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Research Article

Major ginsenosides from *Panax ginseng* promote aerobic cellular respiration and SIRT1-mediated mitochondrial biosynthesis in cardiomyocytes and neurons

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ABSTRACT

Background: Aerobic cellular respiration provides chemical energy, adenosine triphosphate (ATP), to maintain multiple cellular functions. Sirtuin 1 (SIRT1) can deacetylate peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) to promote mitochondrial biosynthesis. Targeting energy metabolism is a potential strategy for the prevention and treatment of various diseases, such as cardiac and neurological disorders. Ginsenosides, one of the major bioactive constituents of *Panax* ginseng, have been extensively used due to their diverse beneficial effects on healthy subjects and patients with different diseases. However, the underlying molecular mechanisms of total ginsenosides (GS) on energy metabolism remain unclear.

Methods: In this study, oxygen consumption rate, ATP production, mitochondrial biosynthesis, glucose metabolism, and SIRT1-PGC-1α pathways in untreated and GS-treated different cells, fly, and mouse models were investigated.

Results: GS pretreatment enhanced mitochondrial respiration capacity and ATP production in aerobic respiration-dominated cardiomyocytes and neurons, and promoted tricarboxylic acid metabolism in cardiomyocytes. Moreover, GS clearly enhanced NAD^+-dependent SIRT1 activation to increase mitochondrial biosynthesis in cardiomyocytes and neurons, which was completely abrogated by nicotinamide. Importantly, ginsenoside monomers, such as Rg1, Re, Rf, Rb1, Rc, Rh1, Rb2, and Rb3, were found to activate SIRT1 and promote energy metabolism.

Conclusion: This study may provide new insights into the extensive application of ginseng for cardiac and neurological protection in healthy subjects and patients.

1. Introduction

Aerobic cellular respiration refers to the process by which organisms convert nutrients into chemical energy, adenosine triphosphate (ATP), using oxygen, which is dominated in highly aerobic tissues, such as cardiac and neural systems [1,2]. During glucose aerobic metabolism, glucose with 6-carbon is broken down into 3-carbon pyruvate by multiple key enzymes such as hexokinase (HK–I/II), phosphofructokinase-1 (PFK-1), and pyruvate kinase (PK) in the cytoplasm. Then pyruvate is transported into the

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mitochondria through the mitochondrial pyruvate carrier (MPC) [3] and metabolized to synthesize acetyl-coenzyme A (CoA) by pyruvate dehydrogenase (PDH) [4]. After the metabolism of tricarboxylic acid (TCA) cycle, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2) from acetyl-CoA can pass their electrons into the mitochondrial electron transport chain (ETC) to produce ATP molecules, with the help of oxygen [5]. During this process, respiratory chain protein complexes I–V embedded in the inner mitochondrial membrane is involved in ATP production [6]. Abnormal energy metabolism is a critical target during the progression of disease, including myocardial ischemia, heart failure [7], stoke [8], or neurodegenerative diseases [9]. During the exposure to hypoxia, cells display decreased carbon flux into the TCA cycle and diminished electron flux through ETC [10]. Moreover, oxygen-glucose deprivation can cause mitochondrial damage and ATP depletion in cardiomyocytes and neurons [11]. Therefore, the enhancements of glucose oxidation and mitochondrial bioenergy have been potential strategies against various disorders, such as ischemic conditions.

Oxidized nicotinamide adenine dinucleotide (NAD^+) is a well-known small molecule that drives energy metabolism through its electron transfer function [12]. Increased NAD^+ level promotes the activity of sirtuin 1 (SIRT1), a strong deacetylase of class III histone deacetylase, which implicates in a number of physical and pathological processes, such as aging-related diseases [13]. SIRT1 deacetylates its substrate, peroxisome proliferator initiated receptor gamma and coactivator 1 alpha (PGC-1α), to regulate mitochondrial biosynthesis and energy metabolism [14]. PGC-1α can bind and co-activate different transcription factors, such as nuclear respiratory factor-1 (Nrf-1) and Nrf-2, in the promoter of mitochondrial transcription factor A (TFAM), which increases the transcription of key mitochondrial enzymes, such as cytochrome *c* oxidase subunits (COXII, COXIV) and ATP synthase β subunit, and drives mitochondrial DNA transcription and replication through the interaction of TFAM [15]. Previous studies have shown that the activation of SIRT1-PGC-1α pathway improves mitochondrial function to prevent and treat ischemic heart or brain disorders [16–19].

Ginsenosides are major active components of *Panax ginseng Meyer*, a medicinal herb as new-resource health food approved by the Ministry of Health, China. They are extensively used for diverse beneficial effects on myocardial infarction, stroke, and ischemia/reperfusion injury [20–22]. Currently, about 250 ginsenosides have been isolated and identified, which are mainly divided into three groups, protopanaxadiol (PPD, such as Rb1, Rb2, Rc, Rd, Rg3), protopanaxatriol (PPT, such as Re, Rf, Rg1, Rh1), and oleanolic acid (OA, such as Ro) [23]. Proteomic analysis has shown that total ginsenosides (GS) can increase the expression of proteins in the TCA cycle to enhance cardiac energy metabolism in the ischemic rat [24]. Meanwhile, ginsenoside Rb1, Rb3, Rg3, Rg1, Rg5, and compound K exert cardioprotective and neuroprotective effects by increasing energy metabolism [25–27], improving mitochondrial dynamics and quality [28,29], or inhibiting mitochondrial apoptosis [30,31]. However, the molecular mechanism underlying the effects of GS and each ginsenoside monomer on mitochondrial aerobic metabolism and biosynthesis in cardiomyocytes and neurons remains unclear and needs to be further investigated.

In this study, we first investigated the effect of GS on mitochondrial oxygen consumption rate (OCR) in a series of cell lines, including aerobic respiration-dominated cardiomyocytes, neuronal cells, and skeletal myoblasts [32], as well as anaerobic respiration-dominated vascular endothelial, epithelial, and stem cells [33,34]. Then ATP production and mitochondrial respiration capacity (MRC) were examined in untreated and GS-treated cardiomyocytes and neurons. Targeted metabolomics and Western blot analysis were performed to observe the effect of GS on the contents of major metabolites and the levels of key multiple enzymes from glycolysis and TCA cycle. Furthermore, NAD^+ level, SIRT1-PGC-1α pathway, and mitochondrial biosynthesis in the cell, Drosophila melanogaster, and mice models were further evaluated to explore the molecular mechanism of GS. Of note, the ginsenoside monomer in GS for promoting SIRT1 activation and mitochondrial energy production was preliminarily identified in cardiomyocytes. This is the first study to explore the molecular mechanism by which GS promote mitochondrial energy metabolism, which may provide new insights into the clinical application of ginseng for the prevention and treatment of cardiac and neurological disorders.

2. Materials and Methods

2.1. Materials and reagents

Total ginsenosides (GS, purity o 80%) and ginsenoside monomers (purity ≥98%), such as Rg1, Re, Rf, Rb1, Rc, Rh1, Rb2, Rb3, Rd, F1, F3, Rk3, S-Rg3, R-Rg3, PPT, Rk1, Rg5, and Rh2, were purchased from Shanghai Yuanye Bio-Technology (Shanghai, China). Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, C2920), and rotenone (R8875) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Oligomycin B (ab143424) and antimycin (ab141904) were obtained from Abcam (Cambridge, MA, USA). Nicotinamide (NAM, HY-B0150) was purchased from MedChemExpress (Shanghai, China).

