TRANSLATIONAL PHYSIOLOGY

A novel estrogen receptor GPER inhibits mitochondria permeability transition pore opening and protects the heart against ischemia-reperfusion injury

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Bopassa JC, Eghbali M, Toro L, Stefani E. A novel estrogen receptor gper inhibits mitochondria permeability transition pore opening and protects the heart against ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 298: H16–H23, 2010. First published October 30, 2009; doi:10.1152/ajpheart.00588.2009.—Several studies have recently demonstrated that G protein-coupled receptor 30 (GPER) can directly bind to estrogen and mediate its action. We investigated the role and the mechanism of estrogen-induced cardioprotection after ischemia-reperfusion using a specific GPER agonist G1. Isolated hearts from male mice were perfused using Langendorff technique with oxygenated (95% O2 and 5% CO2) Krebs-Henseleit buffer (control), with G1 (1 μM), and G1 (1 μM) together with extracellular signal-regulated kinase (Erk) inhibitor PD-98059 (5μM). After 20 min of perfusion, hearts were subjected to 20 min global normothermic (37°C) ischemia followed by 40 min reperfusion. Cardiac function was measured, and myocardial necrosis was evaluated by triphenyltetrazolium chloride staining at the end of the reperfusion. Mitochondria were isolated after 10 min of reperfusion to assess the Ca2+ load required to induce mitochondria permeability transition pore (mPTP) opening. G1-treated hearts developed better functional recovery with higher rate pressure product (RPP, 6140 ± 264 vs. 2,640 ± 334 beats⋅mmHg−1⋅min−1, P < 0.05) compared with the controls. The protective effect of G1 was abolished in the presence of PD-98059 [RPP: 4,120 ± 46 beats⋅mmHg−1⋅min−1, infarct size: 53 ± 2%, and Ca2+ retention capacity: 1.4 ± 0.11 μM/mg mitochondrial protein (P < 0.05)]. These results suggest that GPER activation provides a cardioprotective effect after ischemia-reperfusion by inhibiting the mPTP opening, and this effect is mediated by the Erk pathway.

G protein-coupled receptor 30; mitochondrial permeability transition pore; cardioprotection; infarct size; heart function

It is well established that estrogen has protective effects in cardiovascular function (26). Most of the biological effects of estrogen are mediated by classical estrogen receptors, α (ERα) and β (ERβ) (22, 23). In the classical genomic action, ERα and ERβ act as nuclear transcription factors binding to DNA response elements. Furthermore, activation of estrogen receptors in the cell membrane results in rapid nongenomic actions of estrogen involving cell-signaling cascades (7).

Recent works have demonstrated the existence of a novel G protein coupled receptor 30, GPR30, here referred as GPER, that can bind directly to estrogen and mediates estrogen action (1, 6, 28, 34). Estrogen rapidly activates adenylyl cyclase and the mitogen-activated protein kinases, extracellular signal-regulated kinase (Erk)-1 and Erk-2, through GPER, resulting in mobilization of intracellular Ca2+ concentration store in cancer cells (12, 13). GPER is an integral membrane protein with high affinity for estrogen (1, 11, 31, 32). GPER seems to play only an important role in the rapid estrogen nongenomic signaling events widely observed in cells and tissues (27, 32). The activation of GPER by estrogen resulted in intracellular Ca2+ mobilization and synthesis of phosphatidylinositol 3,4,5-trisphosphate in the nucleus (32, 35).

The potential role and the mechanism of GPER activation in cardioprotection is an important topic under investigation. A recent report showed that GPER activation improves functional recovery and reduces infarct size after ischemia in isolated rat hearts following ischemia and reperfusion and that this protective effect was blocked by the phosphatidylinositol 3-kinase inhibitor wortmannin (10).

The opening of the mitochondria permeability transition pore (mPTP) appears to be a pivotal event in cell death after ischemia-reperfusion (38). During myocardial ischemia, the mPTP remains closed, and it only will open within the first few minutes after myocardial reperfusion in response to mitochondrial Ca2+ overload, oxidative stress, and ATP depletion (24). The inhibition of mPTP opening during the reperfusion exerts myocardial protection (20). However, the upstream mechanisms regulating mPTP opening in the setting of ischemia-reperfusion injury remain to be determined. The aims of this study are to explore in isolated hearts from mice after ischemia-reperfusion the role of GPER activation using G1, a specific GPER agonist (3), in 1) cardioprotection (cardiac function and infarct size), 2) the involvement of the mPTP opening, and 3) the participation of Erk activation.

