GPER modulates tone and coronary vascular reactivity in male and female rats

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Abstract

Compared with age-matched men, premenopausal women are largely protected from coronary artery disease, a difference that is lost after menopause. The effects of oestrogens are mediated by the activation of nuclear receptors (ER α and ER β) and by the G protein-coupled oestrogen receptor (GPER). This study aims to evaluate the potential role of GPER in coronary circulation in female and male rats. The baseline coronary perfusion pressure (CPP) and the concentration–response curve with a GPER agonist (G-1) were evaluated in isolated hearts before and after the blockade of GPER. GPER, superoxide dismutase (SOD-2), catalase and gp91phox protein expression were assessed by Western blotting. Superoxide production was evaluated *'in situ'* via dihydroethidium fluorescence (DHE). GPER blockade significantly increased the CPP in both groups, demonstrating the modulation of coronary tone by GPER. G-1 causes relaxation of the coronary bed in a concentration-dependent manner and was significantly higher in female rats. No differences were detected in GPER, SOD-2 and catalase protein expression. However, gp91phox expression and DHE fluorescence were higher in male rats, indicating elevated superoxide production. Therefore, GPER plays an important role in modulating coronary tone and reactivity in female and male rats. The observed differences in vascular reactivity may be related to the higher superoxide production in male rats. These findings help to elucidate the role of GPER-modulating coronary circulation, providing new information to develop a potential therapeutic target for the treatment of coronary heart disease.

Key Words

- ▶ coronary arteries
- ▶ GPER
- ▶ G-1
- ▶ sex differences
- ▶ vasodilation

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Introduction

It is well known that men are more susceptible to the risk of developing cardiovascular diseases (CVDs) compared with premenopausal women. Despite this sex difference, CVDs are also the leading cause of death among

post-menopausal women (Mosca *et al.* 2004, Heron 2013), possibly because of changes in hormone levels (Reslan & Khalil 2012), specifically the decrease in plasma oestrogen levels (Mendelsohn & Karas 2007). Thus, previous studies

have suggested that oestrogen (primarily 17 β -oestradiol, E2) plays a protective role in the cardiovascular system in women (Kurt & Buyukafsar 2013, Santos *et al.* 2014).

E2 can have genomic (or nuclear) and non-genomic (or extra-nuclear) effects via activation of its classic receptors, the α and β oestrogen receptors (ER α and ER β), and through binding to a membrane receptor known as the G protein-coupled oestrogen receptor (GPER), which is also referred to as GPR30 (Filardo *et al.* 2000, Revankar *et al.* 2005). Currently, to explore the various functions of GPER across multiple systems, including the cardiovascular system, a selective agonist for this receptor (G-1) has been extensively used (Bologa *et al.* 2006, Nilsson *et al.* 2011).

The vascular effects of G-1 have been evaluated in various arteries. This agonist has been found to function as a dilator in internal mammary arteries in humans (Haas *et al.* 2007, 2009), the thoracic aorta in mRen2.Lewis rats (Lindsey *et al.* 2009), the aorta (Jang *et al.* 2013) and the mesenteric artery in rats (Lindsey *et al.* 2011a), the coronary artery in pig (Meyer *et al.* 2010), spontaneously hypertensive rats (De Francesco *et al.* 2013) and the carotid artery in male and female Sprague–Dawley rats, and these effects may be related to significantly decreased NADPH-stimulated superoxide production and the direct action of G-1 as a superoxide scavenger independent of GPER (Broughton *et al.* 2010). In fact, others studies have shown that GPER may mediate effects by changing the oxidative (Lindsey *et al.* 2011b, Liu *et al.* 2016, Yao & Abdel-Rahman 2016) or antioxidative pathways (Kurt *et al.* 2015, 2016, Li *et al.* 2015). However, the relationship between GPER and these pathways has been not well described.

In contrast, Kurt and Buyukafsar (2013) showed that G-1 administered in increasing concentrations triggers vasoconstriction in isolated and perfused kidneys in rats, suggesting that it exhibits diverse functions that may vary depending on the artery bed under investigation.