2.2. OCR assay

OCR is directly related to the function of mitochondrial oxidative phosphorylation [35]. MitoXpress® Xtra probe or Cell Mito Stress Test Kit (Agilent Technologies) was used to assess the effect of GS on the OCR in a variety of cell lines (H9c2, primary neonatal cardiomyocytes, differentiated PC12, primary cortical neurons, L6, C2C12, HUVECs, BMSCs, osteoblast, 16HBE, and THP-1 cells) by a time-resolved fluorescence (TR-F) plate reader (Cytation 5; BioTek, Winooski, VT, USA) or Seahorse XFp analyzer (Agilent Technologies), respectively [36]. After incubation with GS or ginsenoside monomer for 48 h, each well was equilibrated in a CO_2-free incubator at 37 °C for 1 h and measured every 2 min over 2 h to obtain TR-F intensity for calculating the initial slopes (μs min^{-1}), according to the phosphorescence lifetime (τ) values. To further analyze mitochondrial respiration, oligomycin (1 μM), FCCP (5 μM), rotenone (Rot, 1 μM), and antimycin A (AA, 1 μM) were sequentially added to each well with XF base medium (Agilent, pH 7.4) supplemented with 2 mM L-glutamine, 1 mM pyruvate, and 25 mM glucose. After normalization to the intensity of Hoechst 33342 nuclear staining, basal OCR, ATP production, maximal respiratory capacity (MRC), and spare respiratory capacity (SRC) were calculated.

2.3. Metabolite measurements

Major intermediates for glycolysis, TCA cycle, and oxidative-phosphorylation were analyzed with the assistance of Applied Protein Technology Co., Ltd. (Shanghai, China), as previously described [37]. Briefly, 10 samples of untreated and treated cells (H9c2 and HUVECs) were stored in liquid nitrogen and centrifuged at 14,000 rpm for 20 min to collect the supernatants, which were dried by vacuum. Then, cold acetonitrile/H_2O (1:1, v/v) was added to each sample and centrifuged at 14,000 rpm for 20 min to obtain the supernatants, which were separated and analyzed using a UHPLC (1290 Infinity LC system; Agilent Technologies) coupled to the Triple Quad 5500 Mass Spectrometer (QTRAP/MS; AB SCIEX,

Framingham, MA, USA). The raw MS data were processed using Multiquant software based on chromatographic peak area and retention time to identify different energy metabolism-related metabolites.

2.4. Quantification of ATP and NAD/NADH levels

According to the manufacturer's instructions, the contents of ATP, NAD^+, and NADH in the cells of different groups were detected using the ATP Assay System Bioluminescence Detection Kit (FF2000; Promega, Madison, WI, USA) and NAD/NADH-Glo Assay Kit (G9071; Promega). Meanwhile, the levels of cellular ATP, and NAD^+ were measured by the multiple reaction monitoring MS (MRM-MS).

2.5. Drosophila melanogaster activity and climbing distance assays

Wild-type Drosophila melanogaster was maintained on standard medium (50 g/L yeast, 50 g/L glucose, 0.8% agar, and 1% soy flour) at 25–26 °C in 50% relative humidity under a 12:12 h light-dark cycle. The 7-day-old flies were loaded into plastic tubes containing standard food or with GS (1 mg/mL) for 7 days. Then, the sleep-waking activity of 32 flies was consecutively monitored for 24 h using the Drosophila Activity Monitoring System (Trikinetics, Waltham, MA, USA) and recorded as beam-breaks in 1-min bins for generating the number of activities. For the climbing ability assay, 30 flies in the vertical vials (110 × 10 mm) were acclimatized for 15 min and simultaneously tapped to the bottom of the vial for 5 s. The climbing distance of each fly from control or GS pretreatment was recorded to obtain the average of the distance [38].

2.6. Statistical analysis

Data are presented as the mean ± standard deviation or mean ± stanard error of the mean from at least three independent experiments. The unpaired Student's *t*-test for comparing two groups or one-way analysis of variance for comparing multiple groups was used to analyze statistical significance using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Hierarchical cluster analysis of different metabolites was performed by Python. The raw metabolomic intensity of each metabolite from two groups was transformed into the Log^2 value of normalized intensity. For all statistical analyses, a significant difference was accepted at $P < 0.05$.

Additional details of materials preparation and full experimental procedures, including high-performance liquid chromatography and liquid chromatography-mass spectrometry analysis, animal and drug administration, cell culture, measurement of PDH activity, cell viability, scratch assay and reactive oxygen species measurement, immunofluorescence staining, quantitative PCR analysis, Western blot analysis are given in Supplementary date.

3. Results

3.1. GS enhances MRC and ATP production in cardiomyocytes and neurons

To explore the molecular mechanism of GS on extensive cell protection, we first investigated the effects of GS on mitochondrial function by examining OCR. Oxygen consumption probes were used to assess the effect of GS on the rate of basal OCR in a variety of cell lines, including H9c2, primary neonatal cardiomyocytes, differentiated PC12, primary cortical neurons, L6, C2C12, HUVECs, BMSCs, osteoblast, 16HBE, and THP-1 cells. As expected, GS pretreatment for 48 h increased basal oxygen consumption by 2.0-fold in H9c2 cells, 2.8-fold in PC12 cells, 1.5-fold in neurons (Fig. 1A and Fig. S3A), 1.6-fold in C2C12 cells, and 1.5-fold in L6 cells (Fig. S3B). Meanwhile, we found that GS had no effect on basal OCR in HUVECs, BMSCs, osteoblast, 16HBE, and THP-1 cells (Fig. 1B, Fig. S3C-S3F). To further assess the potential function of GS in mitochondrial oxidative capacity, we conducted a mitochondrial stress test in H9c2, PC12, and HUVECs. The pretreatment of GS at 5 μg/mL for 48 h led to increases in basal OCR, MRC, and SRC in H9c2 and PC12 cells (Fig. 1C and D). The similar results for mitochondrial oxidative capacity were detected by Luxcel oxygen consumption probes in H9c2 and PC12 cells (Fig. S4A-S4B). In HUVECs, GS pretreatment had no effects on basal OCR, MRC, and SRC (Fig. S4C). As shown in Fig. 1E, GS pretreatment significantly increased ATP production in cardiomyocytes and neurons in the same manner as OCR. LC-MS analysis showed that GS at 5 μg/mL promoted relative ATP intensity and the ratio of ATP/ADP in H9c2 cells (Fig. 1F). In HUVECs, GS slightly increased ATP content and had no effect on ATP/ADP ratio (Fig. S4D). The results in Fig. 1G and H demonstrated that GS pretreatment for 6 h enhanced basal OCR and ATP content at concentrations of 5 and 10 μg/mL GS. Collectively, GS pretreatment enhanced MRC and ATP production in aerobic respiration-dominated cardiomyocytes and neurons, and had no obvious effect on anaerobic respiration-dominated other cell lines, such as HUVECs and BMSCs.

3.2. GS significantly promote aerobic glucose metabolism in cardiomyocytes

Increased OCR and ATP production are the manifestations of enhanced energy metabolism; thus, we performed targeted metabolomic analysis of glucose metabolites by LC-MS/MS in H9c2 and HUVECs untreated or treated with GS for 48 h (Fig. 2A and Fig. 2B). A total of 15 different intermediates of glycolysis and TCA cycle were identified in these two cell lines. In H9c2 cells, GS led to decreases of seven metabolites and increases of eight metabolites, compared with the control group (Fig. 2C). In HUVECs, only six metabolites were upregulated by GS pretreatment, including glucose-6-phosphate, fructose-6-phosphate, pyruvate, succinate, malate, and fumarate. There were no obvious differences in the other nine metabolites between the control and GS groups (Fig. 2B, Fig. S5A). Collectively, GS had more obvious effects on promoting glucose oxidation in cardiomyocytes than endothelial cells.

