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GPR40-full agonist AM1638 alleviates palmitate-induced oxidative damage in H9c2 cells via an AMPK-dependent pathway

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G protein-coupled receptor 40 (GPR40) is gaining recognition as a potential therapeutic target for several metabolic disturbances, such as hyperglycemia and excessive inflammation. GPR40 is expressed in various tissues, including the heart; however, its specific roles in cardiomyocytes remain unknown. The objective of the present study was to investigate whether treatment with AM1638, a GPR40-full agonist, reduces palmitate-mediated cell damage in H9c2 rat cardiomyocytes. AM1638 treatment increased the phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) and expression levels of the antioxidant molecules heme oxygenase-1 (HO-1) and nicotinamide adenine dinucleotide phosphate: quinone oxidoreductase-1 (NQO1). Palmitate-mediated superoxide production and levels of 4-hydroxynonenal, a biomarker of oxidative stress, decreased after treatment with AM1638. Notably, palmitate-mediated disruption of mitochondrial membrane potential, lower levels of mitochondrial complex protein, and failure of adenosine triphosphate production were all recovered by treatment with AM1638. Moreover, AM1638 blocked palmitate-mediated caspase-3 cleavage and nuclear fragmentation, thereby improving cell viability. However, these AM1638-mediated beneficial effects were abrogated by treatment with Compound C, an AMPK inhibitor. These results demonstrate that AM1638, a GPR40-full agonist, ameliorates palmitate-mediated oxidative stress in H9c2 cells in an AMPK-dependent manner. [BMB Reports 2025; 58(3): 133-139]

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INTRODUCTION

Cardiomyopathy refers to any abnormality of the myocardium leading to the insufficient delivery of blood to the body and can contribute to the development of various cardiovascular diseases (CVD), such as coronary artery disease, arrhythmia, and congestive heart failure (1, 2). High oxidative stress plays a critical role in cardiomyopathy by causing cellular apoptosis and mitochondrial dysfunction (1, 3). Increased levels of reactive oxygen species (ROS) activate several transcription factors that induce apoptosis by damaging DNA and mitochondria (3). Although cardiac cell death leads to increased cardiac fibrosis, inflammation, and eventually cardiac dysfunction, there are no effective therapeutic strategies that can reduce cellular apoptosis by managing oxidative stress in cardiomyocytes.

G protein-coupled receptor 40 (GPR40), also known as free fatty acid receptor 1 (FFAR1), has been identified as a receptor for medium and long-chain FFAs, such as palmitate and oleate (4). Itoh *et al*. reported that long-chain FFAs can induce glucose-stimulated insulin secretion from pancreatic β cells via GPR40-calcium (Ca^{2+}) signaling (4). Therefore, various small chemicals for GPR40 agonism are being developed as novel hyperglycemic agents. Growing evidence suggests that GPR40 activation is associated with beneficial effects against metabolic stress exhibiting diverse action in various tissues and cell types. CNX-011-67, a GPR40 agonist, reduces chronic inflammation-induced endoplasmic reticulum (ER) stress and cell death through cyclic adenosine monophosphate (cAMP)/Ca^{2+} signaling in pancreatic β-cells (5). GPR40 is also expressed in hepatocytes and exerted a beneficial effect in high cholesterol diet-induced hepatic steatosis in C57BL/6 mice (6). In vascular endothelial cells, LY2922470, another GPR40 agonist, modulates inflammatory reactions by inhibiting lipopolysaccharide (LPS)-induced nuclear factor-kappa B (NF-κB) phosphorylation and vascular cell adhesion molecule-1 (VCAM-1) expression (7). Additionally, ultraviolet-induced ROS production and cytotoxicity were attenuated after the addition of GPR40 agonist to epidermal stem cells (8). Although all these studies suggest that GPR40 agonism might be a novel therapeutic target to over-

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come oxidative stress and inflammatory reactions, studies about the function of GPR40 agonists on cardiomyopathy are limited.

