# Inhibition of GPR35 Preserves Mitochondrial Function After Myocardial Infarction by Targeting Calpain 1/2

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**Abstract:** Ischemia and anoxia-induced mitochondrial impairment may be a key factor leading to heart injury during myocardial infarction (MI). Calpain 1 and 2 are involved in the MI-induced mitochondria injury. G protein-coupled receptor 35 (GPR35) could be triggered by hypoxia. Whether or not GPR35 regulates calpain 1/2 in the pathogenesis of MI is still unclear. In this study, we determined that MI increases GPR35 expression in myocardial tissue. Suppression of GPR35 protects heart from MI injury in mice through reduction of reactive oxygen species activity and mitochondria-dependent apoptosis. Further studies show that GPR35 regulates calpain 1/2. Suppression of GPR35 reduces the expression and activity of calpain 1/2, and alleviates calpain 1/2-associated mitochondrial injury to preserve cardiac function. Based on these data, we conclude that a functional inhibition of GPR35 downregulates calpain 1/2 and contributes to maintenance of cardiac function under pathologic conditions with mitochondrial disorder. In conclusion, our study showed that the identified regulation by GPR35 of calpain 1/2 has important implications for the pathogenesis of MI. Targeting the action of GPR35 and calpain 1/2 in mitochondria presents a potential therapeutic intervention for MI.

**Key Words:** myocardial infarction, GPR35, calpain, mitochondria

## INTRODUCTION

Myocardial infarction (MI) is commonly encountered in hospital and outpatient settings and is associated with a high rate of morbidity and mortality. MI injury can often disrupt mitochondrial function and thus contribute to activation of oxidative stress and cardiac energetic metabolism disorder in cardiomyocytes, and lead to cardiomyocyte cell death.[1,2] Preservation of mitochondrial function under hypoxia and ischemic stress represents a potential therapeutic target for protection of cardiomyocytes and treatment of MI.

Calpain 1 and 2, 2 important types of cytosolic cysteine proteases,[3] have been identified to play a key role in the regulation of oxidative metabolism and energy production in mitochondria and are involved in MI or ischemia/reperfusion injuries.[4–6] Previous study showed that the translocation of calpain 1/2 from cytosol to mitochondria elevates intramitochondrial oxidative stress, whereas calpain 1/2 activation enhances the ischemia-induced myocardial injury via increased reactive oxygen species (ROS) generation and cell apoptosis.[7,8] The activation of calpain 1/2 is regulated by intracellular calcium levels.[9] Besides, G protein-coupled receptor 35 (GPR35), an Gi/o coupled orphan receptor that could be triggered by hypoxia and may also be involved in the pathogenesis of MI,[10,11] has been found to regulate the $Ca^{2+}$ transients.[12] Thus, the calpain 1/2 activation may be regulated by GPR35 via $Ca^{2+}$ signaling, and ischemia-induced hyperactivation of GPR35 and calpain 1/2 may result in mitochondrial dysfunction and participate in MI. Here, we tested the hypothesis that inhibition of GPR35 protect against mitochondrial dysfunction following MI. We provide evidence that inhibition of GPR35 preserves mitochondrial function and prevents MI-induced injury. We also find that inhibition of GPR35 suppresses the expression and activation of calpain 1/2, which participates in the protection of mitochondrial in MI.

## METHODS

### Mice MI Model and Treatment

Adult male C57BL/6J mice (6–8 weeks old, Dashuo Biotechnology, Chengdu, China) were housed in a room with a 12 light–dark cycle at 23°C and given free access to standard rodent food and tap water. Mice were randomized to the sham-operated group and MI group, and anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and placed on a heating pad to maintain their body temperature. The mice MI model were preformed as previously described.[13] After intubation via tracheotomy, mice were connected to a Harvard ventilator (Hugo Sachs Elektronik, Hugstetten, Germany) with an inspiratory pressure of 7 mL/kg.

The respiratory rate was set at 100 breaths per minute. After thoracotomy, the left anterior descending coronary artery of MI group was ligated. Moreover, MI groups were divided into the following groups: MI group, scramble siRNA treatment group, GPR35 siRNA treatment group, calpain 1/2 overexpression group, and SNJ-1945 (calpain inhibitor,[14,15] 120 mg/kg, orally, 1 hour before coronary artery ligation, SENJU Pharmaceutical, Kobe, Japan) treatment group. In the siRNA treatment or calpain 1/2 overexpression group, a 100 μL solution of the siRNA (GPR35 siRNA sequence: 5′-CAUUGUGCCUGACUUUAUAdTdT-3′, scrambled siRNA sequence: 5′-TTCGATGCCAGTCGTGCdTdT-3′) or calpain 1/2 plasmids (Addgene, Watertown, MA) in lentivirus vectors was injected into the apex and anterolateral wall with a 30-gauge needle 2 hours before MI. Mice were observed for additional 28 days to evaluate the survival rate.

All animal manipulations were performed strictly according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD). Formal approval to conduct the animal experiments described has been obtained from the Ethics Committee and the Institutional Animal Care and Use Committee of The General Hospital of Western Theater Command. All surgery was performed under anesthesia. All efforts were made to minimize suffering and to use only the number of animals necessary to produce reliable scientific data. No alternatives to animal experimentation are available for this type of experiments.