In addition, GS pretreatment upregulated the levels of HK-II, PFKP, PKM2, PDH, MPC1, MPC2, CS, DLST, and Fumarase, did not change GAPDH, PKM1, IDH1, and IDH2 expression in H9c2 cells (Fig. 2D, Fig. S5B). In addition, LDHA, ACO2 and SDHA were decreased by GS pretreatment (Fig. 2D, Fig. S5B). Importantly, we found a significant induction in PDH activity in GS-treated H9c2 cells and no change of PDH activity in GS-treated HUVECs compared with control cells (Fig. 2E). Taken together, our findings suggest that GS pretreatment mainly accelerates glucose conversion to pyruvate, and increases pyruvate transporting to promote TCA metabolism in the cardiomyocytes. In future experiments, we will focus on the effect and molecular mechanism of GS on cardiomyocytes and neurons dominated by aerobic respiration.

3.3. GS increase mitochondrial biogenesis in cardiomyocytes and neurons

To investigate the role of GS in mitochondrial biogenesis, we first examined mitochondrial count in cardiomyocytes and neurons using the Mitotracker probe. We found that pretreatment with GS induced a significant increase in mitochondrial content in a dose-dependent manner in H9c2 cells (Fig. 3A–B) and primary neurons (Fig. 3A and C). Moreover, high-resolution micrographs of mitochondria were obtained in control and GS-pretreated H9c2

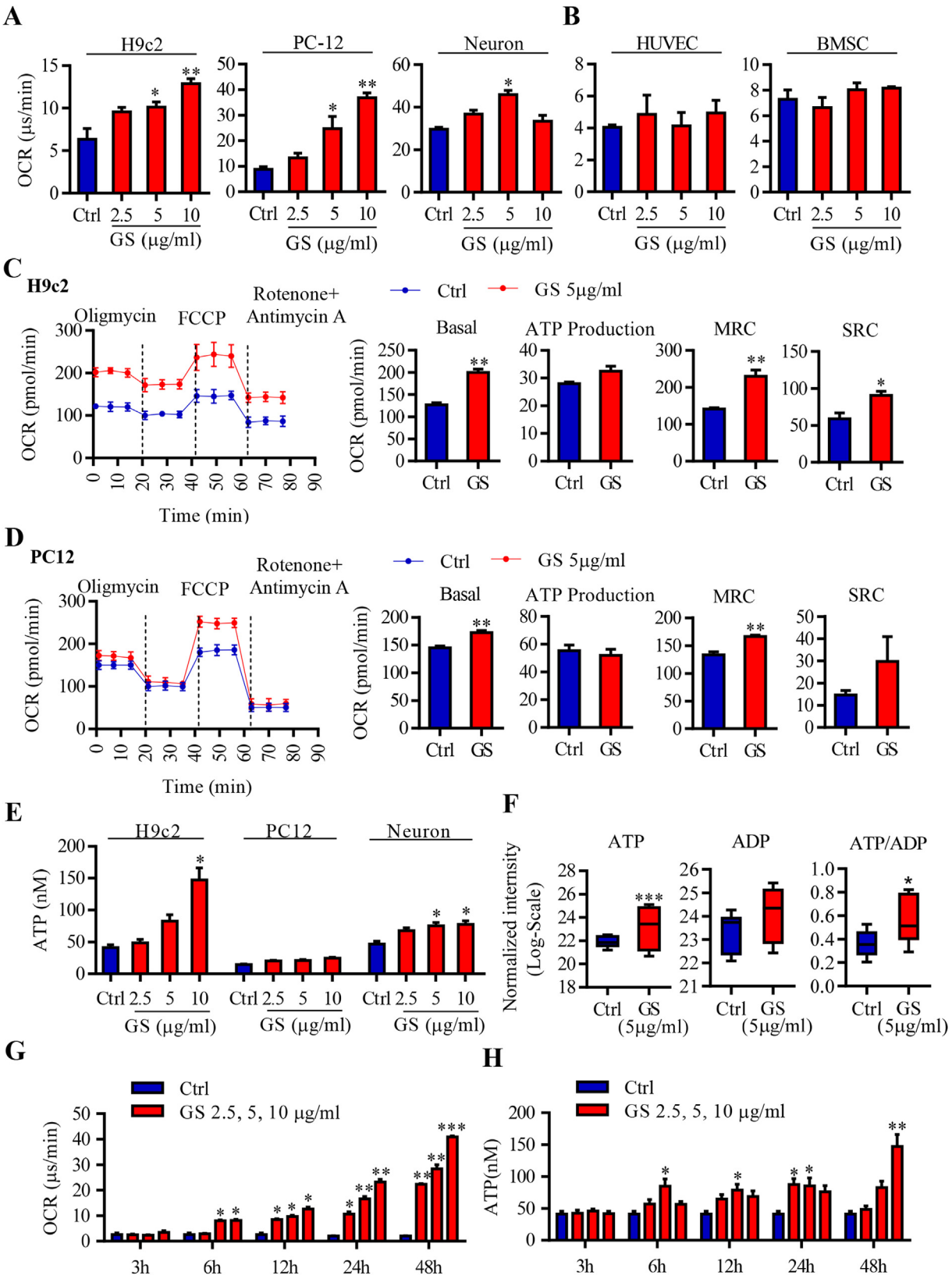
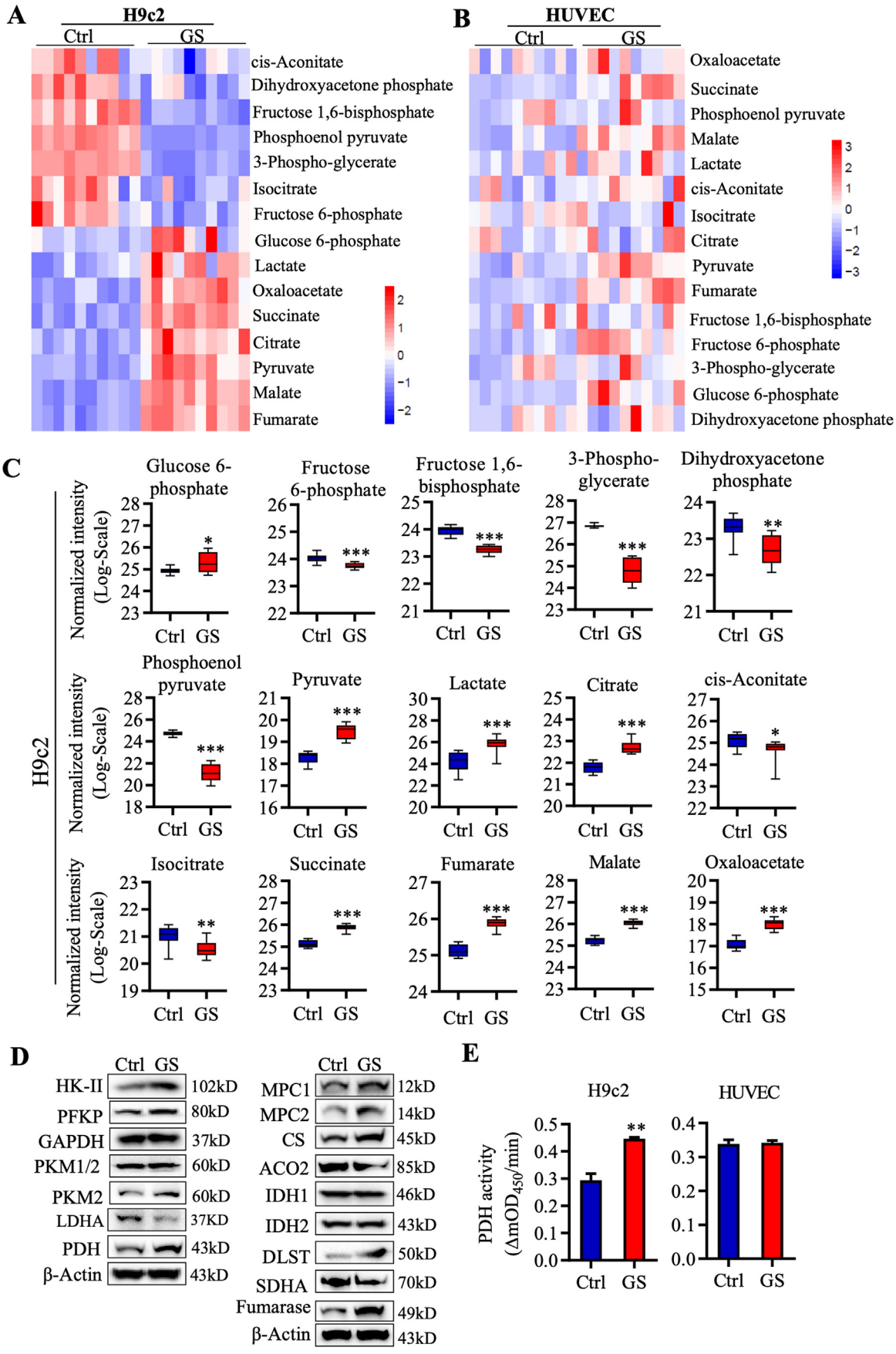