Adenosine monophosphate-activated protein kinase (AMPK) acts as an antioxidant and anti-inflammatory molecule in various tissues, including the heart (9). In diabetic mice, treatment with metformin, an AMPK activator, improves left ventricular ejection fraction (LVEF) and fractional shortening by inhibiting cardiac inflammation and fibrosis (10). In H9c2 cells, the hypoxia-mediated disruption of mitochondrial membrane potential and increased inflammation are reversed by AMPK activation (11). Recently, GPR40 has been identified as a novel target for AMPK activation. Li *et al*. demonstrated that GPR40-mediated calcium efflux from the ER to the cytosol sequentially induces calcium/calmodulin-dependent protein kinase kinase activation and AMPK phosphorylation, thereby blocking the expression of lipogenic genes in HepG2 cells (6). Furthermore, the overexpression of GPR40 in rats fed a high-fat diet, attenuated myocardial hypertrophy and collagen accumulation, whereas sirtuin-1 small interfering RNA prevented these effects (12). GPR40 promoted the expression of sirtuin-1, thus activating the liver kinase B1-AMPK pathway. Based on these findings, we hypothesized that the GPR40-AMPK axis could be a potential therapeutic target for cardiomyopathy via a reduction in oxidative stress, inflammation, and cytotoxicity.

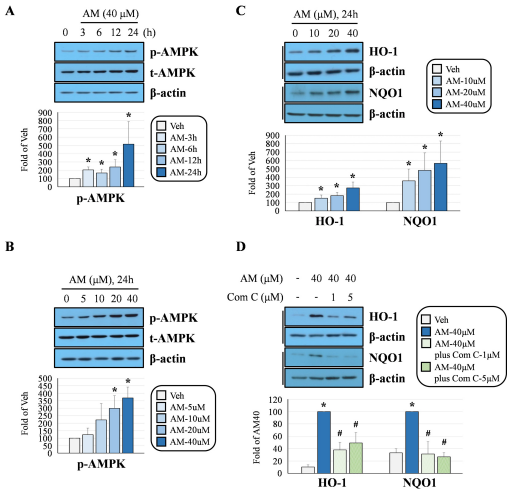

Fig. 1. HO-1 and NQO1 levels were increased by the AM1638-AMPK axis in H9c2 cells. (A, B) H9c2 cells were incubated with 40 μM of AM1638 for the indicated time points (0, 3, 6, 12, and 24 h), and with various doses of AM1638 (0, 5, 10, 20, and 40 μM) for 24 h. The levels of AMPK phosphorylation were analyzed by western blotting. (C, D) Cells were incubated with various doses of AM1638 (10, 20, and 40 μM) or 40 μM of AM1638 plus indicated doses of Compound C (1 and 5 μM) for 24 h. HO-1 and NQO1 levels were analyzed via western blotting. *Veh*, vehicle; *AM*, AM1638; *Com C*, Compound C. The mean ± SD was determined from at least three separate experiments (*P < 0.05 vs. Veh group; #P < 0.05 vs. AM-40 μM group; ANOVA with *post hoc* t-test).

Therefore, this study aims to determine whether AM1638, a novel GPR40-full agonist, exerts protective effects in H9c2 cells against palmitate, which can cause cardiomyopathy-related harmful effects by inducing malfunction of cadimyocytes (13). We assessed whether treatment with AM1638 i) induces AMPK phosphorylation, ii) reduces palmitate-mediated oxidative stress, and iii) inhibits palmitate-mediated mitochondrial dysfunction and cytotoxicity.