METHODS

Animals

Male mice wild types (C57BL/6ScSn), 8–12 wk old were used. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health (NIH Publication No. 85-23, revised 1996). Animal protocol was approved by the University of California Los Angeles School of Medicine Animal Research Committee.
Langendorff Preparation

Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg); heparin (200 IU/kg) was injected to prevent blood coagulation. Hearts were removed and immediately arrested in cold (4°C) Krebs Henseleit bicarbonate buffer solution (KH) (in mM): 11.1 glucose, 118 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 25.0 NaHCO3, and 2 CaCl2 at pH 7.4. The aorta was rapidly cannulated and the heart retrograde-perfused at a constant rate (3 ml/min) in the Langendorff mode using KH solution (control) or with the addition of G1 (1 µM) (Merck, Frankfurt, Germany). G1 is 1-[4-(6-bromo-1H-[1,3]dioxolo[5-yl]-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone, a cell-permeable, nonsteroidal, dihydroquinoline compound that acts as a high-affinity agonist for GPER. G1 (1 µM) should selectively activate GPER without any significant action on ERα and ERβ (3). To investigate the involvement of Erk pathway, G1 (1 µM) together with ERK inhibitor PD-98059 (5 µM) was used. PD-98059 was obtained from Invitrogen. The buffer was bubbled with 95% O2-5% CO2 at 37°C. After 20 min of equilibration, global normothermic ischemia was induced by clamping the aorta for 20 min. Heart function recovery and infarct size were measured during or at the end of a 40-min period of reperfusion after the ischemia, respectively. Mitochondria Ca2+-retention capacity (CRC) was measured after 10 min of reperfusion to measure at an earlier time the initial mitochondria dysfunction preceding cardiac necrosis measured as the infarct size.

Heart Functional Measurements

Left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), and heart rate (HR) were recorded with a pressure transducer (1.4F Millar SPR-671) directly inserted in the left ventricle (LV) after a left atrial incision exposing the mitral annulus (Power Lab; AD Instruments). The left ventricular developed pressure (LVPD) and rate pressure product (RPP) were calculated as LVPD = LVSP – LVEDP, RPP = HR × LVDP. The maximum rate of rise of the LV contraction velocity (dP/dtmax) and the maximum isovolumetric rate of relaxation (dP/dtmin) were directly calculated from the recordings.

Myocardial Infarct Size

Myocardial infarct was assessed by measurement of the infarct size using triphenyltetrazolium chloride (TTC) staining. At the end of reperfusion, the hearts were cut into four transverse slices, parallel to the atrioventricular groove. After removing the right ventricular tissue, the infarcted slices were weighed and incubated for 10 min in 1% TTC in PBS containing 3% sodium deoxycholate (pH 7.4) at 37°C followed by fixation with 4% paraformaldehyde. This procedure differentiates the infarcted (pale) from viable (brick red) myocardial tissue. The slices were photographed using digital microscopic imaging. Extent of the area of necrosis was quantified by computerized planimetry with Adobe Photoshop. Total area of necrosis was calculated and expressed as the percentage of total LV area.

Ca2+-Induced Mitochondrial Permeability Transition

Preparation of isolated mitochondria. Preparation of mitochondria was adapted from a previously described procedure (4). The preparation procedure was carried out at 4°C. Myocardial sections (≈0.15–0.22 g) were placed in isolation buffer A (in mM: 70 sucrose, 210 mannitol, 1 EDTA, and 50 Tris·HCl, pH 7.4). The tissue was finely minced with scissors and homogenized in the same buffer A (1 ml buffer/0.1 g of tissue) using Kontes and Potter-Elvehjem tissue grinders. The homogenate was centrifuged at 1,300 g for 3 min, and the supernatant was filtered through cheesecloth and centrifuged at 10,000 g for 10 min. The mitochondrial pellet was resuspended in isolation buffer B (in mM, 70 sucrose, 210 mannitol, 0.1 EDTA, and 50 Tris·HCl, pH 7.4). Protein concentration was assayed using the Bradford method.