However, whether GPER modulates coronary vascular tone and the potential effects of GPER activation on coronary circulation in the presence of its agonist in male and female rats remains unclear. Therefore, the present study aimed to evaluate potential sex differences in the modulation of basal tone by GPER and the effects exerted by G-1 on the reactivity of the coronary vascular bed.

Materials and methods

Experimental animals

The present study used 10-week-old adult Wistar rats (*Rattus norvegicus albinus*) of both sexes reared at the animal facility

of the Health Sciences Center at the Federal University of Esprito Santo. All procedures were approved by the Ethics Committee on the Use of Experimental Animals of UFES under protocol No. 048/2013. The animals were maintained in group cages with free access to water and food (Purina Labina, So Paulo, Brazil), under controlled conditions of temperature (22–24C) and humidity (40–60%), with a 12/12-h light-darkness cycle.

Experiments in isolated hearts

Animals were anaesthetised with sodium pentobarbital administered intraperitoneally (i.p.) at 50mg/kg, and killed via decapitation. After opening of the thoracic cavity, the heart was exposed, freed from surrounding tissues, and rapidly transferred to an apparatus for perfusion of the isolated heart with aortic cannulation. The coronary vascular bed was examined using the modified Langendorff method (Hugo Sachs Electronics, March–Hugstetten, Germany) as described previously (Santos *et al.* 2004). Briefly, isolated hearts were perfused with modified Krebs solution (120mM NaCl, 1.25mM CaCl₂·2H₂O, 5.4mM KCl, 2.5mM MgSO₄·7H₂O, 2.0mM NaH₂PO₄·H₂O, 27.0mM NaHCO₃, 1.2mM Na₂SO₄, 0.03mM EDTA, and 11.0mM glucose), heated continuously at 37C in a water bath, and gassed with a carbogenic mixture of 95% O₂ and 5% CO₂ in a saturation chamber to maintain a stable pH of 7.4. A constant coronary flow of 10mL/min was maintained using a roller pump (Hugo Sachs), and the changes in coronary perfusion pressure (CPP) were directly associated with changes in vascular resistance.

The baseline CPP was measured using a pressure transducer (ADInstruments MLT0380/A Reusable BP Transducer, ADInstruments, Australia) connected to a digital data acquisition system (Power Lab System, AD Instruments, Bella Vista, New South Wales, Australia). The transducer was fixed near the aortic perfusion cannula, through which the coronary bed was perfused. LabChart software version 7.3.1 (ADInstruments) was employed for reading the recordings obtained with the data acquisition system. A latex balloon located at the end of a steel cannula was inserted into the left ventricle and connected to the pressure transducer to measure the isovolumetric cardiac force. The balloon was inflated with the aid of a glass syringe to maintain a preload of 10mmHg. After a 40-min stabilisation period, the baseline CPP was measured. To evaluate the vascular response to G-1, a dose–response curve (600–10,000nM) for G-1 (Azano Pharmaceuticals, NM, USA) was obtained after *in bolus* infusion. The relaxing response was expressed as the percentage of relaxation and calculated by the following equation:

$$\Delta\% \left(\frac{\text{CPP}_{\text{after infusion}} \times 100}{\text{CPP}_{\text{before infusion}}} \right) - 100$$

In addition, to evaluate GPER modulation of the basal tone of the coronary bed, a selective GPER antagonist (G36 at 1 μM ; Azano Pharmaceuticals, NM, USA) was added to the modified Krebs solution, which was perfused for at least 20 min, and the CPP was measured again. Both G-1 and G36 were diluted in dimethylsulfoxide (<0.01%). The dimethylsulfoxide concentration used as solvent in this study had no effect when used by *in bolus* administration (data not shown).

Dissection of the coronary arteries

The coronary arteries were dissected as described previously (Claudio *et al.* 2013). Briefly, animals were anaesthetised with sodium pentobarbital at 50 mg/kg i.p. and killed via decapitation. The thoracic cavity was opened, and the heart was removed and maintained in a modified Krebs solution (120 mM NaCl, 1.25 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5.4 mM KCl, 2.5 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 27.0 mM NaHCO_3 , 1.2 mM Na_2SO_4 , 0.03 mM EDTA, and 11.0 mM glucose). The anterior descending branch of the left coronary artery and the septal branch were isolated with the aid of a dissecting microscope (M900, F. Vasconcelos, São Paulo, Brazil) and then freed from surrounding muscle tissue and frozen at -80°C until use.