## Echocardiographic Analysis

Transthoracic echocardiography of mice was performed with GE vivid 9D ultrasound 2 weeks after MI. After anesthetizing with sodium pentobarbital (50 mg/kg), 2-dimensional echocardiographic views of the mid-ventricular short axis were obtained at the level of the papillary muscle tips below the mitral valve. Left ventricle fractional shortening (LVFS) and left ventricle ejection fraction (LVEF) was calculated as previously described.[16]

## Masson Staining

The myocardium fibrosis was assessed using a Masson staining kit according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering, Nanjing, China). Briefly, the hearts were isolated, washed several times with PBS, fixed with 4% paraformaldehyde for 24 hours, embedded in paraffin, sectioned (5 μm), and stained with Masson's trichrome for assessments of myocardium fibrosis, which were calculated as a percentage of total LV area.

## Primary NMVMs Isolation, Culture, and Treatment

Neonatal murine ventricular myocytes were isolated from newborn C57BL/6 mice (1–2 days old) with a protocol reported previously.[17–19] After euthanasia, the hearts were removed from the chest and perfused with Tyrode's solution (130 mM NaCl, 5.6 mM KCl, 3.5 mM MgCl₂, 5 mM HEPES buffer, 10 mM glucose, 20 mM taurine, 0.4 mM Na₂HPO₄, pH 7.4). The left ventricular tissues were chopped, triturated,

and digested with collagenase (Invitrogen, 0.5 mg/mL in Dulbecco's modified Eagle's medium, DMEM) at 37°C. After centrifuge, supernatant was removed and cell pellets were resuspended with Tyrode's solution containing 1% BSA and 600 μM calcium. After second centrifuge and wash with 1% BSA supplemented with 1.2 mM calcium, the cells were resuspended in DMEM supplemented with 10% FBS, and plated in culture dishes. The following day, culture media was changed.

Cells were exposed to an anoxic chamber with 5% CO₂ and 95% N₂ at 37°C for 12 or 24 hours and the CID2745678 (100 nM, GPR35 antagonist),[20] Ryan Scientific, Mt pleasant, SC and SNJ-1945 (10 μM) were administrated to the cells 5 minutes after anoxia, and incubated for 24 hours to inhibit GPR35 or calpain.

## Flow Cytometry Analysis

The cell apoptosis was tested using flow cytometry analysis (BD flow cytometry system, San Jose, CA). The NMVMs (1 × 10⁶ per well) were collected, resuspended, and included with 10 μL Annexin V-FITC and 5 μL propidium iodide (Roche Life Science, Basel, Switzerland). The data were analyzed by FlowJo 10.0 software (Ashland, OR).

## ROS Production Detection

Superoxide production in the NMVMs was evaluated with the fluorescent dye dihydroethidium (DHE, Molecular Probes, Eugene, OR). Cells grown on coverslips were stained with 5 μM at 37°C for 30 minutes. The images were taken with an Olympus BX51 Fluorescence Microscope (Olympus America Inc, Center Valley, PA) at an excitation wavelength of 490 nm and an emission wavelength of 590 nm. Moreover, the ROS production was also was measured by lucigenin-enhanced luminescence assay, as described in a previous study.[21]

## Quantification of Intracellular ATP

For ATP quantification, NMVMs (1 × 10⁴) grown in white 96-well plates were lysed and centrifuged. And the ATP content was measured by a luciferase-based ATP assay kit (Beyotime, Chengdu, China) according to the manufacturer's instructions.

## MMP Assay

Mitochondrial membrane potential (MMP) was assessed using JC-1 kit (Invitrogen, Carlsbad, CA). Briefly, cells cultured in 24-well plates after indicated treatments were incubated with an equal volume of JC-1 staining solution (10 μg/mL) for 20 minutes at 37°C in the dark and rinsed twice with buffer (provided as part of the kit and precooled at 4°C). JC-1 fluorescence was measured by a spectrofluorometer (Spectra Max, Atlanta, GA) with an excitation wavelength of 490 nm and emission wavelengths of 530 and 590 nm. Fluorescence intensity values were each expressed as ratios of emission at 590 to that at 530 nm.

## Transmission Electron Microscope Analysis

The transmission electron microscope was used to observe the mitochondrial morphology. The NMVMs (1 × 10⁶) were collected, centrifuged (1000 rpm, 10 minutes),

