GPER-1 Rapid Regulation Influences p-Akt Expression to Resist Stress-Induced Injuries in a Sex-Specific Manner

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Summary

G protein-coupled estrogen receptor 1 (GPER-1) has gained recognition for its role in conferring cardioprotection. However, the extent to which GPER-1 exerts equally important effects in both sexes remains unclear. The study found similar expressions of GPER-1 in rat heart apex in both sexes. In male rats, administering epinephrine (Epi) at a dose of 31.36 μg/100 g resulted in a rapid decline in cardiac function, accompanied by a sharp increase in bax/bcl-2 levels. In contrast, female rats did not display significant changes in cardiac function under the same conditions. Additionally, compared to the injection of Epi alone (at a dose of 15.68 μg/100 g), the administration of G15 (GPER-1 antagonist) further decreased cardiac function in both male and female rats. However, it only increased mortality and lung coefficient in male rats. Conversely, G1 (GPER-1 agonist) administration improved cardiac function in both sexes. Notably, the apex of the male heart exhibited lower levels of inhibitory G protein (Gαi). Furthermore, female and male rats treated with Epi displayed elevated phosphorylated protein kinase B (p-Akt). Compared to their respective Epi groups, the administration of G15 increased p-Akt levels in female rat hearts but decreased them in male rat hearts. Conversely, the administration of G1 decreased p-Akt levels in females but rapidly increased them in male rats. Our study uncovers the vital role of GPER-1 in protecting against stress-induced heart injuries in a sex-specific manner. These findings hold immense potential for advancing targeted cardiac therapies and enhancing outcomes for both females and males.

Key words

GPER-1 • Sex differences • Heart • Stress • p-Akt

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Introduction

Cardiovascular diseases (CVDs) remain the primary cause of death worldwide [1]. Risk factors for CVD include those that are modifiable such as obesity, dietary behavior, smoking and those that are nonmodifiable including age, and gender [2]. Mortality of CVD gradually increases with age and gender disparity also plays an important role in CVDs [1,2]. Men under the age of 65 have a higher risk of CVD than women of similar age and the onset of menopause significantly increases the risk of CVDs in women aged 65 and older [1,2].

The sympathetic nervous system plays a crucial role in regulating cardiac function, and one of its primary mechanisms is the activation of β-adrenergic receptors (βARs) [3-5]. Stress may cause sympathetic overactivity and massive catecholamine releases, such as epinephrine and norepinephrine [3,6]. A higher concentration of catecholamines, primarily epinephrine, which has a higher affinity for β_2AR [7,8], could activate β_2AR to switch its coupling from stimulating G protein (Gαs) to inhibitory G protein (Gαi) during stressful conditions [8,9]. The ratio of β_2AR to β_1AR is higher in apical ventricular cardiomyocytes [10,11]. According to Paur *et al.*, stress cardiomyopathy (SCM) may occur when the Gαi signal pathway is overstimulated by the β_2AR in

response to higher epinephrine concentrations [11].

G protein-coupled estrogen receptor 1 (GPER-1), which mediates a series of non-genomic signals triggered by estrogen [12], has been shown to be expressed in a variety of tissues, such as the brain and heart, independent of species and sex [13-15]. Numerous studies have reported that GPER-1 can mitigate cardiac injury and enhance heart function by facilitating the translocation of proteins, their phosphorylation, and the reinstatement of a typical oxidative equilibrium [9,15-18]. For instance, Adu-Amankwaah *et al.* found that the activation of GPER-1 by estrogen or GPER-1 specific agonist G1 mitigated stress-induced cardiac injury and inflammation by modulating the translocation of ADAM17 [9]. Additionally, Deschamps and Murphy have reported that G1 reduced post-ischemic dysfunction and infarct size after I(ischemia)/R(reperfusion) *via* the phosphorylation of phosphatidylinositol 3-kinase (PI3K) [16].

Our previous studies found that chronic activation of GPER-1 with its agonist G1 attenuates heart failure by normalizing the expression of β_1AR and increasing the expression of β_2AR [19]. We have also shown that estrogen protected the myocardium against SCM by increasing the activity of the β_2AR-Gαs signal pathway in female rats and mice [9,20]. In this current study, we investigated the potential role of GPER-1 in both sexes and whether its effects are equally important or exhibit a sex-specific manner during acute stress.