Western blotting

The coronary arteries were homogenised in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA.2Na, and 1 mM MgCl_2), and a protease inhibitor (Sigma Fast, Sigma) was added. The protein concentration was measured using the Lowry method (Lowry *et al.* 1951), and bovine serum albumin was employed as a standard. Equal amounts of protein (50 μg) were separated via 10% SDS-PAGE. The proteins were subsequently transferred to polyvinylidene difluoride (PVDF) membranes and incubated with a rabbit monoclonal anti-GPER antibody (1:500; Santa Cruz Biotechnology, cat. sc-48524-R; lot #C1414), a rabbit polyclonal anti-SOD-2 antibody (1:1000; Sigma, cat. SAB2102261, lot #QC25874), a rabbit polyclonal anti-gp9phox antibody (1:1000; BD Biosciences, cat. 611414, lot #2146906, NJ, USA), a mouse monoclonal anti-catalase antibody (1:2000; Sigma, cat. C0979, lot. #082M4837) and a mouse monoclonal anti- β -actin antibody (1:5000; Sigma, cat. A5442, lot #061M4808). After washing, the membranes were incubated with

alkaline phosphatase-conjugated anti-rabbit (1:7000, Santa Cruz Biotechnology) or anti-mouse (1:3000, Abcam) secondary antibody. Bands were visualised using a 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) system (Invitrogen Corporation) and quantified with ImageJ software (National Institute of Health, NIH). Protein expression was calculated based on the ratio of the specific density of each protein band vs the corresponding density of β -actin.

Dihydroethidium (DHE) fluorescence

The coronary arteries were isolated, cleaned, freed from surrounding tissues and immediately frozen in cryoprotection liquid (Tissue Tek – OCT). The blocks containing the coronary arteries were stored at -70°C until the preparation of sections using a cryostat (10-mm thick sections prepared at -25°C in the dark). The slides were stored at -20°C until use.

For analysis of DHE fluorescence, the sections were washed with complete modified Hanks' solution (1.6 mM CaCl_2 , 1.0 mM MgCl_2 , 120.0 mM NaCl, 5.0 mM KCl, 0.5 mM NaH_2PO_4 , 10.0 mM glucose and 10.0 mM HEPES at pH 7.4) and subsequently incubated with DHE (which is selective for the superoxide anion, 10 $\mu\text{mol/L}$) for 30 min at room temperature. After the incubation period, the slides were washed, coverslipped with 24 $\times$ 50 mm coverslips (Knittel Glaser) and examined on a confocal microscope (Leica TSC SP2 or SP5) with an oil-immersion objective at a magnification of 63 $\times$. Leica Application Suite–Advanced Fluorescence Lite 2.3.0 (LAS-AF Lite, Leica Microsystems, 1997–2010) analysis software was used to capture images of the sections in xyz mode, with sequence analysis, bidirectional –29.49 analysis at 200 Hz in the 1024 $\times$ 1024 format, a zoom of 1 and a magnification of 63 $\times$ in TCS SP2 or SP5 acquisition mode. Photomicrographs from 3 to 5 animals per group were compared in terms of the same area of vascular smooth muscle (VSM) (rectangles of 1.00 $\times$ 0.60 from 5 different regions of VSM) and analysed with ImageJ software (1.46r, NIH).

Statistical analysis

Data are expressed as the mean $\pm$ S.E.M. To identify possible outlier data, a two-sided Grubbs' test was used to identify whether at least one outlier was present in each data set. When the Grubbs' test identified one outlier, we used an adapted ROUT method to detect any outliers from the column data and removed them according to a Q setting of 1% ($\alpha=0.01$). For each data set, the D'Agostino-Pearson

omnibus normality tests were also performed. Paired or unpaired Student's *t* test was used to analyse coronary protein expression and dihydroethidium fluorescence. CPP data and vascular reactivity to G-1 were analysed by two-way analysis of variance (ANOVA), followed by the Bonferroni *post hoc* test. Each *P* value is adjusted to account for multiple comparisons. Statistical significance was set at *P*<0.05.