Fig. 1. GS enhanced mitochondrial respiration capacity and ATP production in cardiomyocytes and neurons. (A–B) Basal OCR was measured using a MitoXpress® Xtra probe normalized to cell number. **(C)** OCR was analyzed at basal condition and sequential injections of oligomycin (1 μM), FCCP (5 μM), rotenone (Rot, 1 μM) and antimycin A (AA, 1 μM) in control and GS-pretreated H9c2 cells by the MitoStress Test Kit. **(D)** Basal OCR, ATP production, MRC, and SRC were analyzed in PC12 cells. **(E)** ATP levels in H9c2, PC12, and neurons were measured by the Bioluminescence Detection Kit. **(F)** The intensities of ATP and ADP were analyzed by the LC-MS/MS method based on MRM detection. **(G–H)** Basal OCR and ATP content in H9c2 cells treated with different concentrations (2.5, 5, or 10 μg/mL) of GS and incubated for 3, 6, 12, 24, or 48 h were measured as described as the methods above. Ctrl: control group. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ *versus* Ctrl group.


A
H9c2
Ctrl
GS
cis-Aconitate
Dihydroxyacetone phosphate
Fructose 1,6-bisphosphate
Phosphoenol pyruvate
3-Phospho-glycerate
Isocitrate
Fructose 6-phosphate
Glucose 6-phosphate
Lactate
Oxaloacetate
Succinate
Citrate
Pyruvate
Malate
Fumarate
B
HUVEC
Ctrl
GS
Oxaloacetate
Succinate
Phosphoenol pyruvate
Malate
Lactate
cis-Aconitate
Isocitrate
Citrate
Pyruvate
Fumarate
Fructose 1,6-bisphosphate
Fructose 6-phosphate
3-Phospho-glycerate
Glucose 6-phosphate
Dihydroxyacetone phosphate
C
H9c2
Normalized intensity (Log-Scale)
Glucose 6-phosphate
Fructose 6-phosphate
Fructose 1,6-bisphosphate
3-Phospho-glycerate
Dihydroxyacetone phosphate
Phosphoenol pyruvate
Pyruvate
Lactate
Citrate
cis-Aconitate
Isocitrate
Succinate
Fumarate
Malate
Oxaloacetate
Ctrl
GS
D
Ctrl
GS
HK-II
PFKP
GAPDH
PKM1/2
PKM2
LDHA
PDH
β-Actin
102kD
80kD
37kD
60kD
60kD
37KD
43kD
43kD
MPC1
MPC2
CS
ACO2
IDH1
IDH2
DLST
SDHA
Fumarase
β-Actin
12kD
14kD
45kD
85kD
46kD
43kD
50kD
70kD
49kD
43kD
E
H9c2
HUVEC
PDH activity (ΔmOD450/min)
Ctrl
GS

cells using TEM. As shown in Fig. 3D–E, GS pretreatment at 5 μg/mL for 48 h increased the number of mitochondria in the cardiomyocytes and had no effect on the size of myocardial mitochondria, compared with control cells. To determine if the observed changes in mitochondrial respiration and biogenesis translate into altered mitochondrial function, we performed immunoblotting with antibodies against five complexes of the oxidative phosphorylation. Compared with the control group, GS induced increases in complex I-IV levels in H9c2 cells and mainly increased the levels of CII-SDHB and CI-NDUFB8 in PC12 cells (Fig. 3F–G). The enhancement of mitochondrial function can increase ROS generation, which damages the cells, so we examined ROS level in untreated- and GS-treated H9c2 cells. Flow cytometry analysis showed that GS had no significant effect on the production of intracellular and mitochondrial ROS in H9c2 cells (Fig. S6A). Additionally, mitochondrial amount and complex protein level were further investigated in HUVECs. We did not observe the effect of GS pretreatment on mitochondrial mass in HUVECs (Fig. S6B-S6C). Collectively, GS preferentially promote the mitochondrial biosynthesis of cardiomyocytes and neurons, and not that of endothelial cells.

3.4. SIRT1 pathway is involved in GS-enhanced mitochondrial function in cardiomyocytes, neurons, drosophila and mice

NAD^+ level and its dependent deacetylase, SIRT1, play key roles in regulating mitochondrial function [39]. Based on the findings above, we determined if GS could alter the intracellular level of NAD^+ and regulate SIRT1 activation in cardiomyocytes and neurons. Consistent with our hypothesis, the level of NAD^+ luminescence was obviously higher in GS-treated H9c2 and primary neurons than in the control group (Fig. 4A). Moreover, the targeted metabolomic analysis showed that NAD^+ level was significantly increased by GS pretreatment in H9c2 cells (Fig. 4B). As reported, NAD^+-dependent SIRT1 can deacetylate and activate PGC-1α to stimulate mitochondrial biogenesis [14]. As shown in Fig. 4C, qPCR analysis showed that GS incubation for 8 h increased SIRT1 and PGC-1α mRNA levels in H9c2 and primary neurons. Western blot analysis demonstrated that GS pretreatment for 48 h led to increases in SIRT1 and its targets in H9c2 cells (Fig. 4D). Furthermore, similar findings in cardiomyocytes were observed in GS-treated PC12 cells (Fig. 4E). In addition, the wound-healing ability in H9c2 cells and neurons was increased by GS pretreatment, suggesting that GS promoted cell migration (S7A-S7B). Intriguingly, GS were administered to the flies and mice to further validate the function of GS in NAD^+-dependent SIRT1 activation. Consistent with the *in vitro* results, GS administration for 7 days led to a significant increase in the intensity of NAD^+ luminescence and the ratio of NAD^+/NADH in the drosophila brain compared with the control group (Fig. S8A). The increased SIRT1 in response to NAD^+ was confirmed in the brain of drosophila treated with GS (Fig. S8B). Meanwhile, the ATP level, the number of activities and climbing distance of 30 flies fed with GS were greatly increased, compared to the control group with common food (Fig. S8C-S8D). In addition, IHC staining showed that SIRT1 expression was significantly increased in the heart and brain tissues of mice after 21 days of GS administration (Fig. S9E). Together, these *in vitro* and *in vivo* results indicate that GS obviously increase NAD^+-dependent SIRT1 activation.