Materials and Methods

Animals

All animal procedures complied with guidelines of the Animal Ethics Committee of Xuzhou Medical University (China) (permit number: xz11-12540) and with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011).

All rats were kept in standard cages in a room controlled at a temperature of 23 ℃ and a 12-hour/ 12-hour dark-light cycle. Rats were fed on pelleted food and water *ad libitum*. Female (Sprague-Dawley, SD, 200-250 g) and male rats (SD, 250-300 g) were used.

Materials

Epi was purchased from Sigma-Aldrich (St. Louis, MO, USA). G1 (agonist) and G15 (antagonist) of GPER-1 were purchased from Cayman (Ann Arbor, MI, USA). Rat BNP ELISA Kit was purchased from Uscn Life Science Inc. (Wuhan, China). Gαs and Gαi antibodies were purchased from NewEast Biosciences (Malvern, PA, USA). Akt and p-Akt antibodies were purchased from Cell Signaling Technology (Danvers, USA).

In vivo model of stress injury

Adult female and male SD rats were randomly divided into four groups: female and male control, Epi 7.84, 15.68, 31.36 μg/100 g groups. In this study, myocardial injury models were induced by venous epinephrine injection.

The animals were anesthetized and placed in a dorsal position. Body temperatures were maintained at 37±0.5 ℃ throughout the experiment using a thermostatically controlled warming plate. A 1.4-F Millar catheter was inserted through the right carotid artery into the left ventricle to measure hemodynamics. After 10 min of stabilization of the cardiac function, the right jugular vein was dissected to allow the insertion of a catheter for Epi bolus injection. Hemodynamic changes were recorded continuously for an additional 60 min by the Powerlab data acquisition system. After assessment of hemodynamics, the hearts were immediately removed and rinsed in ice-cold 0.9 % saline and stored at -80 ℃.

G1 and G15 treatment to investigate the cardioprotective effects of GPER-1 in female and male rats

Adult Female and male SD rats were divided into four groups: female and male control, Epi, G15+Epi, and G1+Epi groups. GPER-1 was either activated or inhibited using G1 and G15, respectively.

The animals were anesthetized, and the assessment of hemodynamics through left ventricular intubation was performed. After stabilization of the cardiac function for 10 min, Epi (15.68 μg/100 g) was injected through the right jugular vein in female and male Epi groups. In the groups labeled as G15+Epi and G1+Epi for both females and males, Epi was administered 10 min after the administration of G15 and G1, respectively. After an assessment of hemodynamics for 10 min, final blood samples were collected through an abdominal aortic puncture to measure plasma BNP levels. An appropriate amount of heparin was added to all blood samples and centrifuged at 1445.5× g for 15 min. The lungs were removed and weighed. The hearts were immediately removed and rinsed in ice-cold 0.9 % saline and stored at -80 ℃.

Enzyme-linked immunosorbent assay (ELISA)

According to the manufacturer's protocol, plasma

BNP concentration was measured using ELISA kits.

Western blot

The apical tissues of the harvested heart ventricles were homogenized in a lysis buffer containing phosphatase and proteinase cocktail inhibitor in a 100:1:1 ratio, respectively. The concentration of lysates were determined using BCA (Bicinchoninic Acid) Assay kits (Beyotime, China). Lysates' concentrations were normalized and diluted in loading buffer (130 mM Tris-HCl, pH 8.0, 20 % glycerol, 5 % sodium dodecyl sulfate (SDS), 0.02 % bromophenol blue, 2 % DTT) and denatured for 5 min at 95 °C. Equal amount of protein extracts were separated by 10 % polyacrylamide gel electrophoresis in the presence of SDS and then electrophoretically transferred onto nitrocellulose membranes. After incubation in blocking solution (4 % non-fat milk), membranes were then incubated overnight at 4 °C with corresponding primary antibodies against the following: bax and bcl-2 (1:1000, Cell Signaling Technology, USA), GPER-1 (1:250, Abcam, Cambridge, MA, USA), GAPDH (1:1000, Zhongshan, Beijing, China), Gαs and Gαi (1:1000, New East, USA), Akt and phospho-Akt (1:2000, Cell Signaling Technology, USA). This step was followed by incubation with secondary antibodies at room temperature for 1 h. The protein bands were visualized using enhanced chemiluminescence (Millipore, Darmstadt, Germany).