Results

Basal CPP

Basal CPP levels (Fig. 1) were significantly higher in female rats (109 ± 3 mmHg) compared with male rats (69 ± 3 mmHg, *P*<0.0001). Moreover, CPP levels increased significantly in both female (133 ± 10 mmHg, *P*=0.0195) and male rats (83 ± 7 mmHg, *P*=0.044) after the addition of the selective GPER antagonist (G36) to

the modified Krebs solution, demonstrating for the first time important contribution of GPER to the modulation of coronary vascular tone. To confirm the participation of sex hormones in the CPP, the animals were castrated. Castration caused a significant reduction in the baseline CPP only in the female group (108.8 ± 3.4 – 89.73 ± 2.7 mmHg, *P*=0.0156). The use of G36 in castrated animals increased the CPP only in the male group (56.79 ± 5.4 – 84.19 ± 11.8 mmHg, *P*=0.0293; Fig. 1).

Vascular reactivity of the isolated coronary bed

To evaluate the vascular reactivity of the coronary bed in response to the GPER receptor agonist, a dose–response curve for G-1 was constructed (Fig. 2). We observed a progressive, dose-dependent vasodilation of the coronary bed in female rats in response to G-1 (600 nM vs 3000 nM, *P*=0.0340; 1000 nM vs 6000 nM, *P*<0.0001; 3000 nM vs 6000 nM, *P*=0.0079 and 6000 nM vs 10,000 nM, *P*=0.0026). However, in the male group this response was significant only at the highest dose (600 nM vs 10,000 nM, *P*=0.0029 and 1000 nM vs 10,000 nM, *P*=0.0132). Furthermore, the relaxing response was significantly higher in female rats (*P*<0.0001). Nevertheless, in the presence of the inhibitor G36, G-1-induced dilation was abolished in male rats (*P*<0.0001) and reverted to vasoconstriction in female rats (*P*<0.0001).

Figure 1

Basal coronary perfusion pressure (CPP) in isolated hearts before (A) and after (B) the antagonism of GPER with G36 from sham and castrated female and male rats. Data are expressed as the mean $\pm$ S.E.M. (*n*=7 per group). **P*<0.05 vs baseline from sham female rats; **P*<0.05 vs baseline from castrated female rats; +*P*<0.05 vs to the same group under control conditions (before G36, shown in panel A). For the analysis, two-way ANOVA was used, followed by the Bonferroni *post hoc* test.

Figure 2

Dose–response curve for the agonist G-1 (600–10,000 nM) in female rats before (*n*=8, filled circles) and after (*n*=7, open circles) blockade with G36 and in male rats before (*n*=9, filled squares) and after (*n*=7, open squares) the same treatment. Note that the downward deflections (positive values) represent vasorelaxation, while the upward deflections (negative values) represent vasoconstriction. Data are expressed as the mean $\pm$ S.E.M. **P*<0.05 vs the female group before G36, **P*<0.05 vs the male group before G36 and +*P*<0.05 vs the female group after G36. For the analysis, two-way ANOVA was used, followed by the Bonferroni *post hoc* test.

Figure 3

Coronary protein expression of GPER from female ($n=8$) and male rats ($n=7$). Data are expressed as the mean $\pm$ S.E.M. * $P < 0.05$ vs female rats. For the analysis, an unpaired Student's t test was used.

Protein expression

Protein expression in coronary arteries is presented in Figs 3 and 4. GPER (Fig. 3), SOD-2 and catalase expression did not differ significantly between male and female rats (Fig. 4A and B). However, the expression of the membrane-bound subunit of Nox2 (i.e., gp91phox) significantly increased in the coronary arteries of male rats (Fig. 4C; $P < 0.05$).

Measurement of superoxide anion (O_2^-) by DHE fluorescence

The analysis of DHE fluorescence in coronary arteries is summarised in Figs 5 and 6. The results indicate that O_2^- fluorescence levels were higher in male rats (Fig. 5C and D) compared with female rats (Fig. 5A and B). After stimulation with G-1, O_2^- fluorescence levels increased in

male rats (Fig. 6C), while no difference was observed in the female group (Fig. 6G).