3.5. SIRT1 pathway is involved in GS-enhanced mitochondrial function in cardiomyocytes and neurons

We further tested whether mitochondrial function was affected by a SIRT1 inhibitor, nicotinamiede (NAM) combined with GS. Consistent with the results above, we observed increased basal or maximal mitochondrial respiration in H9c2 cells pretreated with GS at the concentration of 5 μg/mL for 48 h, which was significantly blocked by NAM incubation at the concentration of 20 μM for 12 h (Fig. 5A–B). Similarly, GS-mediated increase of ATP content in H9c2 cells was inhibited by NAM (Fig. 5C). For respiratory chain complexes, higher expression of three complexes, CIII-UQCRC2, CIV-MTCO1, and CII-SDHB, induced by GS was inhibited by the combination of GS with NAM (Fig. 5D–E). Importantly, GS combined with NAM significantly reduced GS-induced NAD^+ level in H9c2 cells (Fig. 5F). Then, to further determine whether increased mitochondrial function by GS is mediated by the NAD^+-dependent SIRT1 pathway, we measured protein levels of SIRT1 and its targets in control, GS-, NAM-, or GS + NAM-treated H9c2 cells by Western blot analysis. As expected, GS-mediated activation of SIRT1 and its targets, PGC-1α, Nrf1, and Nrf2, were completely abrogated by the pretreatment of GS and NAM, which was similar to the control or NAM group (Fig. 5G–H). These findings suggest that the SIRT1 pathway is involved in GS-enhanced mitochondrial function in cardiomyocytes.

3.6. Ginsenoside monomers increase mitochondrial function in cardiomyocytes by activating the SIRT1 pathway

As we showed, GS are a complex mixture of ginsenoside monomers of different proportions (Fig. S1-S2). To further gain insight into which ginsenoside in GS promotes mitochondrial function through increased glucose oxidation and SIRT1 activation, we performed a series of experiments to investigate the effects of different ginsenosides on basal OCR, ATP content, and key protein levels in H9c2 cells. Similar to that of GS, different ginsenoside monomers, such as Rg1, Re, Rf, Rb1, Rc, Rh1, Rb2, Rb3, Rd, S-Rg3, R-Rg3, and Rk1, at individual concentrations of GS increased basal OCR in H9c2 cells after 48 h treatment compared with the control group (Fig. 6A). Moreover, ATP content was higher in Re-, Rf-, Rb1-, Rc-, Rh1-, Rb2-, or Rb3-treated H9c2 cells than the control or GS group (Fig. 6B). These results confirm that these ginsenosides can promote mitochondrial energy metabolism. Additionally, key proteins of the glucose oxidation process, HK-II and MPC1, were increased by Rg1, Re, Rf, and Rc, which was similar to GS (Fig. 6C–D). For SIRT1 expression, we found that the level of SIRT1 was upregulated by most of the ginsenoside monomers, with the exception of Rk3 (Fig. 6C–D). Especially, ginsenosides Rg1, Re, Rc, Rh1, Rb2, and Rb3 had a similar role in increasing SIRT1 expression as GS (Fig. 6C–D). Taken together, these results identified the major ginsenosides in GS, including Rg1, Re, Rf, Rb1, Rc, Rh1, Rb2, and Rb3, which promote aerobic cellular respiration and SIRT1-mediated mitochondrial biosynthesis to protect cardiomyocytes.

4. Discussion

Here, we revealed the novel mechanism by which GS promote mitochondrial energy metabolism in cardiomyocytes and neurons, aerobic respiration-dominated cells. In this study, GS pretreatment

Fig. 2. Effect of GS on key metabolites and enzymes of glucose metabolism in H9c2 and HUVECs. (A) The heatmap represents the targeted metabolomic analysis of 15 key metabolites of glucose metabolism in control and GS-pretreated H9c2 cells. **(B)** The heatmap showing 15 metabolites of glucose metabolism in control and GS-pretreated HUVECs. **(C)** Bar graph depicts the quantification of 15 metabolites in H9c2 cells. **(D)** Key enzymes were detected by Western blot analysis (n = 3 replicates per group). **(E)** Pyruvate dehydrogenase (PDH) activities in H9c2 and HUVECs cells pretreated with GS for 48 h were measured. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ *versus* Ctrl group.