Statistical analysis

For each experimental series, data were presented as means ± S.E.M. Statistical analysis was performed with GraphPad Prism software (version 9.5.1). Statistical significance ($P<0.05$) for each variable was estimated by one-way ANOVA followed by Bonferroni *post hoc* tests. The Fisher exact test was used to compare the differences in the probability of mortality occurring. A *t*-test was used to analyze the data between the two groups.

Results

GPER-1 expression in heart apex and stress-induced cardiac injury caused by Epi in different dose groups in female and male rats

We extracted proteins from the tissue and conducted a Western blot analysis. As shown in Figure 1A, there were no differences in GPER-1 expressions in heart apex between female and male rats.

Hemodynamics was monitored in female and male rats treated with the three different Epi doses. Hemodynamics at 60 min in each dose group was measured, and the results are depicted in Figure 1B. With the increase in Epi dose, the LVSP, HR, and |±dP/dt| decreased obviously in the female Epi (31.36 μg/100 g) group, which occurred at a lower Epi dose (15.68 μg/100 g) in male rats.

With the increase in dose, the upregulation of bax/bcl-2 in female rats was not statistically significant. However, bax/bcl-2 upregulation in male rats was notable at an Epi concentration of 31.36 μg/100 g. With the increase in dose, the survival time of rats got shorter. Male rats exposed to Epi at a concentration of 31.36 μg/100 g had a significantly shorter survival time, with most surviving for approximately 6 min. Furthermore, in male rats, the ratio of bax to bcl-2, an indicator of apoptosis, rapidly increased under these conditions. The results are shown in Figure 1C.

GPER-1 improved hemodynamics and reduced mortalities induced by Epi in female and male rats

G15/G1 is a specific antagonist/agonist of GPER-1. We have reported the cardioprotective effect of GPER-1 *via* its chronic activation [19], but few papers have reported its rapid effect. G15 (760 $\mu g/kg^{-1}$)/G1 (480 $\mu g/kg^{-1}$) [17] was administered for 10 min before Epi injection.

Table 1 shows that activation of GPER-1 decreased Epi-induced mortality. There was no noticeable difference in mortality between the Epi and G1+Epi groups compared to the female and male control groups. Administration of G15+Epi caused no statistically significant increase in female rats' mortality, but male rats' mortality increased significantly (75 %) and was higher compared to females (13 %).

As shown in Figure 2A, B, activation of GPER-1 improved the hemodynamics of female and male rats influenced by Epi. The LVSP, LVEDP, and |±dP/dt| change influenced by G15/G1 had no statistical significance. All the hemodynamic indexes increased firstly, then decreased in female and male rats Epi, G15+Epi, and G1+Epi groups. The LVSP, |±dP/dt| of the female and male Epi groups decreased, while the LVEDP remained high. The administration of G15 further decreased the LVSP, and |±dP/dt| in female rats, while the LVEDP increased compared to the administration of Epi alone. However, the administration of G15 sharply decreased the LVSP, LVEDP, and |±dP/dt| in male rats. Treatment with G1 restored all the hemodynamic parameters in female and male rats to base data and