Discussion

The present study aimed to evaluate the role of GPER in modulating basal tone and coronary vascular reactivity in male and female rats. The main findings of this study are as follows: (i) GPER plays an important role in the modulation of coronary basal tone in male and female rats, and (ii) G-1 promotes vasodilation of the coronary bed in male and female rats; this response is more pronounced in female rats, despite the lack of a difference in GPER expression.

Another important observation was the difference in basal CPP levels between the sexes, with significantly higher basal CPP levels being observed in female rats. This result was similar to those of previous studies from our laboratory performed using normotensive animals (Moyses *et al.* 2001, Santos *et al.* 2004, 2016) and spontaneously hypertensive rats (Santos *et al.* 2010). In addition, this difference was maintained even when a selective GPER antagonist was added to the modified Krebs solution.

The mechanisms responsible for the increased CPP levels observed in female rats compared with male rats of the same age have not been fully elucidated. However, Figueroa-Valverde and coworkers (Figueroa-Valverde *et al.* 2011) demonstrated that oestradiol infusion significantly increased the perfusion pressure and coronary resistance of isolated hearts in rats due to an increase in calcium levels via activation of L-type calcium channels through non-genomic molecular mechanisms. In fact, our results show that after castration, the CPP was significantly reduced in the female group. Consistent with the involvement of oestradiol in modulating the CPP, Moyses and coworkers

Figure 4

Coronary protein expression of the antioxidant enzymes SOD-2 (A) and catalase (B) and the gp91phox subunit of the NADPH oxidase enzyme complex (C) in female ($n=8$) and male rats ($n=8$). Data are expressed as the mean $\pm$ S.E.M. * $P < 0.05$ vs female rats. For the analysis, an unpaired Student's t test was used.

Figure 5

Oxidative stress levels in coronary arteries based on dihydroethidium fluorescence (DHE, red staining) in female ($n=4$, A) and male rats ($n=5$, C). Bar graph shows the measurement of fluorescence intensity emitted by DHE in the medial layer of vascular smooth muscle. Data are expressed as the mean $\pm$ S.E.M. * $P < 0.05$ vs female rats. Photomicrographs are related to dichroic phase contrast, identifying the lumen (L), medial layer of vascular smooth muscle (M) and cardiomyocytes (C) from females (B) and males (D). Bar corresponds to 50 μ m. For the analysis, an unpaired Student's t test was used.

(Moyses *et al.* 2001) showed that oestrogen replacement in gonadectomized rats restores the baseline CPP in females and increases the CPP in males to become similar to those found in naive female animals. Indeed, a recent study from our group also showed that gonadectomy reduces the baseline CPP in females and has no effect on the baseline CPP of hearts from male rats (Santos *et al.* 2016). Notably, even after gonadectomy, these differences in the CPP continued to exist between females and males, perhaps because the benefits of oestrogens on the coronary arteries were not completely lost after gonadectomy. Therefore, we suggest that the higher basal tone found in female rats is due to oestrogen. Higher CPP could allow greater vasodilation, thus exerting cardioprotective effects when the supply of oxygen and nutrients are reduced (Santos *et al.* 2016).

After perfusion with G36, the CPP significantly increased in sham animals of both sexes. Although other

studies have used Langendorff's technique to assess the role of GPER in cardiac parameters (Bopassa *et al.* 2010, Kabir *et al.* 2015), to our knowledge, our study is the first to show that GPER plays an important role in the local modulation of the vascular tone of the coronary bed. GPER appears to exert a negative modulatory effect on the elevation of the CPP induced by oestrogen. In fact, after the use of G36 in sham females, the CPP increased significantly, indicating that G36 removes this negative modulatory effect. In castrated females, on the other hand, the effect of CPP elevation induced by oestrogen would not occur, indicating that the use of G36 would not have the same effect as that observed in the sham group, which would explain why we did not observe differences in CPP in castrated females after blockade with G36.

