)</td>
<td>246 ± 8</td>
<td>242 ± 9</td>
<td>244 ± 9</td>
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<tr>
<td>Body weight (g)</td>
<td>232 ± 17</td>
<td>254 ± 17</td>
<td>264 ± 9</td>
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<td>HW/TL (mg/mm)</td>
<td>16.3 ± 0.7</td>
<td>19.6 ± 0.8*</td>
<td>16.6 ± 0.3**</td>
</tr>
<tr>
<td>LVESe (cm)</td>
<td>0.39 ± 0.02</td>
<td>0.39 ± 0.02</td>
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<td>LVEdSe (cm)</td>
<td>0.68 ± 0.02</td>
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<td>RWT</td>
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<td>0.51 ± 0.04**</td>
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<tr>
<td>LV mass</td>
<td>0.36 ± 0.03</td>
<td>0.66 ± 0.06*</td>
<td>0.43 ± 0.06**</td>
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<tr>
<td>Vcf</td>
<td>3.92 ± 0.26</td>
<td>3.63 ± 0.23</td>
<td>3.71 ± 0.24</td>
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</table>

HW/TL, heart weight/tibial length; LVESe, left ventricular end-systolic dimension; LVEdSe, left ventricular end-diastolic dimension; RWT, relative wall thickness; LV mass, left ventricular mass; Vcf, velocity of circumferential fibre shortening. Data are mean ± SEM.

*P < 0.05 vs. sham.

**P < 0.05 vs. OVX-V.
3.4 G-1 inhibited the increase in cardiac NOX4 associated with oestrogen loss

In the hypertrophied hearts from OVX-mRen2.Lewis rats, we found that NOX4 mRNA was increased by 80% when compared with sham-operated rats. Importantly, this increase in cardiac NOX4 mRNA was inhibited by G-1 (Figure 5A). Consistent with these changes in gene levels, cardiac NOX4 protein was also increased with OVX and attenuated by G-1 (Figure 5B and C).

RT–PCR did not reveal differences in the cardiac mRNA levels of NOX2, p22phox, and p47phox (see Supplementary material online, Figure S3).

3.5 Calcium-related gene expression in the heart

Cardiac mRNA levels of sarcoendoplasmic reticulum calcium ATPase (SERCA) 2, PLB, sodium-calcium exchanger 1 (NCX1), NCX3, calsequestrin 1 (CASQ1), CASQ2, triadin, cardiac ryanodine receptor 2 (RyR2), calreticulin, and calmodulin-1 were similar across all groups (see Supplementary material online, Figure S3). Likewise, the PLB-to-SERCA2 protein ratio, an index of SERCA2 inhibition, was not significantly influenced by ovarian hormonal withdrawal or G-1 treatment (see Supplementary material online, Figure S4).
3.6 Protective effects of GPR30 on angiotensin II-induced hypertrophy in H9c2 cardiomyocytes

To better understand the hypertrophic limiting capacity of GPR30, we used H9c2 cardiomyocytes treated with angiotensin II (Ang II) (Figure 6). Consistent with previous reports, Ang II (10^{-7}M) treatment for 24 h induced hypertrophy in H9c2 cardiomyocytes, demonstrated by increases in cell size (Figure 6A), protein content per cell (Figure 6C), and ANF mRNA levels (Figure 6B). These indicators of cell hypertrophy were significantly inhibited following treatment with either E2 or G-1 (10^{-7} M). This anti-remodelling effect of E2 was partially prevented by co-incubation with the GPR30 antagonist G15 (10^{-6}M), while the protective effect of G-1 on cell size was completely blocked by G15 (Figure 6).