RESULTS

AM1638 increases the expression of antioxidant genes in an AMPK-dependent manner in H9c2 cells

Using western blot analyses, we found that AMPK phosphorylation was increased in a time- and dose-dependent manner following AM1638 treatment (Fig. 1A, B). Treatment with AM1638

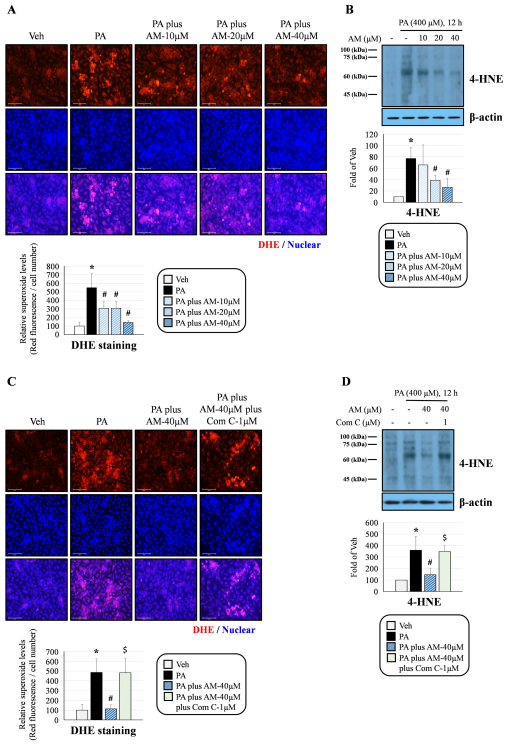

Fig. 2. Palmitate-mediated oxidative stress was inhibited by the AM1638-AMPK axis in H9c2 cells. H9C2 cells were pretreated with various doses of AM1638 (10, 20, and 40 μM) or 40 μM of AM1638 plus Compound C for 24 h, and then stimulated with palmitate for 12 h. (A, C) The stimulated cells were stained with DHE (red fluorescence) and observed under the fluorescence microscope. Scale bar: 125 μm. (B, D) Levels of 4-HNE were analyzed by western blotting. *Veh*, vehicle; *PA*, palmitate; *AM*, AM1638; *Com C*, Compound C. The mean ± SD was determined from at least three separate experiments (*P < 0.05 vs. Veh group; #P < 0.05 vs. PA group; $P < 0.05 vs. PA plus AM-40 μM group; ANOVA with *post hoc* t-test).

enhanced the expressions of antioxidant genes heme oxygenase-1 (HO-1) and nicotinamide adenine dinucleotide phosphate: quinone oxidoreductase-1 (NQO1) (Fig. 1C). However, these increases were abrogated by Compound C, an AMPK inhibitor (Fig. 1D). We expected the Kelch-Like ECH-associated protein 1 (Keap1)-nuclear factor erythroid 2-related factor 2 (NRF2) pathway to be activated by AM1638 treatment because NRF2 is a key transcription factor for HO-1 and NQO1 (14). However, neither Keap1 expression nor NRF2 translocation from the cytoplasm to the nucleus was altered in AM1638-treated H9c2 cells (Supplementary Fig. 1). These results suggest that the GPR40-AMPK axis is involved in the expression of HO-1 and NQO1 in H9c2 cells.

AM1638 ameliorates palmitate-induced oxidative stress in an AMPK-dependent manner in H9c2 cells

We determined the extent of intracellular oxidative stress by measuring superoxide and 4-hydroxynonenal (4-HNE) levels. Dihydroethidium (DHE) staining showed that palmitate-induced superoxide production was decreased by AM1638 treatment (Fig. 2A), and this effect was canceled by treatment with an AMPK inhibitor, compound C (Fig. 2C). Likewise, under palmitate treatment, AM1638 reduced 4-HNE levels in an AMPK-dependent manner (Fig. 2B, D). These results suggest that the GPR40-AMPK axis reduces oxidative stress in palmitate-treated H9c2 cells.