**FIGURE 1.** The effect of MI on the GPR35 expression in myocardium. The GPR35 mRNA (A) and protein (B) expression in myocardium following MI were detected by RT-PCR and immunoblotting (*$P < 0.05$, vs. sham, n = 6).
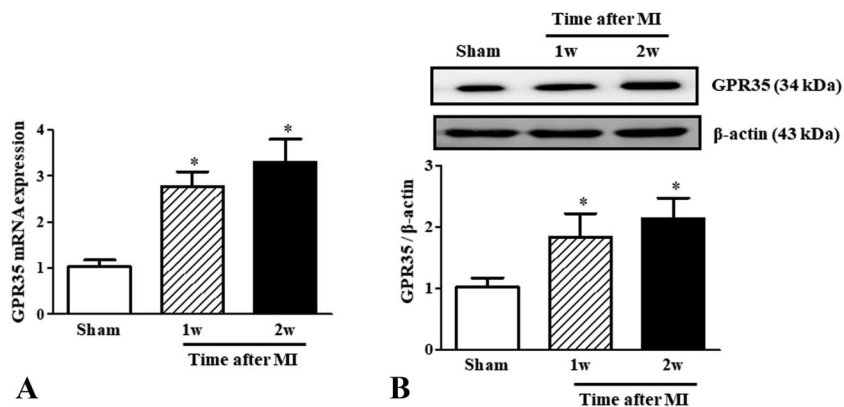
and fixed in 3% glutaraldehyde at 4°C for 2 hours. After postfixing for 1 hour in 1% osmium tetroxide, the cells were dehydrated in graded alcohols and acetones, and embedded in Epon 812. The samples were then sectioned in 80-nm thick slices. After staining with uranyl acetate and lead citrate, the samples were observed and photographed with a JEM-1400 electron microscope (Jeol, Tokyo, Japan).

## Calpain Activity Test

Calpain activity was determined using a fluorescence substrate N-Succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC) with a calpain activity assay kit (Genmed scientifics inc, Shanghai, China) as described in a previous study.[22]

## RNA Extraction and PCR

Total RNA from cells and tissues was extracted by Trizol (Tiangen, Beijing, China). A total 2 μg of RNA was used to synthesize cDNA and served as a template for amplification of GPR35 and calpain 1/2. The primers are as follow: GPR35: the forward primer: 5′-ACCACTGGTGTTGAGACGCC-3′, the reverse primer: 5′-TCTGGGTCTTTGAACTCGCTG-3′; calpain 1: the forward primer: 5′-CACAGCATTTCGAGCAACAGA-3′, 5′-CTTCCGCAGCTCTTTGTTCTC-3′; calpain 2: the forward primer: 5′-GGGACATGTGCAGCCAAGAC-3′, the reverse primer: 5′-TACACCACTTCAATCCACCCA-3′. The amplification was performed using the reverse transcription polymerase chain reaction (RT-PCR) kit (RR086A, TaKaRa, Japan). The relative amount of mRNA was calculated by $2^{-\Delta\Delta CT}$ and was normalized to a housekeeping gene 18s rRNA (forward 5′-CGGTCGGTGTGTGAACGGATTTG-3′ and reverse 5′-TGTAGACCATGTAGTTGAGGTCA-3′). Each sample was run and analyzed in triplicate. Relative expressions of target genes were given as a ratio to control the experiments.

## Immunoblotting

Tissues or cells were washed twice with ice-cold PBS and lysed in lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM PMSF, and 10 mg/mL each leupeptin and aprotinin). The homogenates (20 μg of protein) were separated by 8%–10% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluori membranes (Millipore, Bedford, MA).

The blots were then washed with tris-buffered saline Tween-20 (TBST), blocked with 1% BSA in TBST buffer for 1 hour, and incubated with the appropriate primary antibody at dilutions recommended by the supplier. Transblots were probed with the rabbit anti-GPR35 (cat No. PA5-23237, 1:500, Thermo Fisher Scientific, Rockford, IL), calpain 1/2 antibody (cat No. sc-58326, 1:500, Santa Cruz, Dallas, TX), Caspase-3 (cat No. ab13847, 1:300, Abcam, Cambridge, United Kingdom), cleaved Caspase-3 (cat No. ab2302, 1:300, Abcam), Caspase-9 (cat No. ab32539, 1:300, Abcam), and cleaved Caspase-9 (cat No. ab2324, 1:300, Abcam) overnight at 4°C, respectively. Then, the primary antibodies were detected with goat antirabbit-IgG (cat No. 111-005-003, 1:5000, Jackson ImmunoResearch Laboratory, PA) conjugated to horseradish peroxidase for 1 hour at room temperature, and the bands were visualized using a super signal chemiluminescence detection kit (Thermo Scientific, Waltham, MA). The amount of protein transferred onto the membranes was verified by immunoblotting for β-actin (cat No. SAB5600204, 1:3000; Sigma, St. Louis, MO).

## Statistical Analysis

The statistical analyses were conducted by SPSS 22.0 statistics software (IBM SPSS Inc, Chicago, IL). The data are expressed as mean ± SE. Comparison within groups was made by analysis of variance for repeated measures (or paired *t* test when only 2 groups were compared), and comparison among groups was made by analysis of variance with Holm–Sidak test (or *t* test when only 2 groups were compared). The least-significant difference was used for the post-hoc test. Survival curves were created by the method of Kaplan and Meier, and compared by a log-rank test. A value of *$P < 0.05$* was considered significant.

