Received: 27 May 2021 | Revised: 21 October 2021 | Accepted: 3 November 2021

DOI: 10.1096/fj.202100882R

THE FASEB JOURNAL

RESEARCH ARTICLE

LPL/AQP7/GPD2 promotes glycerol metabolism under hypoxia and prevents cardiac dysfunction during ischemia

Sohta Ishihama[1] | Satoya Yoshida[1] | Tatsuya Yoshida[1] | Yu Mori[1] | Noriyuki Ouchi[2] | Shunsuke Eguchi[1] | Teruhiro Sakaguchi[1] | Takuma Tsuda[1] | Katsuhiro Kato[1] | Yuuki Shimizu[1] | Koji Ohashi[2] | Takahiro Okumura[1] | Yasuko K. Bando[1] | Hiroaki Yagyu[3] | Nina Wettschureck[4] | Naoto Kubota[5] | Stefan Offermanns[4] | Takashi Kadowaki[5] | Toyoaki Murohara[1] | Mikito Takefuji[1]

[1]Department of Cardiology, Nagoya University School of Medicine, Nagoya, Japan

[2]Department of Molecular Medicine and Cardiology, Nagoya University School of Medicine, Nagoya, Japan

[3]Division of Endocrinology and Metabolism, Department of Internal Medicine, Jichi Medical University, Shimotsuke, Japan

[4]Department of Pharmacology, Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany

[5]Department of Diabetes and Metabolic Diseases Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Correspondence
Mikito Takefuji, Department of Cardiology, Nagoya University School of Medicine, 65 Tsurumai, Showa, Nagoya, 466-8550, Japan.
Email: takefuji@med.nagoya-u.ac.jp

Funding information
Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan

Abstract

In the heart, fatty acid is a major energy substrate to fuel contraction under aerobic conditions. Ischemia downregulates fatty acid metabolism to adapt to the limited oxygen supply, making glucose the preferred substrate. However, the mechanism underlying the myocardial metabolic shift during ischemia remains unknown. Here, we show that lipoprotein lipase (LPL) expression in cardiomyocytes, a principal enzyme that converts triglycerides to free fatty acids and glycerol, increases during myocardial infarction (MI). Cardiomyocyte-specific LPL deficiency enhanced cardiac dysfunction and apoptosis following MI. Deficiency of aquaporin 7 (AQP7), a glycerol channel in cardiomyocytes, increased the myocardial infarct size and apoptosis in response to ischemia. Ischemic conditions activated glycerol-3-phosphate dehydrogenase 2 (GPD2), which converts glycerol-3-phosphate into dihydroxyacetone phosphate to facilitate adenosine triphosphate (ATP) synthesis from glycerol. Conversely, GPD2 deficiency exacerbated cardiac dysfunction after acute MI. Moreover, cardiomyocyte-specific LPL deficiency suppressed the effectiveness of peroxisome proliferator-activated receptor alpha (PPARα) agonist treatment for MI-induced cardiac dysfunction. These results suggest that LPL/AQP7/GPD2-mediated glycerol metabolism plays an important role in preventing myocardial ischemia-related damage.

KEYWORDS
aquaporins, glycerol, myocardial infarction

Abbreviations: AMPK, AMP-activated protein kinase; ANP, atrial natriuretic peptide; AQP7, aquaporin 7; ATP, adenosine triphosphate; DHAP, dihydroxyacetone phosphate; DMEM, Dulbecco's Modified Eagle Medium; EDTA, ethylenediaminetetraacetic acid; FFA, free fatty acids; G3P, glycerol-3-phosphate; GK, glycerol kinase; GPD2, glycerol-3-phosphate dehydrogenase 2; KO, knock out; LPL, lipoprotein lipase; MEM, minimum essential medium; MI, myocardial infarction; PPARα, peroxisome proliferator-activated receptor alpha; qPCR, quantitative polymerase chain reaction; WT, wild-type.

Sohta Ishihama, Satoya Yoshida, and Tatsuya Yoshida contributed equally to this paper.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.
© 2021 The Authors. *The FASEB Journal* published by Wiley Periodicals LLC on behalf of Federation of American Societies for Experimental Biology

1 | INTRODUCTION

The heart has a high adenosine triphosphate (ATP) demand to sustain contractile activity and maintain tissue perfusion. Approximately 60%–90% of cardiac ATP is produced via the oxidation of fatty acids, whereas the remaining 10%–40% is from the oxidation of glucose, lactate, ketone bodies and amino acids.[1] Thus, although fatty acids constitute the predominant substrate for energy in the heart, the cardiac metabolic network is highly flexible in terms of using other substrates depending on physiological and pathological stress, such as exercise, pregnancy, myocardial infarction (MI), and heart failure.[2] The reciprocal relationship between fatty acid and glucose metabolism was first described in the 1960s.[3] Specifically, the increased generation of acetyl CoA derived from fatty acid oxidation decreases glucose oxidation in the heart by inhibiting pyruvate dehydrogenase, the enzyme that catalyzes pyruvate decarboxylation, which is a key irreversible step in glucose oxidation.[4] Conversely, increased acetyl CoA generation from glucose oxidation hinders fatty acid oxidation by suppressing carnitine palmitoyltransferase 1, which enhances fatty acid transport into the mitochondria.