AM1638 alleviates palmitate-mediated mitochondrial damage in an AMPK-dependent manner in H9c2 cells

We measured mitochondrial membrane potential using a JC-1 dye. Flow cytometry and fluorescence images showed that healthy mitochondria (red fluorescence) were increased after treatment with AM1638 in palmitate-exposed H9c2 cells (Fig. 3A, Supplementary Fig. 2A). Adenosine triphosphate (ATP) level was decreased by palmitate, and this effect was reversed after treatment with AM1638 (Fig. 3B). Furthermore, we identified mitochondrial oxidative phosphorylation (OXPHOS) levels using western blot analyses. Treatment with AM1638 mitigated the palmitate-mediated reduction of complex I, II, III, and IV levels, but the level of complex V was unaffected by palmitate and AM1638 (Fig. 3C). The beneficial effects of AM1638, such as restoration of mitochondrial membrane potential, increased ATP production, and enhanced levels of OXPHOS complex I-IV, disappeared with the inhibition of AMPK (Fig. 3D-F, Supplementary Fig. 2B). These results suggest that the GPR40-AMPK axis improves mitochondrial function in palmitate-stimulated H9c2 cells.

AM1638 inhibits palmitate-mediated cytotoxicity in an AMPK pathway-dependent manner in H9c2 cells

The reduction of cytotoxicity under palmitate treatment was facilitated by the AM1638-mediated inhibition of oxidative stress and the improvement of mitochondrial function. AM1638 treatment inhibited palmitate-mediated caspase3 cleavage (Fig. 4A), decreased cell viability (Fig. 4B), and DNA fragmentation (Fig. 4C). Treatment with an AMPK inhibitor reversed these beneficial effects of AM1638 (Fig. 4D-F). These results suggest that the GPR40-AMPK axis reduces palmitate-induced cell damage in H9c2 cells.

DISCUSSION

In this study, the following results were obtained in H9c2 cells treated with AM1638. AM1638 i) enhanced AMPK phosphorylation and expression levels of the antioxidant molecules HO-1 and NQO1; ii) reduced palmitate-mediated oxidative stress; iii) improved palmitate-induced mitochondrial dysfunction; iv) inhibited palmitate-mediated cytotoxicity; and v) Compound C, an AMPK inhibitor, nullified all these AM1638-mediated beneficial effects.

Ho *et al*. discovered two ligand-binding regions in GPR40 via X-ray crystal analysis, which they named the first (A1) and second (A2) allosteric sites (15). GPR40-partial agonist TAK875 binds to the A1 and activates only the G-protein subunit alpha q (Gαq)/Ca^{2+} pathway, whereas GPR40-full agonist AM1638 binds to the A2 and activates both the Gαs/cAMP and Gαq/Ca^{2+}

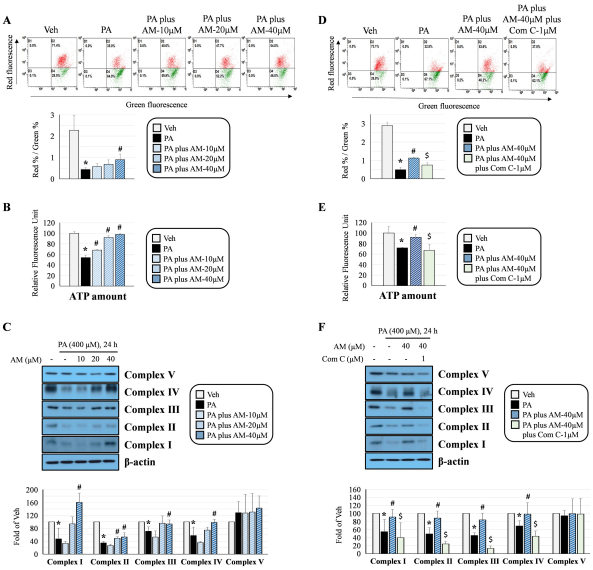

Fig. 3. Palmitate-induced mitochondrial damage was reduced by the AM1638-AMPK axis in H9c2 cells. H9c2 cells were pretreated with various doses of AM1638 (0, 10, 20, and 40 μM) or 40 μM of AM1638 plus Compound C for 24 h, and then stimulated with palmitate for 24 h. (A, D) The stimulated cells were stained using a JC-1 dye and analyzed by flow cytometry. (B, E) ATP levels were measured using an ATP assay kit. (C, F) Mitochondrial OXPHOS levels were determined by western blotting. *Veh*, vehicle; *PA*, palmitate; *AM*, AM1638; *Com C*, Compound C. The mean ± SD was determined from at least three separate experiments (*$P < 0.05$ vs. Veh group; $^{\#}P < 0.05$ vs. PA group; $^{\$}P < 0.05$ vs. PA plus AM-40 μM group; ANOVA with *post hoc* t-test).