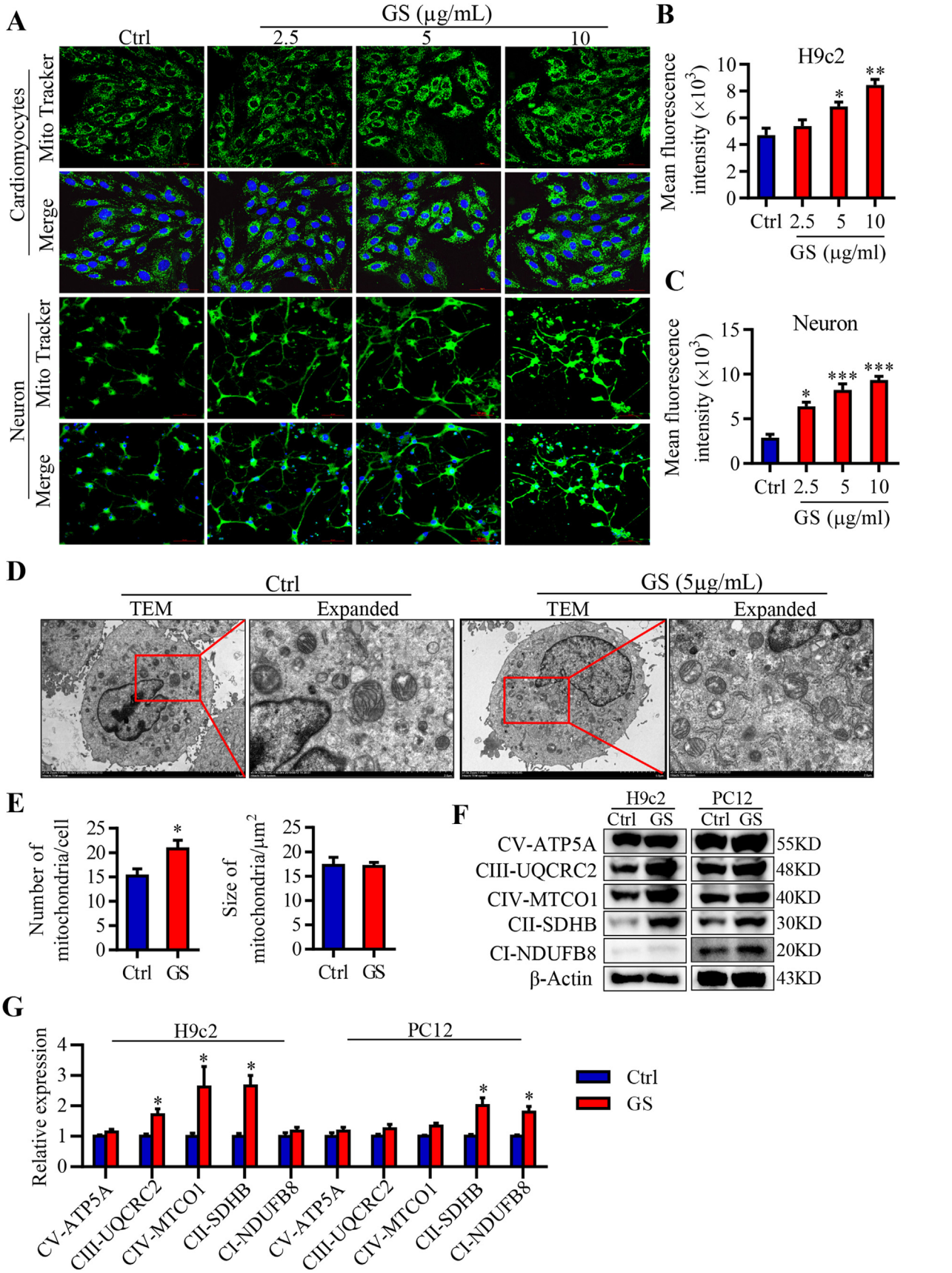


Fig. 3. GS increase mitochondrial biogenesis in cardiomyocytes and neurons. (A) H9c2 and neurons were co-stained with MitoTracker Green probe and Hoechst 33342 to analyze mitochondria amount by confocal microscopy. **(B–C)** Quantitative analysis from (A). **(D–E)** Mitochondrial structure was observed by a transmission electron microscope. **(F)** The levels of mitochondrial complexes I–V were detected by Western blot analysis (n = 3 replicates per group). **(G)** Relative expressions from (F) were quantified. *$P < 0.05$, **$P < 0.01$,

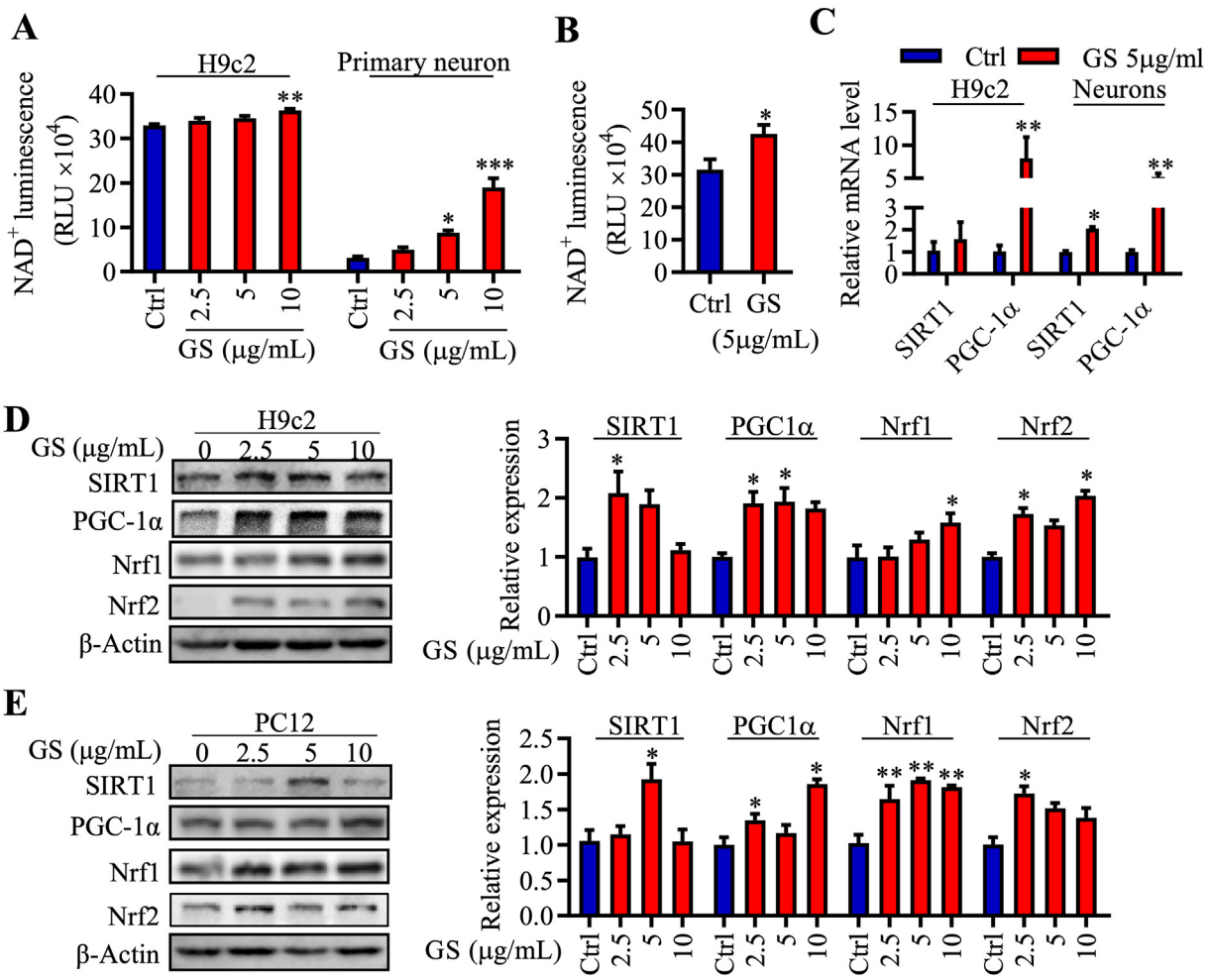


Fig. 4. GS promote NAD $^{+}$ -dependent SIRT1 activation in cardiomyocytes and neurons. **(A)** The NAD^{+} levels were measured by a luminescence -based kit. **(B)** The NAD level was analyzed by the LC-MS/MS method. **(C)** The SIRT1 and PGC-1α mRNA levels were analyzed by qPCR analysis. **(D–E)** The levels of SIRT1, PGC-1α, Nrf1, and Nrf2 were examined by Western blot analysis. Semi-quantitative analysis of relative expression for each protein is shown on the right (n = 3 replicates per group). Ctrl: control group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *versus* Ctrl group.

increased mitochondrial aerobic respiration, ATP production, and cellular NAD^{+} level. Moreover, GS activated the SIRT1-PGC-1α pathway to promote mitochondrial biosynthesis and ETC complex II-IV levels, which was inhibited by NAM. In hypoxia- and OGD-induced cell injuries, GS also increased ATP content and OXPHOS protein levels by promoting the activation of the SIRT1-PGC-1α pathway. Finally, major ginsenoside monomers in GS were identified for increasing HK-II, MPC1, and SIRT1 levels to promote mitochondrial energy promotion. These findings confirmed the potential role of GS for energy metabolism, which supported the clinical application of ginseng for the benefit of human health and ischemic disorders (Fig. S9).