MI, which is defined as myocardial cell death owing to prolonged ischemia, remains the leading cause of mortality worldwide.[5] The onset of myocardial ischemia results from an imbalance between oxygen supply and demand. It is generally accepted that under hypoxic conditions, cardiac metabolism shifts from fatty acids to glucose,[1] which is more efficient with respect to ATP production per O_2 consumed.[6]

Accumulating evidence suggests that modulating cardiac energy metabolism by increasing glucose oxidation and decreasing fatty acid oxidation can improve cardiac function in heart diseases[7]; however, various factors increase the concentration of plasma free fatty acids (FFA), such as the hormonal state in response to myocardial ischemia.[3] This contradiction between the detrimental effects of fatty acid oxidation and increased plasma FFA levels in MI suggests that lipoprotein lipase (LPL), which is the principal enzyme that converts triglycerides in the circulation to FFA,[8] mediates other metabolic pathways in MI. LPL is produced from cardiomyocytes, skeletal muscles and adipose tissues to control local fatty acid uptake,[9] and a genetic study indicates that LPL activation reduces the risk of coronary artery disease.[10] Glycerol is generated during the LPL-catalyzed breakdown of the triglyceride component of lipoproteins to provide fatty acids.[11] Aquaporin 7 (AQP7) is an aquaglyceroporin that facilitates glycerol transport across cell membranes into the heart.[12,13] AQP7 deficiency reduces glycerol uptake in the heart and exacerbates pressure overload-induced heart failure[14]; however, the role of glycerol as a substrate for energy production in cardiomyocytes under hypoxic conditions remains unclear.

2 | MATERIALS AND METHODS

2.1 | Materials and chemicals

Antibodies against the following proteins were used: α-actinin (Cat#CST-3134; Cell Signaling Technology, Beverly, MA) and LPL (Cat#ab21356; Abcam, Cambridge, UK). To detect apoptosis, heart sections were assessed by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining using the In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN).

2.2 | Isolation of adult murine cardiomyocytes

The protocol for isolation of adult murine cardiomyocytes was modified from that described by Ackers-Johnson et al.[15] Male mice (8–10 weeks old) were anesthetized with hydrochloric acid medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol tartrate (5 mg/kg). The chest was opened to fully expose the heart. The inferior vena cava was cut, and ethylenediaminetetraacetic acid (EDTA) buffer was immediately injected into the right ventricle. Hearts were quickly removed, cannulated from the aorta with a blunted 24-gauge needle, and then connected to a perfusion apparatus for retrograde perfusion. EDTA buffer and collagenase buffer (type II and IV collagenase: Worthington; protease XIV: Sigma-Aldrich, Burlington, MA) were perfused via the aortic cannula. After the heart was removed from the perfusion apparatus, the atria were removed, and the ventricles were cut and gently separated into small pieces with forceps and dissociated by gentle pipetting. Stop Buffer was added, the cell suspension was passed through a 100-μm strainer, and the myocytes were allowed to settle by gravity for 20 min. The cell suspension was subjected to four sequential rounds of gravity settling using three intermediate Ca^{2+} reintroduction buffers to gradually restore the extracellular Ca^{2+} concentration to 1.8 mM. Settled cells were then plated in plating buffer (minimum essential medium [MEM] containing 5% fetal bovine serum [FBS], 2.0 mM L-glutamine, and 10 nM 2, 3-butanedione monoxime) for 1 h, at which point the medium was removed and adherent cells were resuspended in culture medium (MEM containing 0.1% fatty acid free bovine serum albumin [BSA], 2.0 mM L-glutamine, 10 nM 2,3-butanedione monoxime, 10 μg/ml insulin, 5.5 μg/ml transferrin, and 5.0 ng/ml selenium) for 2 h. The culture medium was then changed to each

study-conditioned medium, and cells were cultured under each study condition. The glucose-free culture media consisted of Dulbecco's Modified Eagle Medium-no glucose containing 0.1% fatty acid-free BSA, 2.0 mM L-glutamine, 10 nM 2,3-butanedione monoxime, 10 μg/ml insulin, 5.5 μg/ml transferrin, and 5.0 ng/ml selenium. To establish culture media with each indicated glycerol concentration, the appropriate amount of glycerol was added to the glucose-free culture medium. For hypoxic studies, cells were exposed to hypoxic conditions generated using an AnaeroPack System (Mitsubishi GAS Chemical, Tokyo, Japan).