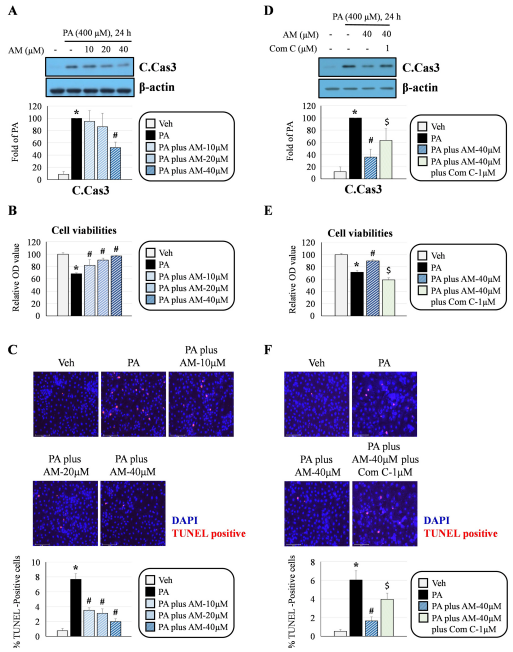

Fig. 4. Palmitate-induced cell death was decreased by the AM1638-AMPK axis in H9c2 cells. H9c2 cells were pretreated with various doses of AM1638 (0, 10, 20, and 40 μM) or 40 μM of AM1638 plus Compound C for 24 h, and then stimulated with palmitate for 24 h. (A, D) The level of C.Cas3 was determined by western blotting. (B, E) Cell viabilities were measured using an EZ-CYTOX solution. (C, F) DNA fragmentation was stained using a TUNEL assay kit. Scale bar: 125 μm. TUNEL-positive cells (red fluorescence) were observed and counted under a fluorescence microscope. *Veh*, vehicle; *PA*, palmitate; *AM*, AM1638; *Com C*, Compound C; *C.Cas3*, cleaved caspase 3. The mean ± SD was determined from at least three separate experiments (*$P < 0.05$ vs. Veh group; $^{\#}P < 0.05$ vs. PA group; $^{\$}P < 0.05$ vs. PA plus AM-40 μM group; ANOVA with *post hoc* t-test).

pathways (15). Hauge *et al*. reported that treatment with two GPR40-full agonists, AM1638 and AM5262, increased glucagon-like peptide-1 (GLP-1) expression and secretion in murine colonic crypts in a Gαs/cAMP-dependent manner. However, GPR40-partial agonist-mediated Gαq/Ca^{2+} signaling was not involved in GLP-1 expression (16). GLP-1 receptors, which are widely distributed in the body, including cardiomyocytes and blood vessels (17), have attracted attention as a novel therapeutic target for CVD including cardiomyopathy (17). Hwang *et al*. showed that AM1638 inhibits palmitate-mediated ROS production, ER stress, and cytotoxicity through the NRF2-HO-1 axis in vascular endothelial cells. However, the GPR40-partial agonists LY2922470 and TAK875 did not affect NRF2 activation, HO-1 expression, and ROS reduction (18). The ligand-dependent conflicting action of GPR40 has also been observed in cardiomyocytes. AM1638 treatment reduced palmitate-mediated oxidative stress via the AMPK/HO-1 axis in H9c2 cells (Fig. 1, 2). However, TAK875 did not affect the AMPK/HO-1 pathway. TAK875 treatment increased AMPK phosphorylation but not HO-1 expression (Supplementary Fig. 3). Unlike in endothelial cells, NRF2 signaling was not activated in H9c2 cells treated with AM1638 (Supplementary Fig. 1). Based on these results, we believed that GPR40-full agonists might be a better therapeutic candidate for cardiomyopathy than GPR40-partial agonists. Therefore, this study focused on the cardioprotective effects of GPR40-full agonism.