Several studies have demonstrated that neurons depend on mitochondria, and endothelial cells rely on glycolysis, whereas skeletal cells utilize both OXPHOS and glycolysis for energy supply [34,40]. For the first time, we chose a couple of types of cell lines such as cardiomyocytes (H9c2, primary cardiac myocytes), neurons (differentiated PC12, primary neurons), bone marrow-derived stem cells, endotheliocytes (HUVECS), and stem cells to determine the effect of GS on energy metabolism. The analysis of basal OCR demonstrated that GS preferably increased oxygen consumption in cells, cardiomyocytes, and neurons, which were highly dependent on mitochondria, not those cell lines dependent on glycolysis for energy production, such as endothelial cells, or stem cells. Furthermore, ATP measurement and mitochondrial stress assay confirm the preferable effect of GS on mitochondrial respiration-dominant cardiomyocytes and neurons [41].

Glucose 6-phosphate is an essential metabolite in the first step of glucose metabolism, which is converted from glucose by hexokinases [42]. During the early stage of glycolysis, the content of glucose-6-phosphate was increased by GS in both cell lines. However, other major metabolites before pyruvate were obviously decreased in cardiomyocytes and not in endothelial cells. It suggests that the rapid metabolic process from fructose 6-phosphate to pyruvate was induced by GS in cardiomyocytes, which may be related to GS-mediated high expression of key rate-limiting enzymes, HK-II, PFKP, and PKM2. PDH is a vital regulatory enzyme for the connection of glycolysis to the aerobic TCA cycle, which was

$^{***}P < 0.001$ *versus* Ctrl group. CI-NDUFB8: NADH dehydrogenase 1 beta subcomplex subunit 8, CII-SDHB: succinate dehydrogenase iron-sulfur subunit, CIII-UQCRC2: ubiquinol-cytochrome-c reductase complex core protein 2, CIV-MTCO1: mitochondrially encoded cytochrome C oxidase I, CV-ATP5A: ATP synthase F1 subunit α.

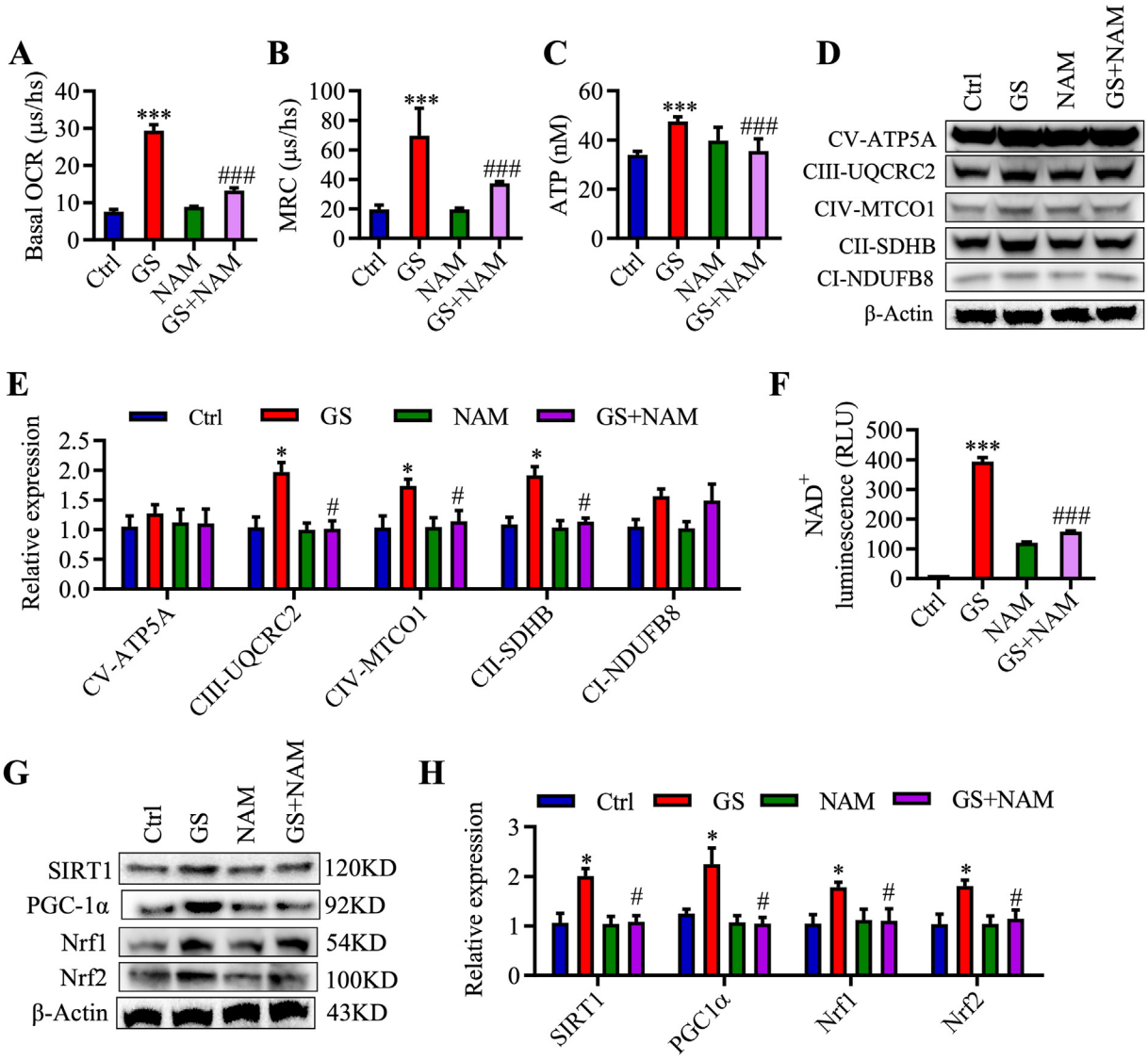


Fig. 5. The effect of GS on mitochondrial function was abrogated by NAM, a SIRT1 inhibitor. (A–B) Basal OCR and MRC in H9c2 cells were measured. **(C)** H9c2 cells were treated with GS and/or NAM for 48 h to measure ATP level by a bioluminescence detection kit. **(D)** The expressions of mitochondrial complexes in H9c2 cells treated with GS and/or NAM for 48 h were detected by Western blot (n = 3 replicates per group). **(E)** Relative levels from (D) are shown, after the quantification by ImageJ. **(F)** The effect of GS combined with NAM on the NAD^+ level was analyzed by a NAD/NADH-Glo assay kit. **(G–H)** Total proteins of H9c2 cells treated with GS and/or NAM for 48 h were prepared and followed by Western blot analysis (n = 3 replicates per group). $^*P < 0.05$ and $^{***}P < 0.001$ *versus* Ctrl group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *versus* GS + NAM group.

activated by GS in cardiomyocytes, not in endothelial cells. Compared with current reports, our findings clearly explore the role of GS on energy metabolism that GS pretreatment preferably promotes glucose metabolism into the TCA cycle for providing energy in cardiomyocytes, and has a slight function in endothelial cells through regulating different key enzymes in a series of steps of glucose metabolism into the TCA cycle. However, the effect of GS on the whole landscape and metabolic pattern of glucose involving multiple metabolic pathways in cardiomyocytes and neurons are still unclear, which are essential for addressing the detrimental effects of GS pretreatment on glucose metabolism. We need to perform the ^{13}C-based metabolic flux analysis to identify the accurate mapping of GS-mediated aerobic glucose oxidation in the future.