CRC. The installation of mPTP opening was assessed following in vitro Ca2+ overload as previously described (15). Free Ca2+ concentra-
gel. Protein was electrotransferred to a nitrocellulose membrane and then blocked with 5% nonfat dry milk in 20 mM of TBS with 0.1% Tween. After blocking, the membrane was incubated overnight at 4°C with either anti-GPER primary antibody (Ab) (sc-48524, lot F0208, 1:200 dilution; Santa Cruz Biotechnology) or GPER Ab + antigen (sc-48524 P, lot no. C2307, 10 times) and with the corresponding secondary Ab of 1:100,000 (Li-Cor) at room temperature. After washing three times, bands were visualized using an infrared fluorescence system (Odyssey Imaging System; Li-Cor).

**Quantitative Real-Time PCR**

Total RNA was isolated using Trizol (Invitrogen) and reverse transcribed with gene-specific primers for real-time PCR. The transcript level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference gene.

**Statistical Analysis**

All values are expressed as means ± SE. Comparisons among groups were performed with two-way ANOVA. Statistical significance of differences between groups was defined as a value of *P* < 0.05.

![Fig. 2. Heart function recovery in the specific GPER agonist G1 and control group after ischemia-reperfusion.](http://ajpheart.physiology.org/)

A and B: heart function recordings showing the left ventricular developed pressure (LVDP) and the maximum velocity of contraction and relaxation (dP/dt) in control (A) and with 1 μM G1 (B). HH, Krebs Henseleit bicarbonate buffer solution. C–F: graphs representing the values before ischemia and their time course after ischemia-perfusion (time 0) of dP/dt_max (C), −dP/dt_min (D), rate pressure product (RPP) (E), and LVDP (F). Values are expressed as means ± SE; *P* < 0.05 vs. control (n = 5–8/group). Note that, in control and after G1 treatment, values before ischemia are very similar, whereas after ischemia-reperfusion heart functional recovery was dramatically improved by G1 treatment. G1 (1 μM) activates GPER without a significant action on estrogen receptor α and β (3).
GPER ACTIVATION PROVIDES CARDIOPROTECTION BY INHIBITING mPTP OPENING

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To activate GPER, Figure 2, A and B, shows examples of recording of isolated heart subjected to ischemia-reperfusion in an ex vivo model using the Langendorff system in the absence (control, A) and in the presence (B) of 1 µM G1. We selected an ischemic period of 20 min that in the mouse model typically shows ~50 of infarct size in control conditions (see Fig. 3).

There was no difference in cardiac function in two groups before ischemia, since dP/dt max (Fig. 2C), -dP/dt min (Fig. 2D), RPP (Fig. 2E), and LVDP (Fig. 2F) were similar. LV function after 40 min of reperfusion postischemia was significantly decreased from the baseline in control and G1-treated hearts. However, the functional recovery was significantly higher with G1 treatment (Fig. 2, C-F, and Table 1). In summary, heart functional parameters were similar in control and after G1 treatment and were much better in G1-treated hearts at all times after ischemia-reperfusion, which demonstrates that activation of GPER by G1 improves functional recovery after ischemia-reperfusion.

Myocardial Infarct Size Reduction by G1 Treatment After Ischemia-Reperfusion Injury

There was no infarct size in nonischemic groups after 20 min of initial perfusion in the presence or absence of G1 (n = 8/group). Figure 3A shows typical heart cross sections at different levels from control and G1 treatment after TTC staining. The red area represents the viable region and the white area the infarct size. The infarct size in the G1-treated group was reduced significantly by about one-half compared with control at the end of 40 min reperfusion (46 ± 5 vs. 22 ± 2%, P < 0.001, n = 5–8/group, Fig. 3B).

Increased Resistance by G1 Treatment of Ca2+ Induced mPTP Opening

To investigate the role of GPER activation in mitochondria function, we compared the sensitivity of mPTP opening by

Table 1. Heart functional parameters recovery improved with G1 treatment

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Values are means ± SE; n = 5–8 mice/group. G1, specific G protein-coupled receptor 3 agonist. Cardiac functional parameters recovery of left ventricular systolic pressure (LVSP); left ventricular end-diastolic pressure (LVEDP); left ventricular developed pressure (LVDP), and heart rate (HR) before ischemia (sham control and sham G1) and during different times of reperfusion in G1-treated and control group. *P < 0.05 vs. control.