2.3 | LPL activity assay

After the culture media were removed, adult murine cardiomyocytes were cultured under normoxic or hypoxic conditions. After 1 h, heparin (5 U/ml) was added to each medium. At 10 min later, the medium was collected, and LPL activity was assayed using Lipoprotein Lipase Activity Assay Kit (INC STA-610; CELL BIOLABS, San Diego, CA) following the manufacturer's protocol.

2.4 | Cell viability

After 1 h of culture under normoxic (O_2 concentration: 21%) or hypoxic (O_2 concentration: <1%) conditions, a cell viability assay was conducted using trypan blue staining, following a previously described method.[16]

2.5 | Intracellular ATP concentration

Cardiomyocyte intracellular ATP concentration was measured using Cell Titer Glo 2.0 (Promega, Madison, WI). The glycerol-3-phosphate dehydrogenase 2 (GPD2) inhibitor (Cat#530655; Sigma-Aldrich) was dissolved in Dimethyl sulfoxide (DMSO, final concentration < 0.1%).

2.6 | GPD2 enzymatic activity

Mitochondria were isolated from cardiomyocytes or heart tissues using the Mitochondrial Isolation Kit (Cat#ab110168; Abcam) according to the manufacturer's instructions. GPD2 enzymatic activity was measured as the rate of reduction of cytochrome C following the addition of glycerophosphate. Mitochondria were isolated for less than 3 h before activity assays were performed on fresh mouse hearts. Measurement of GPD2 activity using murine cardiac mitochondrial lysate preparations were performed using cytochrome C as the electron acceptor and a measuring absorbance gain of 550 nm. The reaction buffer consisted of 10 mM Tris-HCl, 50 μM cytochrome C, 25 μM sodium azide, 1 mM EDTA, and 50 mM KCl. The reactions were conducted at 37°C.

2.7 | Mice

The GPD2 (mGPDH)-KO mouse line was obtained from Tokyo University.[17] The AQP7-KO mouse line (RBRC06294) was obtained from RIKEN BRC.[18] Mice carrying floxed alleles of *Lpl* were obtained from Columbia University.[19] MI was induced in 8- to 10-week-old male mice as described previously.[16] For isolation of adult murine cardiomyocytes, 8–10-week-old male mice were also used. All procedures for animal care and use were approved by the Animal Ethics Review Board of Nagoya University School of Medicine.

2.8 | Generation of inducible LPL knockout (KO) mice

Tamoxifen-inducible, cardiomyocyte-specific LPL knockout mice (cmc-LPL-KOs) were generated by intercrossing the αMHC–CreERT2 line to $LPL^{flox/flox}$ mice.[19,20] Cre-mediated recombination of floxed alleles was induced via intraperitoneal injection of 1 mg tamoxifen dissolved in 100 μl Miglyol for five consecutive days. Vehicle-treated mice received Miglyol only, and αMHC–$CreERT2^{+/-}$-$LPL^{wt/wt}$ mice were used as controls. Experiments were performed 5–7 days after the end of induction.

2.9 | MI mouse model

MI was induced in 8- to 10-week-old male mice as described previously.[16] After the left anterior descending artery was ligated, the mice were sacrificed at 1, 7, or 28 days following surgery. Before sacrifice, the mice were fasted overnight. Echocardiography was performed using a Vevo 1100 (Fujifilm VisualSonica Inc., Bothell, WA). Glycerol (2 g/kg; Sigma-Aldrich) was orally administered at 0, 8, and 16 h after the left anterior descending artery was ligated, and echocardiography was performed at 24 h after the surgery. Fenofibrate (800 mg/kg/day; Sigma-Aldrich) was orally administered for five consecutive days before the left anterior descending artery was ligated and echocardiography was performed at 24 h after the surgery.