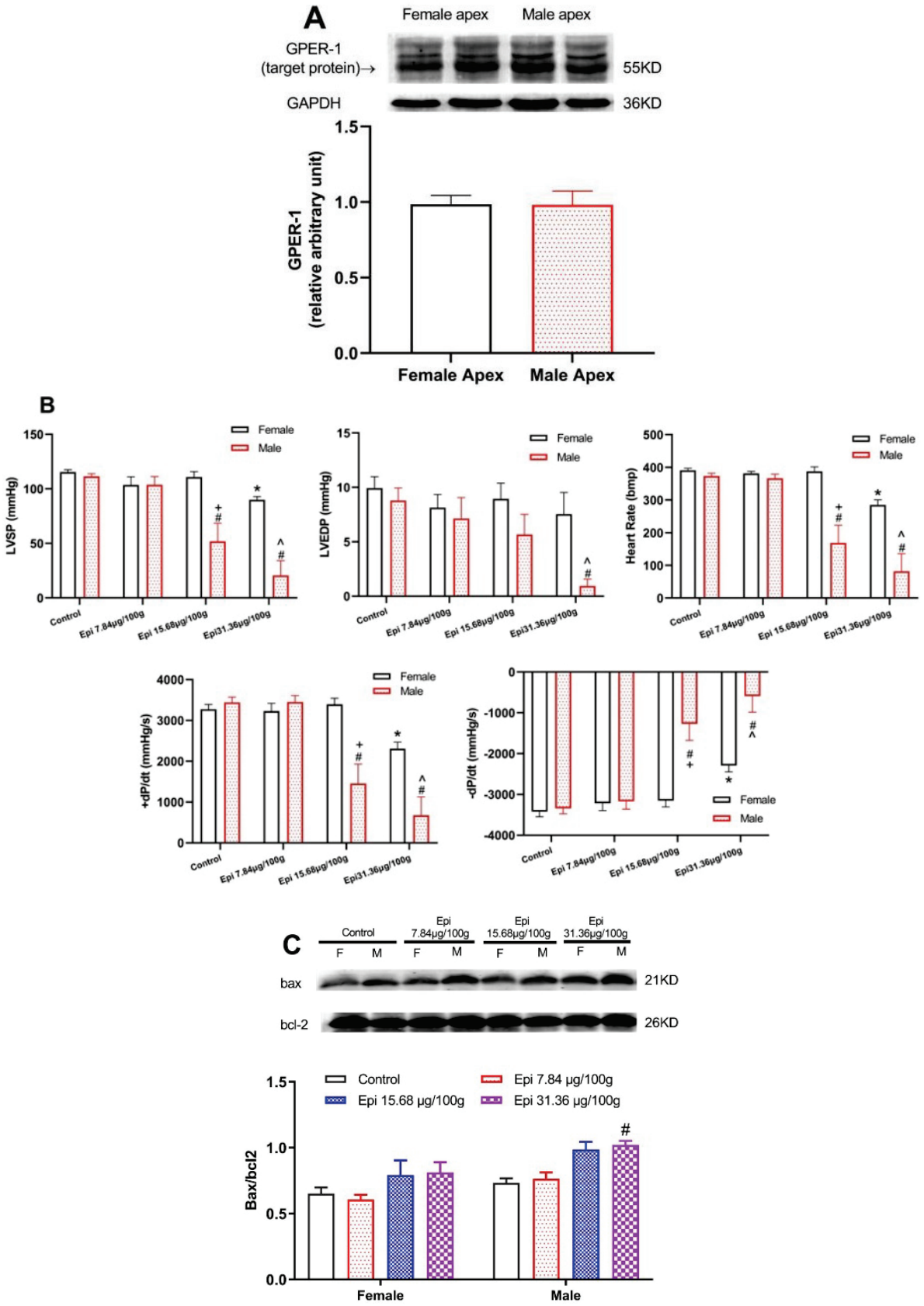

Fig. 1. (**A**) GPER-1 expression in heart's apex in female and male rats; (**B**) Hemodynamics at 60 min after Epi injection. HR, heart rate; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; +dP/dt, rate of pressure development; -dP/dt, rate of pressure decay; (**C**) bax/bcl-2 protein expression of cardiac apex in each dose group. Control groups (Normal Saline). Each value represents the means ± S.E.M., (A) n=6, (B) n=9, (C) n=5. Female (F), Male (M). * P<0.05 vs. Female control group; # P<0.05 vs. Male control group; + P<0.05 vs. Female Epi 15.68 μg/100 g group; ^ P<0.05 vs. Female Epi 31.36 μg/100 g group.

Table 1. GPER-1 mediated mortality changes in female and male rats.