RESULTS

Expression of GPER Transcripts in Whole Heart Lysate

Quantitative real-time PCR experiments and Western blot analysis demonstrate that GPER is expressed in adult mouse heart. Figure 1A shows the fluorescence-cycle number curves for GAPDH, GPER, and Kv4.3, which is the molecular correlate of the fast transient repolarizing current in mouse heart. The housekeeping gene GAPDH transcript level (cycle threshold ~9) was more abundant when compared with GPER (cycle threshold ~21) and the ion channel Kv4.3 (cycle threshold ~18). These data are the mean of n = 3, each mouse triplicate.

Western Blot Analysis

In agreement with transcript determinations, Western blot analysis demonstrates that anti-GPER Ab labels a predominant band of the expected molecular size (38 kDa) of GPER that are blocked when the anti-GPER Ab was preincubated overnight with GPER antigenic peptide (Fig. 1B). This result indicates the presence of GPER in the heart. In summary, GPER levels in heart should be sufficient to modulate function. For Western blot, we have used n = 3, each mouse in duplicate.

G1 Treatment Improves Heart Function After Ischemia-Reperfusion Injury

To investigate the role of GPER in cardioprotection, heart function was recorded in both control and G1 treatment to
Ca$^{2+}$ overload in isolated mitochondria from control and G1-treated hearts. Figure 4A shows the time course of Ca$^{2+}$ concentration in the solution surrounding the mitochondria. The initial Ca$^{2+}$ concentration in the buffer was set to Ca$^{2+}$ contaminant that declined after adding mitochondria due to the mitochondria Ca$^{2+}$ uptake. The initial sharp decay is because of a global reduction of the solution optical transmittance by the increase of turbidity after the mitochondria loading. The following slower phase before the addition of the Ca$^{2+}$ pulses reflects the rate of Ca$^{2+}$ uptake by the mitochondria. Note the faster Ca$^{2+}$ uptake with G1 treatment when compared with control that is already indicating a more robust Ca$^{2+}$ uptake after G1 treatment. Data in Fig. 4A indicate the establishment of mPTP opening defined when there is a beginning (represented by a two time increase of fluorescence spot in a minute) of spontaneous increase of Ca$^{2+}$ fluorescence after the preceding Ca$^{2+}$ pulse.

In isolated mitochondria from control hearts, eight 100 nM Ca$^{2+}$ pulses were required to induce mPTP opening. The number of pulses to induce mPTP opening increased to 13 when using mitochondria from hearts perfused with 1 μM G1 (Fig. 4A).

In nonischemic control, CRC was 3.5 ± 0.11 μM/mg mitochondrial proteins. The CRC value was significantly reduced to 1.6 ± 0.7 μM/mg mitochondrial proteins after ischemia-reperfusion and it was recovered to 2.6 ± 0.5 μM/mg mitochondrial proteins after G1 treatment. As in functional studies, CRC values are very similar in nonischemic conditions in control and after ischemia-reperfusion after G1 treatment. Most importantly, G1 treatment after ischemia-reperfusion induced a much larger CRC value that is consistent with the
inhibition of the mPTP opening and improved mitochondria function (Fig. 4B).

**Erk Pathway Inhibitor (PD-98059) Prevents Cardioprotective Effect of G1 After Ischemia-Reperfusion**

We investigated whether Erk signaling is involved in GPER-mediated cardioprotection as a mechanism leading to the inhibition of mPTP opening by using PD-98059 (5 μM), a known blocker of Erk signaling. PD-98059 prevents the improvement of the heart function recovery and the reduction of infarct size caused by G1 treatment after ischemia-reperfusion (Figs. 5–7). Heart function recovery was much lower in heart perfused with G1 + PD-98059 compared with G1 alone: RPP 4,421 ± 76 vs. 9,420 ± 264 (beats·mmHg⁻¹·min⁻¹, n = 3 P < 0.05) (Fig. 6A), dP/dt max 927 ± 138 vs. 1,695 ± 128 mmHg/s (P < 0.01, n = 3/group) (Fig. 6B), and −dP/dt min −506 ± 53 vs. −762 ± 86 mmHg/s (P < 0.05, n = 3/group) (Fig. 6C). The infarct size increased in the G1 + PD-98059 group (53 ± 4 vs. 20 ± 2%) compared with the G1-treated group (n = 3 P < 0.01) (Fig. 7). The mitochondrial CRC was reduced in the G1 + PD-98059 group compared with the G1-treated group (1.4 ± 0.2 vs. 2.4 ± 0.1 μM/mg mitochondrial proteins, n = 3 P < 0.01, Fig. 8). These data strongly support the involvement of Erk pathway in the cardioprotective action of G1.