## RESULTS

## Myocardial GPR35 Expression Increases After MI

We first quantified the expression of GPR35 in myocardium tissues following MI in a mouse model. As shown in Figure 1, the mRNA and protein expression of GPR35 were significantly increased when compared with sham mice at 1–2 weeks post-MI. In addition to the mouse

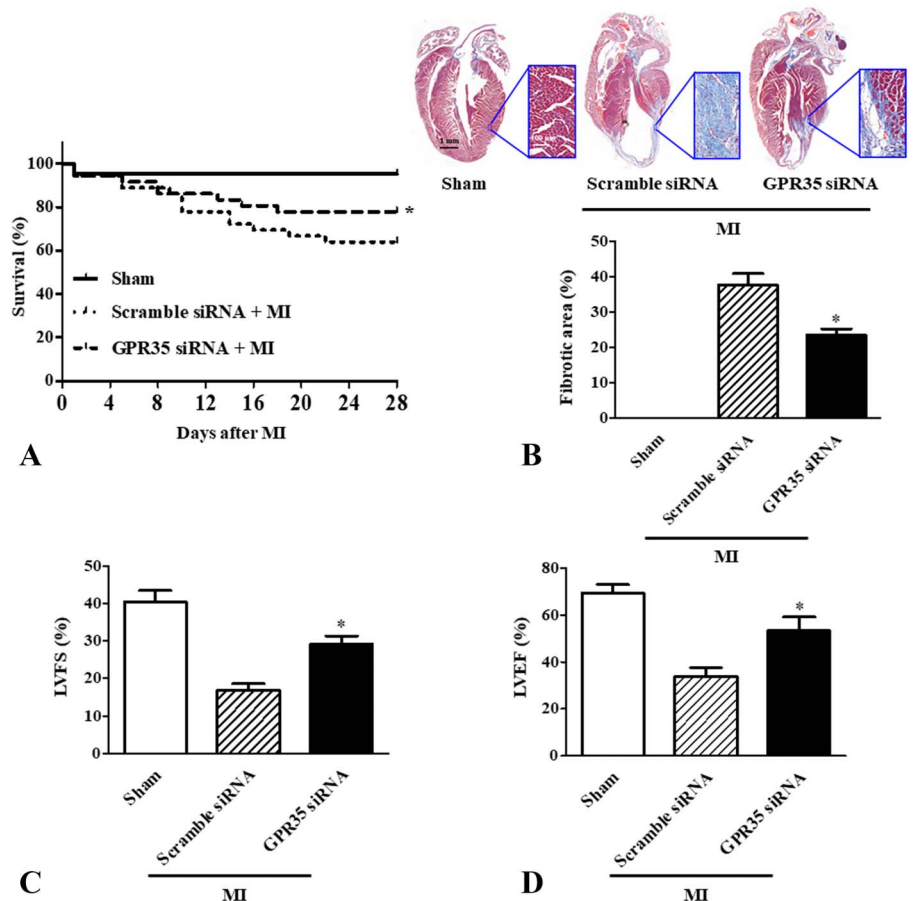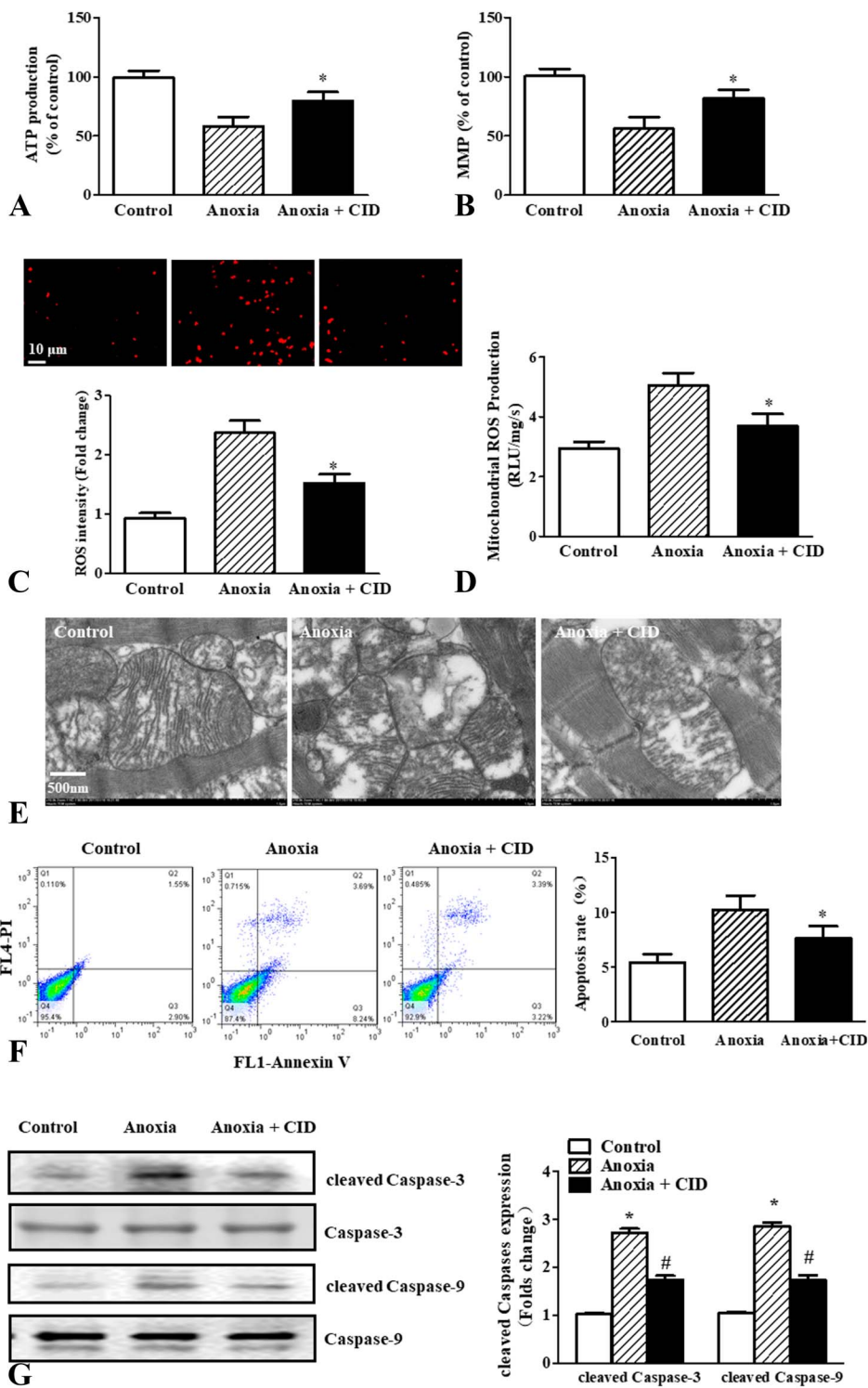**FIGURE 2.** The effect of anoxia on the GPR35 expression in NMVMs. The GPR35 mRNA (A) and protein (B) expression in NMVMs after 12 or 24-hour anoxia were detected by RT-PCR and immunoblotting (*$P < 0.05$, vs. control, n = 6).
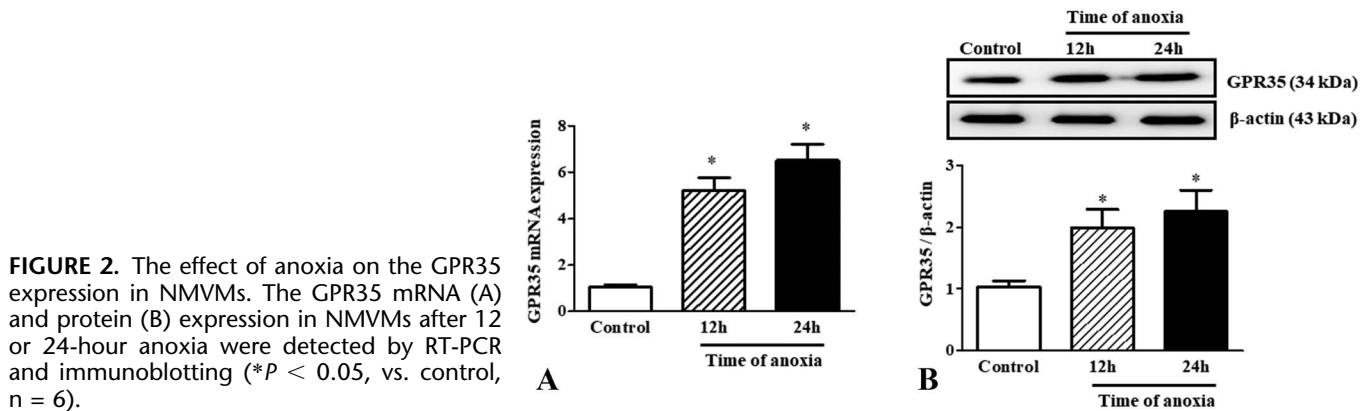
studies, we conducted experiments with NMVMs, and similar observations were made with the NMVMs subjected to 12- and 24-hour anoxia (Fig. 2).