4. Discussion

The most important findings of this study are that chronic GPR30 activation by its agonist G-1 for 2 weeks attenuated the adverse effects of ovariectomy on diastolic function and LV remodelling in the female mRen2.Lewis rat. Importantly, this GPR30-mediated lusitropic and anti-remodelling effect occurred in the absence of a significant reduction in systolic blood pressure. Together with our previous data from the oestrogen-intact-mRen2.Lewis rat, these observations provide evidence that an increase in GPR30 receptor expression in the heart may contribute to cardiac functional and structural stability during periods of physiological (e.g. oestrogen loss) and pathophysiological stress (e.g. high salt intake). Given the cardioprotective reports of G-1 in other animal models and strains, our findings are unlikely exclusive to the female mRen2.Lewis rat.

The adult mRen2.Lewis female rat, a congenic rodent model of angiotensin II- and oestrogen-dependent hypertension, consistently manifest elevations in systolic blood pressure, LV hypertrophy, and diastolic functional impairments when ovariectomy occurs between 4 and 5 weeks of age. Our approach of initiating treatment after the establishment of the exacerbated hypertension is practical as it emulates the cardiovascular phenotype of both pre- and post-menopausal women after the surgical or natural cessation of ovarian hormone production. While others have shown that non-selective ER stimulation using oestradiol attenuates the development of cardiac hypertrophy and fibrosis, our study using the selective agonist G-1 strongly suggests that the cardiac GPR30 receptor is involved in the maintenance of LV structure and function, particularly in females. LV hypertrophy commonly occurs in women after the cessation of ovarian oestrogen production and is an independent risk factor for the development of ventricular stiffness and impaired myocardial relaxation. In rodent studies, cardioprotective and anti-hypertrophic oestrogenic effects have been well described. In adult rats, ovariectomy inhibits female-specific protection against pressure-induced hypertrophic remodelling and attenuates the effect of age on ventricular remodelling, while oestradiol replacement reverses these effects. Consistent with these studies and our own, OVX-mRen2.Lewis rats exhibited increased myocyte cell size, ventricular wall thickness, and LV weight compared with oestrogen-intact littermates. One striking and novel finding from our in vivo study was that administration of G-1, the selective agonist for the G protein-coupled membrane ER GPR30, attenuated hypertrophic remodelling...
without affecting blood pressure. These favourable effects of GPR30 activation were further corroborated in H9c2 cultured cardiomyocytes where G-1 reversed Ang-II mediated increases in cell size, ANF expression, and cell protein content. While additional studies are needed to determine the signalling pathways associated with GPR30-mediated reductions in cardiomyocyte size, our data strongly suggest that GPR30 activation with G-1 preserves diastolic function after oestrogen loss, in part by attenuating hypertrophic remodelling.

Cardiac collagen deposition not only contributes to hypertrophic remodelling, it directly influences LV compliance and, subsequently, diastolic function. In this study and our previous studies, oestrogen loss or G-1 administration. Indeed, other mechanisms that modulate LV relaxation, such as cardiac bioenergetics, conformation of the cytoskeleton, or titin isoform expression could be involved in the improved lusitropic function induced by G-1,1,5,2

The downstream signalling mechanisms of GPR30 in the heart are still unclear. Acute ex vivo studies show that G-1 inhibits Akt and Erk pathways which become activated by ischaemia and reperfusion injury.20 The present study shows that G-1 attenuated the up-regulation of NOX4 in OVX-rat hearts. NOX4 expression is increased by hypertrophic stimuli, including angiostatin II, and mediates cardiac hypertrophy and heart failure induced by pressure overload and chronic angiotensin II treatment.53 Therefore, NOX4 may also play an important role in LV remodelling in the female mRen2-Lewis rat, an established model of oestrogen and angiotensin II-sensitive hypertension.32 Since NOX4 is known to be a significant source of reactive oxygen species (ROS) in the failing heart, future studies evaluating cardiac ROS production will be required to determine whether the loss of endogenous activation of cardiac GPR30 or the interaction between angiotensin II and cardiac GPR30 signalling leads to an increase in oxidative stress, and subsequent NOX4-related hypertrophic remodelling.