	Female				Male			
	Control	**Epi**	**G15+Epi**	**G1+Epi**	**Control**	**Epi**	**G15+Epi**	**G1+Epi**
Survival	8	8	7	8	8	7	2	8
Death	0	0	1	0	0	1	6	0
Total	8	8	8	8	8	8	8	8
Mortality	0	0	13 %	0	0	13 %	75 %$^{*,\#,+}$	0

Each value represents the means ± S.E.M. n=8. * P<0.05 vs. Male control group; $^{\#}$ P<0.05 vs. Male Epi group; $^{+}$ P<0.05 vs. Female G15+Epi group.

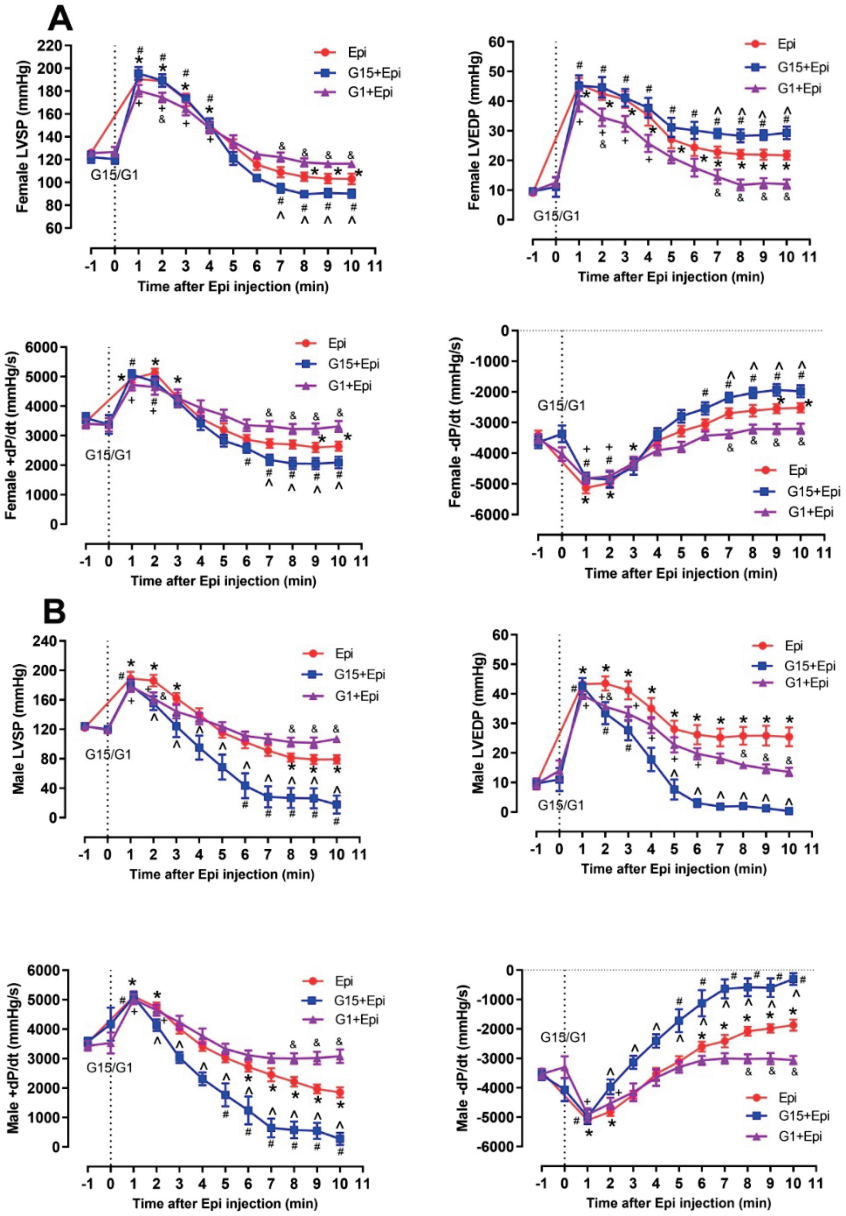

Fig. 2. GPER-1 mediated hemodynamic changes in female (**A**) and male (**B**) rats during Epi treatment. Each value represents the means ± S.E.M. n=8. LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; +dP/dt, rate of pressure development; -dP/dt, rate of pressure decay. * P<0.05 vs. base data in Epi group; $^{\#}$ P<0.05 vs. base data in G15+Epi group; $^{+}$ P<0.05 vs. base data in G1+Epi group; $^{\wedge}$ P<0.05, G15+Epi group vs. Epi group; $^{\&}$ P<0.05, G1+Epi group vs. Epi group.

decreased the LVSP and LVEDP at 2 min compared with the Epi groups.