## GPR35 Inhibition Protects Against MI-Induced Myocardium Injury by Targeting Mitochondria

Because MI causes elevation of GPR35 expression in heart tissue, we further used GPR35 siRNA to treat mice intramyocardially 48 hours before MI (see Figure S1,

**Supplemental Digital Content 1**, http://links.lww.com/JCVP/A450). We found that GPR35 siRNA improved the survival of mice that were subjected to MI (Fig. 3A). To quantify the effect of GPR35 inhibition on myocardial fibrosis, Masson staining was performed. Clearly, GPR35 siRNA treatment reduced myocardial fibrosis in mice subjected with MI injury (Fig. 3B). The echocardiographic analysis showed that the LVFS and LVEF was decreased in MI mice, whereas inhibition of GPR35 significantly recovered this hallmark of heart function (Figs. 3C, D).



**FIGURE 3.** GPR35 inhibition protected against MI in the mouse heart. A, The animal survival rate after MI with GPR35 siRNA treatment (*$P < 0.05$, vs. others; n = 21). B, The fibrosis of myocardium after MI was detected by Masson staining. The fibrotic area (blue) were calculated as a percentage of total LV area (*$P < 0.05$, vs. others; n = 6, scale bar = 1 mm). C and D, The cardiac function parameters including LVFS (C) and LVEF (D) were measured by echocardiography (*$P < 0.05$, vs. others; n = 6).