NAD^+ can modulate glycolysis in the cytoplasm and the TCA cycle/OXPHOS in the mitochondria, and activate sirtuins to maintain energy homeostasis [43]. In our study, NAD^+ levels in the cardiomyocytes, primary neurons, and fly brains were increased by GS treatment, which can activate SIRT1 to match cellular energy requirements [44]. SIRT1-mediated deacetylation leads to higher levels of PGC-1a to induce the nuclear transcription of Nrf1/2 for mitochondrial biogenesis [44]. In different models, we found that SIRT1 and its downstream pathway were obviously activated by GS, which may be the molecular mechanism of GS on the induction of mitochondrial mass. As we showed, GS-mediated mitochondrial respiration, ATP production, and higher levels of ETC proteins were abolished by NAM, which confirm that GS promote mitochondrial biosynthesis and function through the activation of SIRT1 pathway. Our results together with previous findings can verify the beneficial functions of ginsenosides on mitochondrial energy metabolism without ROS injury.

In our study, we found that most ginsenosides can activate SIRT1 and enhance ATP content, similar to previous findings. According to the content percentage of each ginsenoside in GS, we found that Rh1, Rb3, Rb2, or Rf at the concentration of lower than 0.2 μg/mL had a good effect on SIRT1 activation and mitochondrial

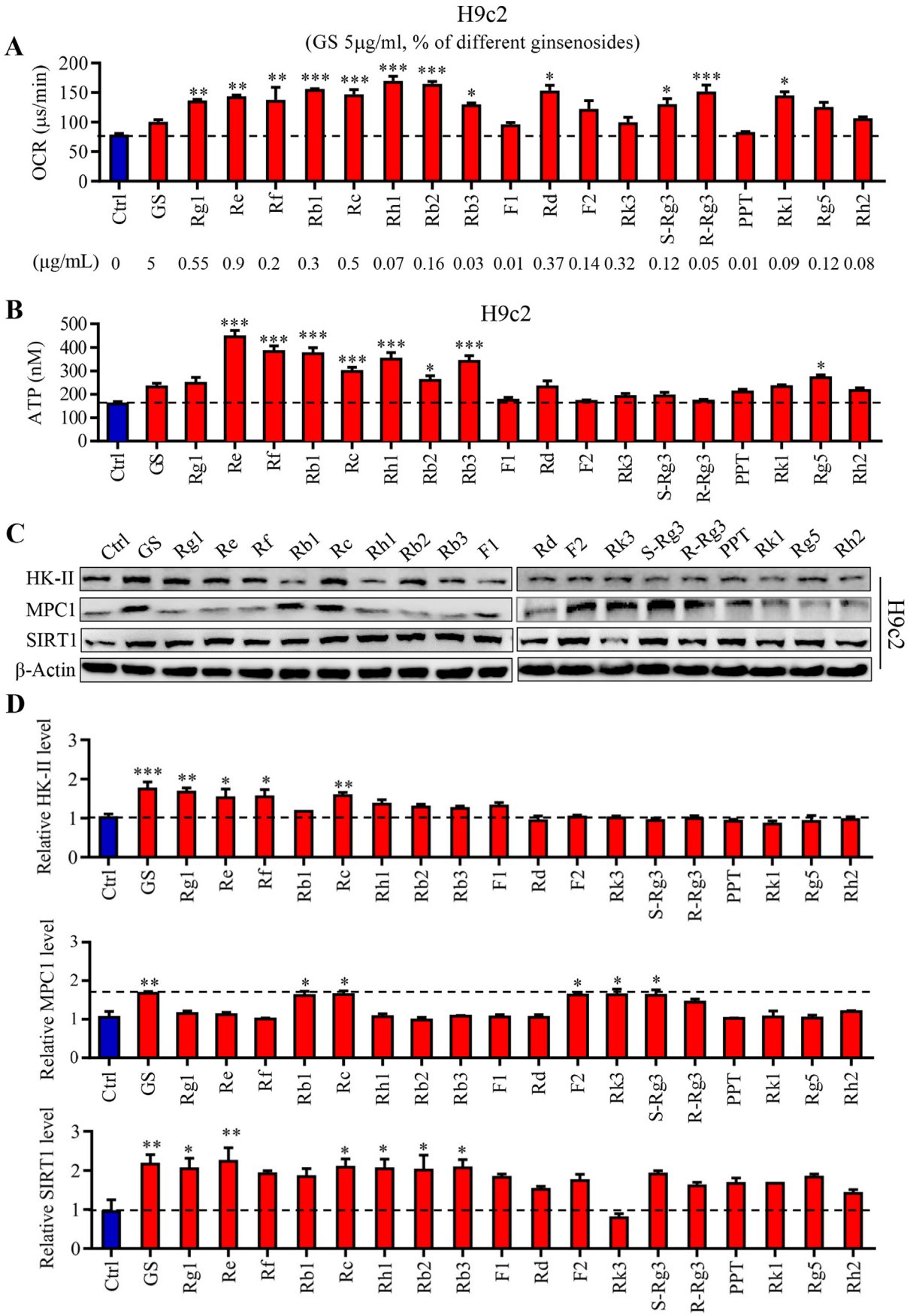


Fig. 6. The effects of main ginsenosides monomers on SIRT1-mediated mitochondrial function in H9c2 cells. (A–B) Basal OCR and ATP production were detected as described in Materials and Methods. **(C–D)** The levels of HK-II, MPC1 and SIRT1 in H9c2 cells were detected by Western blot (n = 3 replicates per group). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ *versus* Ctrl group.

respiration. Moreover, the analysis from published findings speculated that two glucopyranosyl groups on the C-3 position of ginsenosides are critical for SIRT1 activity [45,46]. Therefore, the binding mechanism of ginsenosides on the SIRT1 and their activity need to be well studied to explore the sites of key amino acids in SIRT1 protein using molecular docking, protein interaction, and mutation techniques. More importantly, recent study found that Rd combined with Re as neuroprotectant compounds for Parkinson's disease prevention and treatment through improving mitochondrial integrity and functions, and inhibiting mitochondrial apoptosis [47]. GRh2 combined with GRg3 or GRh2 combined with PPD induce mitochondrial-associated apoptosis by increasing mitochondrial ROS in human leukemia Jurkat cells or human gastric cancer cells [16,48]. These observations highlight the combinatory or synergistic effect of the ginsenoside monomers for regulating mitochondrial function. Therefore, the combinatory and synergistic effects of different ginsenosides targeting potential molecules of glucose metabolism and SIRT1 pathway could be investigated in the future.

In summary, *in vitro* and *in vivo* data provided evidence that GS from *P. ginseng* promoted aerobic cellular respiration and SIRT1-mediated mitochondrial biosynthesis in cardiomyocytes and neurons. Major ginsenoside monomers, such as Rb1, Rb2, Rb3, Rc, Rd, Re, Rf, or Rg1, in GS were found to activate mitochondrial respiration and SIRT1 pathway to promote energy metabolism. This study provides new insights into the extensive application of ginseng for cardiac and neurological protection in healthy subjects and patients with ischemic disorders.

Author contributions

Q. Zhao and X. Li conceived and designed this study. L. Sun, Q. Zhao and X. Li participated in the study design and interpreted data. Q. Huang and X. Li prepared the draft manuscript. Q. Huang, T. Lou, J. Lu, M. Wang, X. Chen, L. Xue, X. Tang, W. Q, Z. Zhang, H, Su, W. Jin, and C. Jing participated in the data collection and analysis. All authors have read and approved final manuscript.

Declaration of competing interest

All authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2022.02.002.

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