GPER-1 attenuated cardiac injury induced by Epi in female and male rats

BNP levels are shown in Figure 3A. The BNP levels in plasma were significantly increased in Epi groups of female and male rats, and administration of G15 further elevated the levels. Compared with the Epi group, administration of G1 reduced the levels of BNP. There were no obvious differences between female and male rats in each treatment group.

Lung coefficients are shown in Figure 3B. There was no difference among each female group. Administration of G15+Epi increased the lung coefficient in male rats compared with their control, Epi groups, and female rats. Lung coefficient = Lung wet weight (mg)/Bodyweight (g)×100 %.

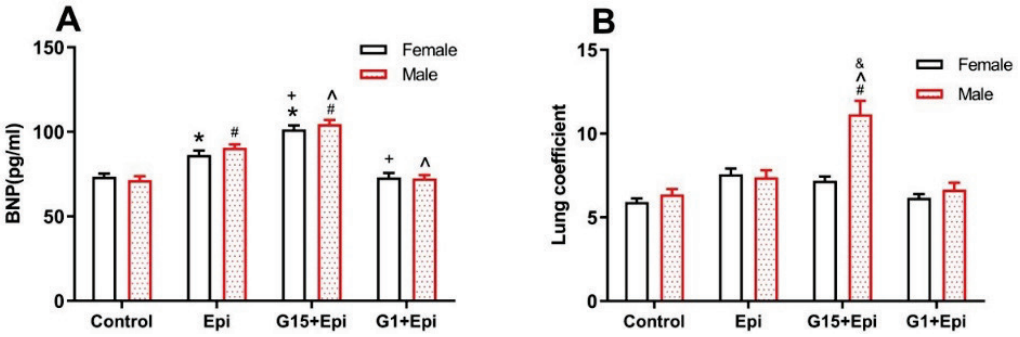

Fig. 3. The BNP (**A**) and the lung coefficient (**B**) changes of female and male rats induced by Epi. Each value represents the means ± S.E.M. n=8. * P<0.05 vs. Female Control group; # P<0.05 vs. Male Control group; + P<0.05 vs. Female Epi group; ^ P<0.05 vs. Male Epi group; & P<0.05 vs. Female G15+Epi group.

Gαs and Gαi levels showed sex-specific differences, and GPER-1 affected p-Akt expression in the heart apex of female and male rats exposed to Epi

We were interested in determining the differences in the mechanism involved in the GPER-1-mediated effect between female and male rats. β_1AR couples with Gαs to play a positive inotropic effect, and β_2AR couples not only with Gαs but also with Gαi to inhibit contraction. As shown in Figure 4A, the Gαi expressions in the female heart apex were higher than in the male rats. The Gαs and Gαi expressions had no obvious differences in female rats, but the Gαs expression was higher than Gαi in male rats.

Activation of GPER-1 has been shown to trigger the PI3K pathway, leading to a regulation in the phosphorylation of downstream kinases such as Akt [9]. As shown in Figure 4B, Epi treatment increased p-Akt expression in female and male rats. Administration of G15 further increased p-Akt expression, and treatment with G1 decreased its expression compared to the Epi group in female rats. However, compared with female rats, administration of G15 decreased p-Akt expression, and the treatment with G1 increased its expression compared to the Epi group in male rats.