**FIGURE 4.** GPR35 inhibition protected against anoxia injury in NMVMs. A, Mitochondrial ATP production was checked by an assay kit. The result was expressed as a percentage of control (*$P$ < 0.05, vs. others; n = 6). B, MMP was assessed using JC-1 kit. Fluorescence intensity values were each expressed as ratios of emission at 590 to that at 530 nm. The result was expressed as a percentage of control (*$P$ < 0.05, vs. others; n = 6). C and D, The extent of ROS production in NMVMs was determined by DHE staining (C) and lucigenin-enhanced luminescence assay (D) (*$P$ < 0.05, vs. others; n = 6, scale bar = 10 μm). E, The mitochondrial morphology was observed by electron microscopy. Anoxia led to a marked swollen and disruption of cristae of mitochondrial in NMVMs, and GPR35 antagonist, CID2745678, alleviated the MI-induced mitochondrial injury (scale bar = 500 nm). F, The cell apoptosis was tested using flow cytometry analysis after Annexin V/PI staining. The data were analyzed by FlowJo 10.0 software (*$P$ < 0.05, vs. others; n = 6). G, Anoxia treatment enhanced caspase-3 and -9 activation in NMVMs, whereas CID2745678 treatment reduced anoxia-induced caspase-3 and -9 activation (*$P$ < 0.05, vs. others, #$P$ < 0.05, vs. anoxia group; n = 6).

As a key factor in MI, mitochondria dysfunction leads to cellular energy depletion, ROS generation, and mitochondria-induced cellular apoptosis.[23,24] The MMP and ATP production were decreased in anoxia-injured cells, and GPR35 inhibition increased MMP and ATP production in NMVMs subjected to anoxia (Figs. 4A, B). We also found that NMVMs subjected to anoxia showed enhanced DHE fluorescent intensity, indicative of ROS elevation, whereas CID2745678 treatment significantly reduced ROS in IR injured heart tissue (Fig. 4C). Similar observations were made
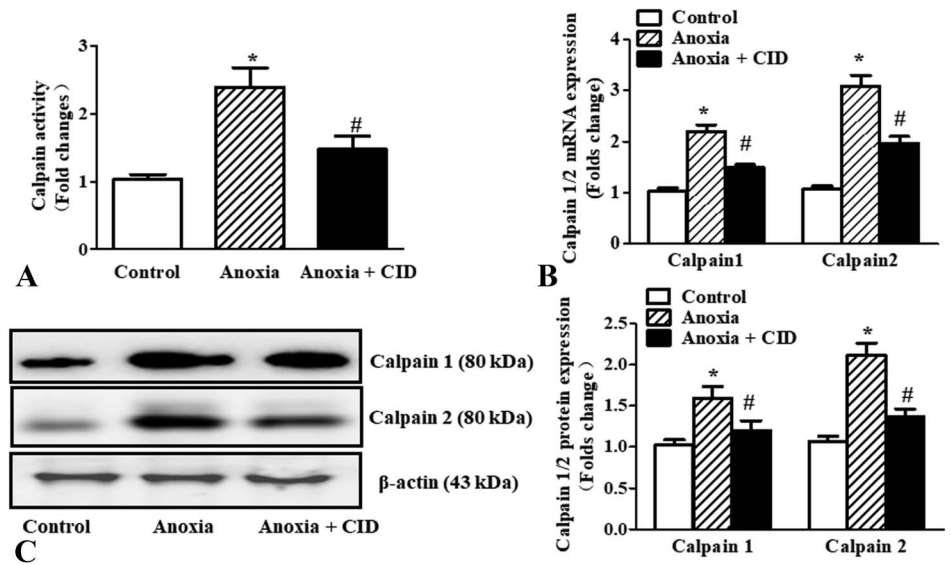
**FIGURE 5.** GPR35 inhibition suppresses anoxia-induced calpain 1/2 activation. A, The calpain activation was checked by a fluorescence substrate kit (*$P < 0.05$, vs. others, #$P < 0.05$, vs. anoxia group; n = 6). B and C, GPR35 inhibition suppresses the mRNA (B) and protein (C) expression of calpain 1/2 after anoxia treatment (*$P < 0.05$, vs. others, #$P < 0.05$, vs. anoxia group; n = 6).

with NMVMs by a ROS measurement assay (Fig. 4D). And the electron microscopy analysis showed that anoxia led to a marked swollen and disruption of cristae of mitochondrial in NMVMs, and GPR35 antagonist, CID2745678, alleviated the anoxia-induced mitochondrial injury (Fig. 4E). Besides, the effect of GPR35 antagonist in prevention of apoptotic cell death was evident in flow cytometry analysis. Figure 4F showed that anoxia increased NMVMs apoptosis, whereas GPR35 inhibition led to significant reduction of apoptosis rate in anoxia-injured cells. Figure 4G showed that caspase-3 and caspase-9 were activated in anoxia-treated NMVMs (represented by the cleavage product of caspase-3 and caspase-9), and administration of GPR35 antagonist, CID2745678, led to significant reduction of caspase-3 and -9 activity in anoxia injured cells. Overall, these results suggest that GPR35 inhibition has beneficial effects in preservation of heart function following MI injury, which involves mitochondria-dependent apoptosis.