Frozen mouse heart samples were embedded in optimal cutting temperature compound (Sakura Finetek, Tokyo, Japan). The samples obtained from at

least six sites, from below the ligation site to the apex, were sectioned to 6-μm thickness, and then stained with PicroSirius red to assess cardiac fibrosis. Infarct size measurement was obtained according to length measurement.[21]

2.10 | Immunostaining assay

Samples for immunohistochemistry were fixed with 4% paraformaldehyde at 4°C for 1 h and washed thoroughly in phosphate-buffered saline (PBS). Next, 0.5% Triton X-100 in PBS was applied for 30 min, and samples were blocked with Protein Block Serum-Free (Agilent Technologies, Santa Clara, CA) for 30 min. Thereafter, they were incubated overnight at 4°C with the primary antibodies and then reacted with Alexa-conjugated secondary antibodies (Alexa Fluor 488 and 647; Thermo Fisher Scientific, Waltham, MA). Nuclei were visualized using Cellstain DAPI Solution (Cat#340-07971; Wako, Tokyo, Japan). The isolated cardiomyocytes and the heart sections were observed using a BZ-X microscope or a Zeiss LSM780 confocal laser scanning microscope using the ZEN software (Zeiss, Oberkochen, Germany).

To assess the expression of LPL on the cell surface, isolated cardiomyocytes without permeabilization were incubated with the anti-LPL primary antibodies. After five washes in PBS, Triton X-100 was added and the cardiomyocytes were incubated with anti-α-actinin primary antibodies. LPL expression on the cell surface of isolated cardiomyocytes was counted in 10 groups of 20 random individual cells using Image J (NIH, Bethesda, MD). High expression was defined as the cell exhibiting over five particles. The fluorescence intensity of LPL at the infarct or non-infarct area was quantitated using Image J.

2.11 | Quantitative reverse transcription-PCR

To analyze mRNA expression, RNA was extracted from the left ventricle of C57BL/6 mice using an RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol and reverse-transcribed using the ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Quantitative reverse transcription-PCR analysis was performed using SYBER Premix Ex Taq II (TaKaRa Bio, Kusatsu, Japan) and the following primers: *Nppa* (ANP) 5′-TGA CAG GAT TGG AGC CCA GA-3′, 5′-GAC ACA CCA CCA GGG CTT AGG A-3′; *Rps18* 5′-TTC TGG CCA ACG GTC TAG ACA AC-3′, 5′-CCA GTG GTC TTG GTG TGC TGA-3′; and *Gk* (glycerol kinase) 5′-TGC ATG ATC CTC TAA GCA GAC C-3′, 5′-ACA TCA TGA CAG TGG AGG CA-3′. Data are presented following normalization to *Rps18* expression.

2.12 | Quantification and statistical analysis

Data are presented as the mean ± SEM in all experiments. All statistical analyses for animal experiments were performed using GraphPad Prism 9 (GraphPad Software Inc., La Jolla, CA). Data were analyzed with the Student's unpaired *t*-test to compare means when there were two experimental groups, with one-way ANOVA followed by Tukey's post-hoc test to compare means between > 2 groups, and with two-way ANOVA with Tukey's post-hoc test or two-way repeated-measures ANOVA with Bonferroni post-hoc test when there were two or more independent variables. "*n*" refers to the number of independent experiments or mice per group. Significance was defined as $p < .05$.

3 | RESULTS

3.1 | Hypoxia enhances cardiomyocyte LPL expression both in vitro and in vivo

To investigate whether acute MI increases cardiac LPL expression, the left coronary artery in wild-type (WT) mice was ligated. LPL was assayed by immunostaining of sections using an antibody against LPL at 1 h after the ligation (Figure 1A,B). MI increased the intensity of LPL in the infarct area, suggesting that the ischemic conditions enhanced LPL expression in vivo. To investigate whether hypoxic conditions affect LPL expression on isolated adult murine cardiomyocytes, LPL expression on these cells under 1-h of culture under hypoxic conditions was determined by immunostaining (Figure 1C,D). Hypoxic conditions significantly increased the expression level of LPL on the cardiomyocyte surface, suggesting that hypoxia enhanced cardiac LPL expression. We also examined LPL activity in the culture medium, and our findings showed that hypoxic conditions significantly increased LPL activity (Figure 1E). To examine the functional significance of cardiac LPL under ischemic conditions in vivo, we used mice with tamoxifen-inducible cardiomyocyte-specific deficiency for LPL (cmc-LPL KO; Figure S1A,B). No differences were observed in basal cardiac function between cmc-LPL WT and cmc-LPL KO mice (Figure S1C). Notably, cardiomyocyte-specific LPL deficiency suppressed MI-induced LPL expression in the heart, suggesting that LPL is synthesized in cardiomyocytes under ischemic conditions (Figure 1F). We performed echocardiography to