To our knowledge, this study is the first to demonstrate the vasodilatory effect of G-1 in the coronary vascular bed, since the vascular action of this compound has

Figure 6

Oxidative stress levels in coronary arteries based on dihydroethidium fluorescence (DHE, red staining) after G-1 stimulation in female ($n=4$, A, B, C and D) and male rats ($n=5$, E, F, G and H). Bar graph represents the variation (percentage) from the baseline of fluorescence intensity emitted by DHE (% Δ basal) in the media layer of vascular smooth muscle. Data are expressed as the mean $\pm$ S.E.M. * $P < 0.05$ vs female rats. Photomicrographs are related to dichroic phase contrast, identifying the lumen (L), medial layer of vascular smooth muscle (M) and cardiomyocytes (C) from females (B and D) and males (F and H). Bar corresponds to 25 μ m. For the analysis, an unpaired Student's t test was used.

been evaluated previously only in isolated vessels, as shown by Lindsey and coworkers (Lindsey *et al.* 2011a). In our study, the acute administration of G-1 promoted vasodilation in the coronary vascular beds of isolated hearts from male and female rats. Our previous studies have already demonstrated that E2 is able to induce a relaxing response in the coronary vessel (Santos *et al.* 2004, 2010, 2012, 2016). However, such actions could not be directly attributed to GPER, since oestradiol also acts on intracellular receptors (ER α and ER β). Indeed, G-1 has been shown to induce endothelium-dependent dilation that is not affected by the concomitant blockade of ER α and ER β (Meyer *et al.* 2010).

Our study also revealed sex differences in G-1-induced relaxation. Lindsey and coworkers (Lindsey *et al.* 2013) reported a similar difference using G-1 as a vasodilator agonist; however, this study was performed in mesenteric arteries. In resistance arteries obtained from subcutaneous tissues of humans, G-1-induced relaxation was found to be significantly greater among post-menopausal women compared with men from the same age group (Arefin *et al.* 2014). In contrast, in carotid arteries (Broughton *et al.* 2010) and cerebral arterioles of Sprague–Dawley rats (Murata *et al.* 2013), no differences in G-1-induced relaxation were observed between the sexes.

To evaluate whether the increased vasodilator response to G-1 found in female rats was associated with higher GPER protein expression in coronary arteries, we measured the protein expression of this receptor; however, no significant differences were observed between the groups. Other studies have shown similar results, such as no differences in GPER expression in the brains (Matsuda *et al.* 2008) and cardiomyocytes (Deschamps & Murphy 2009) of male and female Sprague–Dawley rats. In contrast, Lindsey and coworkers (Lindsey *et al.* 2013) reported increased GPER expression in mesenteric arteries of female mRen2.Lewis rats.

Considering that no differences in GPER expression were found in coronary arteries in male and female rats, we evaluated whether the weaker dilation observed in male rats was due to a greater basal production of reactive oxygen species (ROS) compared with female rats according to our DHE results. Indeed, O $_2^-$ generation was higher in males even after stimulation with G-1. ROS production can impair the vascular response by interacting with the main factors responsible for vascular relaxation (Zou & Ulrich 1996, Liu *et al.* 2002, Fösterman 2010).

Accordingly, we evaluated the expression of antioxidant enzymes such as SOD-2 and catalase.

Furthermore, we analysed the gp91phox isoform of the NADPH oxidase enzyme complex. Our results indicate that SOD-2 and catalase protein expression was similar in both sexes; however, gp91phox expression was higher in male rats. These results support our hypothesis that higher oxidative stress in males may have contributed to the weaker response to G-1 in this group compared with females. In male animals, aside from the lower oestrogen levels, testosterone activates NADPH oxidase via the renin-angiotensin system (Fischer *et al.* 2002, Costa *et al.* 2015), contributing to high O $_2^-$ generation, one of the most important ROS (Chignalia *et al.* 2012). Superoxide, in turn, quickly reacts with NO and reduces its bioavailability, which may explain the lower relaxing response in the male group. In addition, the product of the reaction between NO and O $_2^-$ i.e., peroxynitrite (ONOO $^-$), is able to reduce the synthesis of prostacyclin (PGI $_2$), even at lower concentrations (10 $^{-8}$ M) (Zou & Ulrich 1996). Furthermore, ONOO $^-$ also impairs EDHF-mediated vasodilation reducing K $^+$ channel activity (Liu *et al.* 2002). The effect of GPER on oxidative stress is not fully elucidated. Indeed, Yao and Abdel-Rahman (2016) showed that acute GPER blockade with G15 attenuated ethanol-evoked increases in myocardial ROS. On the other hand, Broughton and coworkers (Broughton *et al.* 2010), also using an acute analysis protocol, examined whether G-1 directly scavenges superoxide using a cell-free enzyme assay. They found that in the presence of the GPER agonist G-1, NADPH-stimulated superoxide in the carotid and intracranial cerebral arteries was ~30–40% lower than levels generated by arteries treated with vehicle. On the other hand, a recent study demonstrated that GPER contributes to increased oxidative stress, thereby regulating NADPH oxidase (Nox), specifically Nox1, even without stimulation by oestrogen (Meyer *et al.* 2016). When we analysed chronic G-1 treatment, the actions of this GPER agonist seemed to be more homogeneous. In fact, several studies have demonstrated beneficial effects for chronic G-1 administration (Lindsey *et al.* 2009, Jessup *et al.* 2010, Li *et al.* 2012). Lindsey and coworkers (Lindsey *et al.* 2009) showed that chronic G-1 treatment significantly reduces hypertension in oestrogen-depleted mRen2.Lewis congenic rats. In addition, Jessup and coworkers (Jessup *et al.* 2010) found that chronic GPER activation improved myocardial relaxation in hypertensive female mRen2.Lewis rats and reduced cardiac myocyte hypertrophy and wall thickness in rats fed a high-salt diet. Nevertheless, Li and coworkers (Li *et al.* 2012) demonstrated that following chronic administration of