## GPR35 Inhibition Decreases Calpain to Preserve Mitochondrial Function Following MI

Although calpains control mitochondrial function in several tissues,[5,25] we then explore the contribution of calpain in the anoxia-induced mitochondria dysfunction in heart. We used SNP-1945, a known inhibitor of calpain,[14,26] to treat mice orally (120 mg/kg) 1 hour before heart ischemia. The SNJ-1945 significantly preserved the heart function after MI, and reduced the MI-induced cardiac fibrosis (see **Figure S2A–S2C**, **Supplemental Digital Content 2**, http://links.lww.com/JCVP/A451). And we also showed that SNJ-1945 improved the reduced mitochondrial function in anoxia-injured NMVMs (see Figure S2D–S2F, **Supplemental Digital Content 2**, http://links.lww.com/JCVP/A451).

Toward understanding the potential role of calpain in GPR35 regulating mitochondrial function, we used

CID2745678 to inhibit GPR35 and found GPR35 inhibition reduced the calpain activity (Fig. 5A). Compared with the other members of the calpain family, calpain 1 and 2 are the predominant isoforms expressed within mitochondria,[27] and calpain 1/2 are responsible for the mitochondria-associated cell apoptosis.[4,28,29] Thus, we test the calpain 1/2 mRNA and protein expression by real-time PCR and Western blot and found the NMVMs calpain 1/2 expression significantly increased after anoxia, and GPR35 antagonist, CID2745678, decreased the mRNA and protein expression of calpain 1/2 (Figs. 5B, C).

Moreover, we induced the endogenous calpain 1/2 overexpression by pCMV-HA plasmid[30] in mice heart and NMVMs. The overexpression of calpain 1/2 were verified by fluorescence microscopy and immunoblotting (see **Figures S3A and S2B**, **Supplemental Digital Contents 2 and 3**, http://links.lww.com/JCVP/A452 and http://links.lww.com/JCVP/A451). We found that calpain 1/2 overexpression impaired heart function and led to mitochondria disorder and ROS elevation, whereas GPR35 inhibition, by siRNA or antagonist, restored heart function (Fig. 6) and significantly ameliorated the hallmarks of mitochondria function, including ATP production, MMP, and ROS elevation (Fig. 7).

## DISCUSSION

Data presented in this study support the concept that inhibiting GPR35 alleviates MI injury through preservation of mitochondrial function by suppressing calpain 1/2. We determined that MI increases GPR35 expression in myocardial tissue. Suppression of GPR35 protects heart from MI injury in mice through reduction of ROS activity and mitochondria-dependent apoptosis. Further studies show that GPR35 regulates calpain 1/2. Suppression of GPR35 reduces the expression and activity of calpain 1/2, and alleviates calpain 1/2-associated mitochondrial injury to preserve
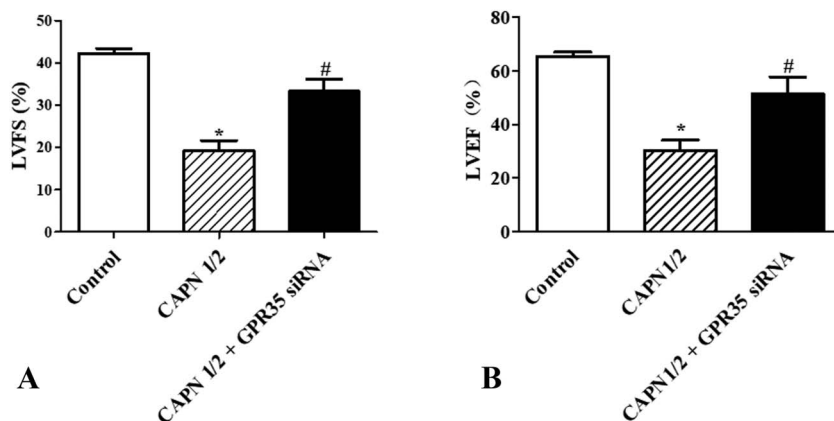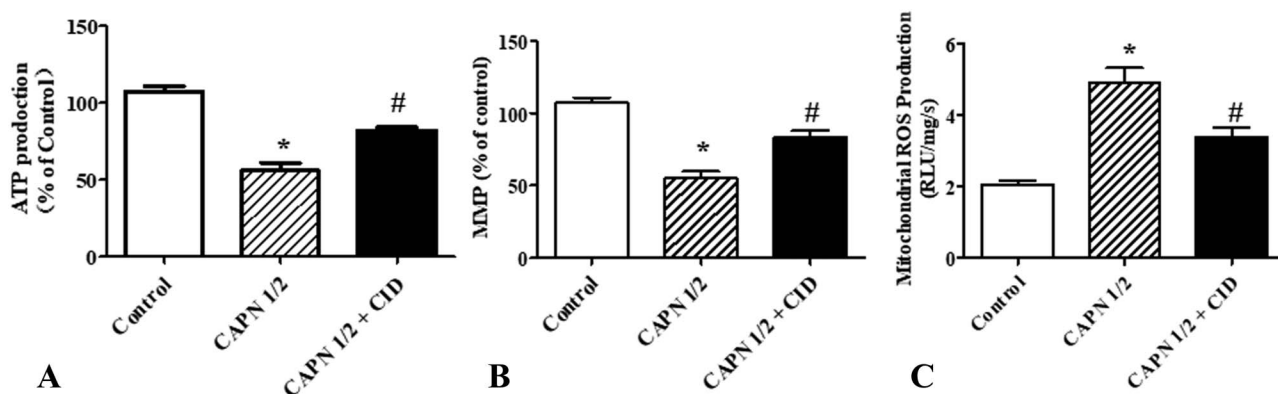
**FIGURE 6.** GPR35 inhibition suppresses calpain 1/2 overexpression-induced cardiac dysfunction. The cardiac function parameters including LVFS (A) and LVEF (B) were measured by echocardiography (*$P < 0.05$, vs. others, #$P < 0.05$, vs. calpain 1/2 overexpression group; n = 6).