HO-1 and NQO-1 are representative cardioprotective molecules. HO-1 breaks down heme, an iron-containing protein, into carbon monoxide, ferrous ions, and biliverdin, which are involved in anti-inflammatory and antioxidative responses (19), and NQO-1 protects cells by breaking down quinones that produce free radicals (20). In H9c2 cells exposed to hypoxia, HO-1 overexpression enhances autophagy, thereby inhibiting ROS production and mitochondrial dysfunction and improving cell viability (21). Administration of dunnione, an NQO1 substrate, restored left ventricular internal dimensions, fractional shortening, and heart mass in Adriamycin-injected mice; however, these effects were canceled by NQO1 deficiency (22). In the present study, we showed that AM1638 enhanced the expression of HO-1 and NQO1 in an AMPK-dependent manner in H9c2 cells (Fig. 1). Palmitate-mediated superoxide production and 4-HNE levels were decreased after treatment with AM1638, and these antioxidant responses were attenuated by AMPK inhibition (Fig. 2). Notably, 4-HNE is a product of lipid peroxidation (LPO), a type of oxidative stress (23). Therefore, we suggest that GPR40-full agonism may have an antioxidant function via the AMPK/HO-1/NQO-1 axis in cardiomyocytes. Consistent with our results, GPR40 agonism triggered antioxidant responses in several cell types. In vascular endothelial cells, AM1638 treatment inhibited palmitate-induced superoxide generation via the NRF-HO-1 axis (18). Verma *et al*. reported that pancreatic GPR40 can be responsible for antioxidant effects as well as insulin secretion (5). Furthermore, SCO-267, another GPR40-full agonist, reduced the expression of cytochrome b-245-beta polypeptide (Cybb) and neutrophil cytosol factor1 (Ncf1), which are involved in ROS production, in the liver of obese mice (24).

Importantly, the present study also showed that AM1638 improved impaired mitochondrial membrane potential (Fig. 3A), decreased ATP production (Fig. 3B), and reduced OXPHOS (Fig. 3C), in palmitate-stimulated H9c2 cells, and these effects were all attenuated by AMPK inhibition (Fig. 3D-F). Mitochondria are important cellular organelles responsible for energy metabolism (25). Cardiac mitochondria provide ATP to cardiomyocytes to maintain a constant heart performance (26). When cardiomyocytes fail to produce sufficient ATP or die, the heart loses its ability to circulate blood (26, 27). Therefore, mitochondrial abnormalities are particularly relevant to cardiovascular diseases including heart failure, atherosclerosis, ischemia-reperfusion injury, and cardiomyopathy. Patients with chronic heart failure have lower mitochondrial oxidative capacities (28). In a myocardial infarction model, healthy mitochondria

were observed to be strongly involved in the reduction of infarct size and improvement of cardiac actions, such as LVEF and fractional shortening (29). Ablation of mitochondrial transcription factor A (Tfam) decreases mitochondrial gene expression and induces mitochondrial Ca^{2+} overload, thereby attenuating heart function (30). Peoples *et al*. reported that cardiac mitochondrial abnormalities are strongly associated with enhanced ROS production and limited ROS detoxification, suggesting that mitochondria-targeted antioxidant therapies are important for improving cardiac performance (31). Even though the preservation of mitochondrial function is considered an important therapeutic target for myocardial dysfunction, only a few studies have focused on the role of GPR40 in mitochondrial function. SCO-267 administration increases Tfam expression and β-oxidation pathway in the liver (24). Sunil *et al*. demonstrated that treatment with CNX-011-67, a GPR40-partial agonist, increases ATP levels in the islets of diabetic rats (32). Considering both previous studies and our results, GPR40-full agonism preserved mitochondrial function via the resolution of oxidative stress in an AMPK-dependent manner in cardiomyocytes.