G-1 (200 µg/kg i.p., 2 times per day for 8 weeks), ACh-induced relaxation in the aortic segments from the diabetic OVX rats was significantly enhanced. Although chronic G-1 treatment appears not to be controversial, the exact mechanics related to the chronic actions of this GPER agonist have not yet been fully elucidated.

To confirm that the vasodilator response in the coronary bed was mediated by GPER, this receptor was blocked with G36. Indeed, this antagonist completely abolished G-1-induced relaxation in both sexes confirming the involvement of GPER. Nevertheless, GPER antagonism surprisingly converted the vasodilator response to a vasoconstrictor response in female rats. Kurt and Buyukafsar (2013) also demonstrated that G-1 is able to induce vasoconstriction. However, these authors observed this response before the antagonism of GPER. Therefore, the mechanisms involved in the vasoconstrictor response in female rats following the antagonism of GPER remain unclear, and further studies are necessary.

Recent studies using G15, another GPER antagonist that is less selective than G36 (Dennis *et al.* 2011), have indicated that pharmacological blockade of GPER attenuates the vascular relaxation induced by E2 and G-1 in aortic rings of female mRen2.Lewis rats (Lindsey *et al.* 2011a) and attenuates the relaxation of the coronary arteries in pigs (Yu *et al.* 2011).

In conclusion, our results indicate that GPER modulates basal tone and coronary vascular reactivity in male and female rats and that this modulation is more pronounced in female rats. However, although GPER protein expression in coronary arteries was similar between the sexes, the decreased response observed in male rats appears to be associated with higher oxidative stress in this group, likely due to higher expression of the pro-oxidant enzyme gp91phox. Notably, our findings may help elucidate the role of G-1 in coronary arteries, thereby contributing to the following: (i) our understanding of the actions of G-1 on the coronary arteries and (ii) the elucidation of the sex differences in the actions of this GPER agonist. Characterising these mechanisms may lead to a better understanding of GPER actions in the cardiovascular system and perhaps to the development of improved therapeutic strategies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

A R D conceived and designed the experiments, performed the experiments, analysed the data, contributed reagents/materials/analysis tools and wrote the paper. W N R and N T B D performed the experiments, analysed the data, contributed reagents/materials/analysis tools and wrote the paper. V M performed the experiments and contributed reagents/materials/analysis tools. E R G C performed the experiments, analysed the data, contributed reagents/materials/analysis tools, wrote the paper and critically read the manuscript. L P performed the experiments, contributed reagents/materials/analysis tools, and critically read the manuscript. L M B contributed reagents/materials/analysis tools and critically read the manuscript. M R M conceived and designed the experiments, performed the experiments, analysed the data, contributed reagents/materials/analysis tools, wrote the paper, and critically read the manuscript. R L S conceived and designed the experiments, performed the experiments, analysed the data, contributed reagents/materials/analysis tools, wrote the paper, and critically read the manuscript.

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