Discussion

We simulated an acute stress state characterized by excessive release of catecholamines through intravenous administration of Epi bolus. To verify the sex-specific differences in the heart during various stress levels, different doses of Epi were administered. We conducted statistical analysis of the hemodynamic parameters 60 min following the administration of Epi, as well as the levels of apoptotic proteins, specifically bax/bcl-2 expressions. As the dose of Epi increased, the cardiac function of female rats declined notably when Epi was given at a concentration of 31.36 μg/100 g, and during this condition, the levels of bax/bcl-2 did not show a significant increase. In male rats, as the Epi dose increased, the reduction in cardiac function became more significant, especially when Epi was administered at a concentration of 15.68 μg/100 g. Male rats exhibited a significant increase in mortality, with most male rats dying within 5 or 6 min after the administration of Epi at a concentration of 31.36 μg/100 g, due to a rapid increase in bax/bcl-2 levels. Perhaps this can explain why men have a higher risk of CVD during stressful conditions.

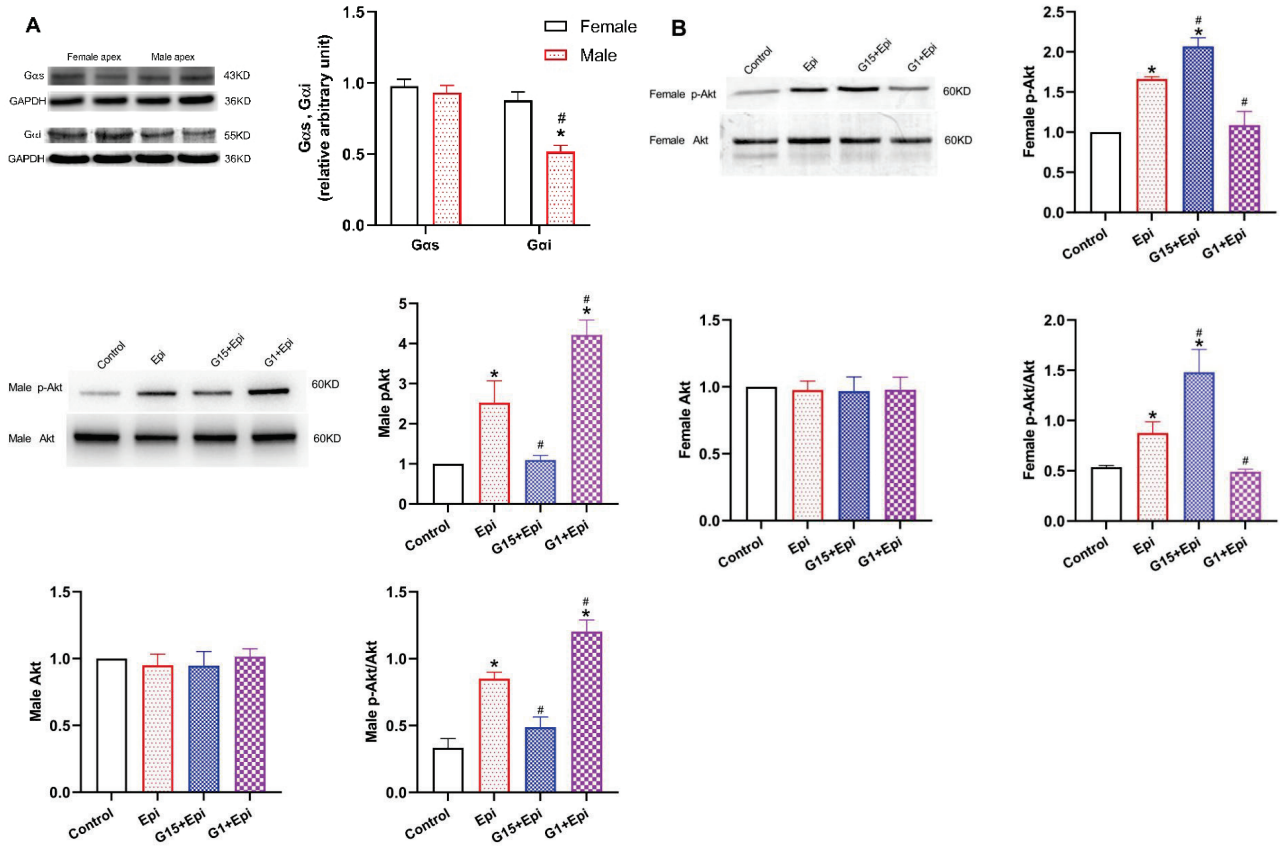

Fig. 4. (**A**) Gαs, Gαi protein expression in cardiac apex. Each value represents the means ± S.E.M., n=6. * P<0.05 vs. Female Gαi; # P<0.05 vs. Male Gαs. (**B**) GPER-1 mediated the p-Akt changes in Epi-treated female and male rats heart apex. Each value represents the means ± S.E.M. n=5. * P<0.05 vs. Control group; # P<0.05 vs. Epi group.

In recent years, studies about the cardioprotective effect of GPER-1 have increased. A study by Haas *et al.* revealed that GPER-1 plays a role in both sexes [21], facilitating estrogen cardioprotective activity *via* its rapid effects [22]. Consequently, we explored the GPER-1 protein levels specifically in the apical region of the heart. Our results indicated that the levels of GPER-1 expression in the apex of the heart were not significantly different between female and male rats. According to our findings, inhibiting GPER-1 signaling with G15 only increased male rat mortality, indicating different outcomes between female and male rats. This was demonstrated by comparing hemodynamics, BNP levels, and pulmonary coefficients. The levels of BNP are often used as indicators of cardiac injury [23]. Its elevation indicated that female and male rats had acute heart failure symptoms. However, heart failure in male rats increased lung coefficient compared to female rats. Also, the male rats exhibited pink foam in their mouths, a sign of acute pulmonary congestion caused by severe heart failure. This condition was irreversible and ultimately had fatal consequences for the male rats. GPER-1 plays an equally important protective role in female and male rats, and to further elucidate this protective role, agonist G1 was administered. Administration of G1 alleviated the increase of shrinkage induced by Epi at 2 min in female and male rats and male rats and ultimately improved heart function. G1's intervention could further activate GPER-1 to counteract Epi's storm.