The loss of mitochondrial membrane potential can induce cytochrome C release from mitochondrial intermembrane space to the cytosol and activate caspase cascades, leading to reduced cell viability (33). Similarly, in the present study, GPR40-mediated reduction of oxidative stress and improvement of mitochondrial actions led to increased cell viability. AM1638 treatment reduced caspase-3 cleavage, enhanced cell viability, and decreased DNA fragmentation in palmitate-stimulated H9c2 cells in an AMPK-dependent manner (Fig. 4). Excessive loss of cardiomyocytes results in a decrease in cardiac performance and collagen accumulation in the myocardium, a process known as cardiac fibrosis (34). As advanced fibrosis is irreversible, preserving the cardiomyocyte population is an important therapeutic strategy for heart dysfunction (34).

In conclusion, we describe for the first time that AM1638, a GPR40-full agonist, reduced ROS production, improved mitochondrial actions, and inhibited cell death in palmitate-stimulated H9c2 cells in an AMPK-dependent manner (Supplementary Fig. 4). These results show that GPR40-full agonism can become a novel therapeutic target to overcome myocardial dysfunction, such as cardiomyopathy.

MATERIALS AND METHODS

Cell culture and reagents

H9c2 cells were obtained from the Korean Cell Line Bank (KCLB, Seoul, South Korea) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L D-glucose, 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, and 50 g/ml streptomycin (Gibco, NY, USA) at 37°C and 5% CO_2. A GPR40-full agonist, AM1638 (MedChemExpress, NJ, USA), and an AMPK inhibitor, Compound C (Sigma-Aldrich, MO, USA), were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). Palmitic acid (Sigma-Aldrich) was dissolved in ethanol and conjugated with bovine serum albumin (Gibco).

Western blotting

Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose (NC) membranes (Amersham Bioscience, Westborough, MA, USA). The NC membrane was sequentially incubated with a blocking solution (5% non-fat dry milk dissolved in 0.05% TBST), blocking solution containing primary antibody, and blocking solution containing horseradish peroxidase (HRP)-conjugated secondary antibodies (Vector Laboratories, CA, USA). The immunoreactive bands were detected using a chemiluminescent solution (Bio-Rad Laboratories, CA, USA). The band density was measured using ImageJ software (National Institutes of Health, MD, USA).

DHE staining

H9c2 cells were stimulated with DMEM containing 10 μM DHE (red fluorescence, Invitrogen) for 30 min, and then stained in DMEM containing 1 μg/ml Hoechst (blue fluorescence, Sigma-Aldrich) for 10 min. Intracellular superoxide levels were observed under a fluorescence microscope (Olympus, Japan) and quantified using ImageJ software (National Institutes of Health).

JC-1 staining

H9c2 cells were stained in DMEM containing 1 μg/ml JC-1 (BioVision, CA, USA) for 30 min. To quantify healthy mitochondrial levels (red fluorescence), JC-1-stained cells were analyzed using a Cytomics FC500 Flow Cytometer (Beckman Coulter, CA, USA). Mitochondrial membrane potential was calculated by the ratio of red to green fluorescence.

ATP assay

ATP levels were measured using an EZ-ATP Assay Kit (Dogen Bio, Seoul, South Korea) according to the manufacturer's instructions. A fluorescence microplate reader (Bio-Rad Laboratories) was used to obtain fluorescence units (excitation, 535 nm; emission, 595 nm).

Cell viability assay

Cell viability was measured using EZ-CYTOX solution (Dogen Bio) according to the user manual. The optical density (OD, 450 nm) was measured using a microplate reader (Bio-Rad Laboratories).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

To assess DNA fragmentation, a TUNEL assay kit (MyBioSource, CA, USA) was used according to the manufacturer's instructions. TUNEL-positive cells (red fluorescence) were observed and counted under a fluorescence microscope (Olympus).

Statistical analysis

Differences between groups were checked for statistical significance using analysis of variance (ANOVA) with *post hoc* t-test. All graphs were obtained from at least three experiments and are presented as the mean ± standard deviation (SD). Results were considered significant at $P < 0.05$.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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