cardiac function. Based on these data, we conclude that functional inhibition of GPR35 downregulates calpain 1/2 and contributes to maintenance of cardiac function under pathologic conditions with mitochondrial disorder.

There is increasing evidence that GPR35 plays an important role in the pathogenesis of cardiovascular diseases.[10,31,32] A genetic epidemiological study including 1241 hypertensive non-Hispanic white participants has indicated that GPR35 is associated with coronary artery disease.[11] Although a global gene expression microarray analysis showed the GPR35 expression in myocardial tissues from heart failure patients was significantly upregulated, compared with the healthy control,[33] similar result was also found in the present study. We also provided evidence to show GPR35 expression is increased in MI-injured myocardium. Because GPR35 has been identified as a marker of hypoxia,[10] we present data to show that myocardium ischemia and anoxia during MI lead to the increased expression of GPR35. And suppressing the expression of GPR35 could improve the cardiac function and survival following MI. However, the

underlying mechanisms by which elevation of GPR35 results in myocardial injury remain largely unknown.

Mitochondrial dysfunction is a key factor in MI injury, and impaired mitochondrial integrity may predispose cells to energy depletion, free radical generation, and eventual cell death.[1,24] Little is known on the role of GPR35 in MI-induced myocardial mitochondrial injury. Here, we present evidence that inhibition of GPR35 can suppress MI-induced reduction of ATP production and elevation of ROS. Thus, GPR35 antagonist can potentially be used as a prophylactic agent in the medical settings of anticipated MI injury. The effect of GPR35 on MI involves calpain-related mitochondria dysfunction. Mitochondrial calpain plays important roles in regulating cellular metabolic function and its response to physiologic or pathologic stresses.[6,34,35] Although published papers indicated that calpains shows a negative effect in the pathogenesis of MI,[14,36] our data also found inhibition of calpains improves the cardiac function and attenuates the MI-induced mitochondrial injury. There are 15 calpain subtypes. Among them, calpain 1 and 2 have been considered as mainly cytoplasmic



**FIGURE 7.** GPR35 inhibition suppresses calpain 1/2 overexpression-induced mitochondrial dysfunction in NMVMs. A, Mitochondrial ATP production was checked by an assay kit. The result was expressed as a percentage of control (*$P < 0.05$, vs. others, #$P < 0.05$, vs. calpain 1/2 overexpression group; n = 6). B, MMP was assessed using JC-1 kit. Fluorescence intensity values were each expressed as ratios of emission at 590 to that at 530 nm. The result was expressed as a percentage of control (*$P < 0.05$, vs. others, #$P < 0.05$, vs. calpain 1/2 overexpression group; n = 6). C, The extent of ROS production in NMVMs was determined by lucigenin-enhanced luminescence assay (*$P < 0.05$, vs. others, #$P < 0.05$, vs. calpain 1/2 overexpression group; n = 6).

enzymes; they are also present in mitochondria.[3,5,8,37] Because calpain 1 and calpain 2 are biologically active as proteases not as monomers, but only as dimers,[38] we further checked the role of calpain 1/2 dimers in MI. And our present study showed the regulation of GPR35 to calpain 1/2. The inhibition of GPR35 is able to suppress the expression of calpain 1/2 and restore the mitochondrial function, thus facilitating the protection of heart after MI. However, the underlying mechanisms remain largely unknown. The activation of calpain 1/2 is regulated by the intracellular calcium,[9,39] whereas GPR35 has been found to regulate $Ca^{2+}$ transients,[12] indicating that calcium may be involved as a signaling molecule in the GPR35-mediated regulation of calpain 1/2 expression and function. Our further researches will focus on the role of GPR35 in regulation of calpain 1/2.

In conclusion, our study showed that the identified signaling of GPR35 to calpain 1/2 has important implications for the pathogenesis of MI. One can envision that therapeutic means targeting GPR35-calpain 1/2 in modulating mitochondria-dependent ROS signaling and cell apoptosis may have translational value for treating MI.

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