**DISCUSSION**

In this study, we report that the activation of GPER by the specific agonist GPER protects the heart against ischemia-reperfusion injury, inhibiting Ca²⁺-induced mPTP opening. GPER has been shown to be localized in the endoplasmic reticulum (28, 32) and plasma membrane (11) of reproductive organs, uterus, and mammary gland as well as in the hippocampal regions (25). Our results show the expression of GPER both at the mRNA and protein levels in the mouse adult heart.Recently, activation of GPER by acute infusion of G1 has been
shown to be involved in reduction of mean arterial blood pressure (17). Haas and coworkers (17) concluded that GPER contributes to regulation of blood pressure and vascular tone, suggesting the possibility that some of the known vasculoprotective effects of estrogen involve GPER activation. Here, we found that the acute G1 treatment significantly reduced the infarct size after ischemia-reperfusion. In a similar manner, acute treatment of 17β-estradiol (40 nM) reduced the infarct size in male mouse isolated hearts (unpublished data) as previously reported by others (14, 36). Thus the vasculoprotective action of estrogen may involve the activation of GPER.

We have shown that acute G1 treatment considerably improves the cardiac function recovery after ischemia-reperfusion. Our data strongly support the view that activation of GPER induces a cardioprotective effect against ischemia-reperfusion injury. Similarly, a recent report showed that G1 treatment of isolated rat heart is cardioprotective during ischemia-reperfusion (10).

Crompton et al.’s (8) laboratory, in the late 1980s, first proposed the role of the mPTP as a mediator of lethal ischemia-reperfusion injury. The role of mPTP opening in ischemia-reperfusion was later confirmed by other groups (19, 33). Several studies have demonstrated the involvement of the inhibition of the mPTP in ischemic and pharmacological preconditioning (18, 30), postconditioning (2), and controlled reperfusion (5). The mPTP opening occurred in response to Ca2+ overload, oxidative stress, inorganic phosphate, and relative ATP depletion, conditions that are recreated during ischemia-reperfusion (8), and cyclosporin A could inhibit mPTP opening and confer cardioprotection (9, 16). In this study, we show that G1 treatment leads to mPTP opening inhibition after ischemia-reperfusion. This is the first observation linking the GPER and the mPTP opening. These results allow better understanding of the GPER activation-induced cardioprotection after ischemia-reperfusion. However, the mechanism through which the activation of GPER can cause the mPTP opening remains to be investigated.

It has been shown that the activation of Erk during ischemia and reperfusion plays an important role in modulation of the cardioprotection occurring by preconditioning (21). Erk activation has been shown to result in inhibition of the mPTP opening (37). Our results show that, in the presence of the Erk inhibitor PD-98059, G1 effect on the heart function recovery, infarct size, and the mitochondria CRC were all abolished. These data indicate that GPER activation by G1-induced cardioprotection is mediated by the Erk pathway, leading to the inhibition of mPTP opening. The participation of the Erk pathway in cardioprotection agrees with the recent findings that G1 treatment in rat heart increases Erk phosphorylation, which was prevented by PD-98059 (10). However, they did not observe a significant effect of PD-98059 in preventing G1 cardioprotection in heart function and in infarct size. The lack of effect of PD-98059 on G1-induced cardioprotection could be tentatively explained by a relative short time (20 min) of ischemia used in the isolated rat heart subjected to ischemia-reperfusion. In fact, in the rat model, 20 min of ischemia induced a small reversible functional damage subjected to variations of function recovery (29).

In conclusion, the present study demonstrates that GPER activation improves cardiac functional recovery, induces reduction of infarct size, and inhibits the mPTP opening in isolated mice hearts subjected to ischemia-reperfusion. This cardioprotective effect of GPER activation is mediated by the Erk pathway.

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GRANTS
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DISCLOSURES
No conflicts of interest are declared by the authors.

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