**4.1 Limitations of this study**

Subcutaneous administration of a selective GPR30 agonist was used to determine the role of this receptor in the maintenance of LV structure and diastolic function. Although our dose of G-1 had no overt effect on blood pressure, we cannot exclude the possibility that G-1 has some other systemic action that could indirectly alter vascular haemodynamics and subsequently improve cardiac structure and function.
diastolic function. The presence and content of G-1 in the heart after subcutaneous administration was not determined in this study. However, evidence strongly suggests that the cardiac effects of G-1 occur mainly through its activation of GPR30 in the heart: (i) data show the presence of GPR30 in the heart (see Supplementary material, Figure S1 and Ref. 17) and the high affinity and specificity of G-1 to GPR30; and (ii) exogenous administration of G-1 is commonly used to study the roles of cardiac GPR30 in both ex vivo studies and in vivo studies, without leading to confounding factors as reported in the GPR30 knockout model. Although no specific myocardial phenotypic changes were reported in the global GPR30 knockout mice, studies using conditional cardiomyocyte-specific GPR30 knockout animals will be needed to determine the exact physiological functions of GPR30 in the female heart. A second limitation is that early ovariectomy in the mRen2.Lewis female does not address the age-related changes that also influence the cardiac diastolic dysfunction phenotype of older women. Indeed, a more hormonally relevant model mimicking human menopause might be useful in future studies. Thirdly, although tissue Doppler echocardiography is a well-accepted methodology for the clinical assessment of lusitropic function, other load-independent approaches, such as cardiac catheterization with pressure–volume analyses, could provide more quantitative information of diastolic function in the experimental setting.

4.2 Clinical implications
Preclinical studies support the potential benefits of oestrogen therapy in reducing the risk of cardiovascular diseases. Oestrogen replacement therapy in hypertensive post-menopausal women has also been shown to diminish cardiac fibrosis and hypertrophy. However, oestrogen replacement therapy remains controversial given that data from the Women’s Health Initiative (WHI) and the Heart and Estrogen/Progestin Replacement Study (HERS) did not show protective cardiovascular benefits in older women who received conjugated equine oestrogen (Premarin® Pfizer Pharmaceuticals, Inc., New York, NY, USA) with or without synthetic progestins (medroxyprogesterone acetate) well after the onset of menopause. Prior to halting the hormone replacement arms of the WHI study, there was an increased risk for myocardial events and strokes among the oestrogen-replete post-menopausal women. Similarly, the HERS study found an increased risk for thromboembolic events in a large group of hormone-replaced post-menopausal women with established coronary disease. Fortunately, it does not appear that the increased cardiovascular risks, including myocardial infarction, stroke, pulmonary embolism, and venous thrombosis persist once hormone replacement is discontinued. Preclinical data also suggest that late-in-life oestrogen may adversely influence cardiovascular risk. Specifically, ovariectomy in mid-aged mRen2.Lewis female rats conveyed protection against salt-induced renal damage when compared with oestrogen-intact littermates. Certainly, in order to ascertain the appropriate composition of oestrogens, source, dosage, and the therapeutic window for perimenopausal women, more studies focused on the dynamic relationship between oestrogens and the cardiovascular system are needed. Despite a disease continuum of great clinical importance and urgency, diastolic dysfunction in post-menopausal women is not well understood and its medical management remains largely empirical. Herein, using a reverse translational rodent model that emulates the cardiovascular phenotype of women after the surgical or natural cessation of ovarian hormonal production, we conclude that the loss of endogenous GPR30 activation in the heart could provoke enhanced deposition of interstitial collagen and cardiomyocyte hypertrophy, eventually leading to diastolic dysfunction. The improved myocardial relaxation, reduced filling pressures, and anti-remodelling that occurred with the administration of a highly specific GPR30 agonist, late after ovariectomy, further reveals the importance of the GPR30 receptor in the maintenance of female-sex-specific cardiac function and structure.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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Conflict of interest: none declared.

References


Kuroda T, Ago T, Matushima S, Zhai P, Schneider MD, Sadashima J. NADPH oxidase (Nox4) is a major source of oxidative stress in the failing heart. *Proc Natl Acad Sci U S A* 2010;107:15565–15570.