The downstream pathways of GPER-1, such as Gαs and Gαi also played crucial regulatory roles. Studies in our laboratory have demonstrated that the activation of GPER-1 can adaptively modulate the Gαi/cAMP ratio [17]. Additionally, by balancing the activation of the Gαs and Gαi signaling pathways under stressful circumstances, GPER-1 mediates estrogen cardioprotection [9]. In this study, we explored the potential sex-related variations in the expressions of Gαs and Gαi in the apical region of the heart and examined whether GPER-1 can mitigate cardiac damage by promptly modulating p-Akt in female and male rats during stress. Gαi in female rats heart apex were highly expressed, while the expression of Gαs in male rats' hearts was superior.

P-Akt is the Gαi downstream signaling molecule [9]. Its increase has a protective effect on the heart, but overexpression may cause excessive inhibition of the systole [24]. Exposure to an Epi storm resulted in significant p-Akt

level increases in both male and female rats. Intriguingly, administering G15 led to elevated p-Akt in female rats but a reduction in male rats. The activation of GPER-1 decreased p-Akt in female rats and increased it in male rats, suggesting a reversed regulatory effect of GPER-1 during stress in females and males. In brief, based on the higher expression of Gαi in female rats, an Epi storm can also activate β_2AR to switch its coupling from Gαs to Gαi, which results in the substantial p-Akt levels. GPER-1 activation induces the Gαs-PKA-cAMP pathway [17], to balance the over activation of Gαi-p-Akt signaling, effectively counteracting excessive systolic inhibition. Conversely, in male rats, the prevailing Gαs advantage during an Epi storm leads to heightened activation of Gαs and its downstream pathways, resulting in excessive myocardial contraction. GPER-1 activation in this context further stimulates Gαi-p-Akt, providing resistance against severe myocardial injury.

Conclusions

Based on our findings, we can conclude that whether in females or males, GPER-1 emerges as a key player in stress, which is *via* sex-dependent mechanism. In females, activating GPER-1 resisted over-excited p-Akt. However, in males, GPER-1 activated p-Akt pathway to improve heart function and decrease mortality.

Conflict of Interest

There is no conflict of interest.

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