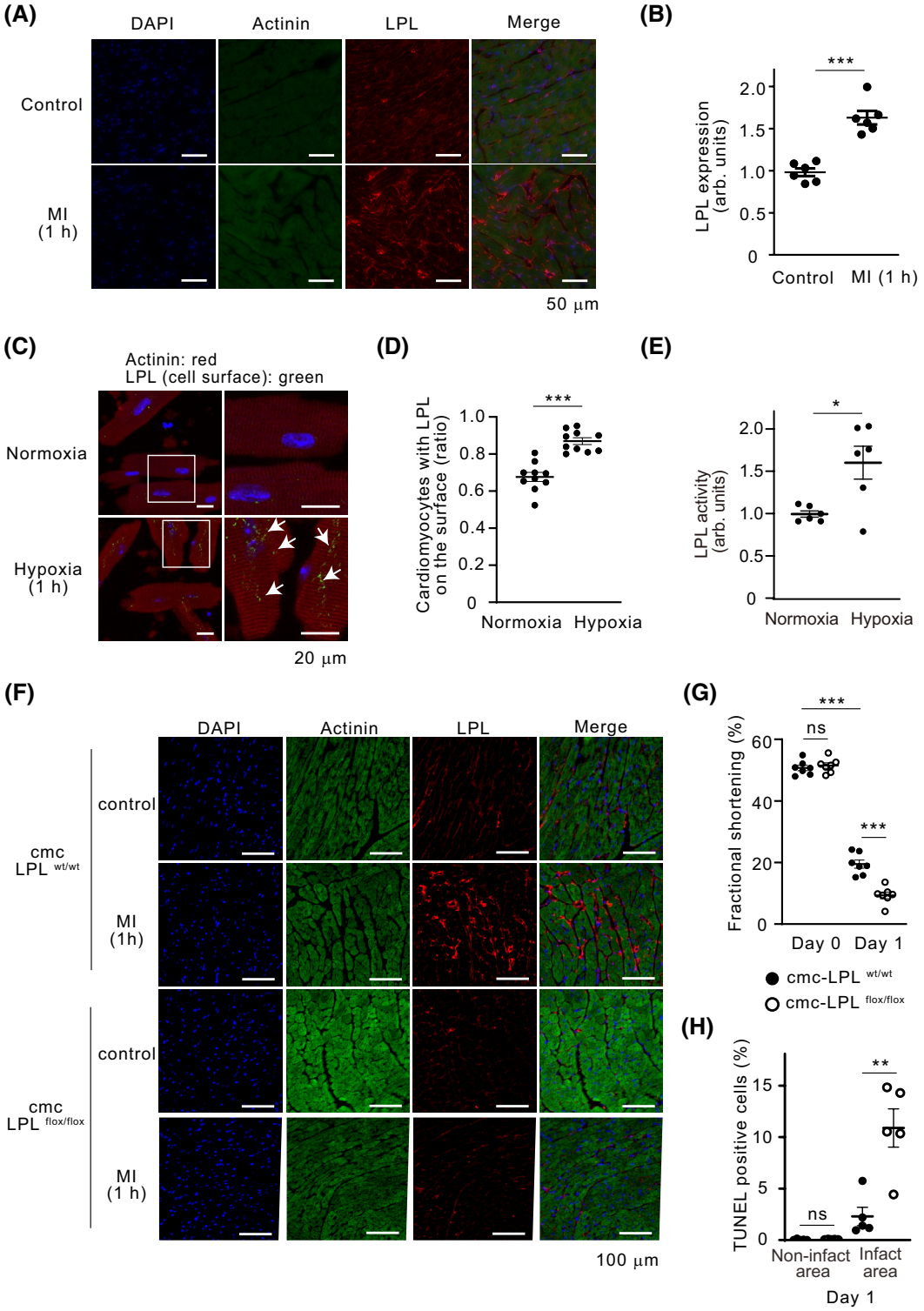

FIGURE 1 Myocardial infarction (MI) increases lipoprotein lipase (LPL) expression in the heart. (A) Immunostaining showing LPL expression in the left ventricle after 1-h MI (blue, DAPI; green, actinin; red, LPL; scale bar, 50 μm). (B) Statistical evaluation of (A) ($n = 6$). (C) Hypoxic conditions increase LPL expression on the surface of isolated murine cardiomyocytes (red, actin; green, LPL; blue, DAPI; scale bar, 20 μm). *Right*, magnified image of the boxed area in the panel. (D) Statistical evaluation of (C) ($n = 10$). (E) Hypoxic conditions increased LPL activity in the culture medium of isolated murine cardiomyocytes ($n = 6$). (F) LPL expression in the left ventricle after 1-h MI in cardiac-specific $LPL^{wt/wt}$ and $LPL^{flox/flox}$ mice (blue, DAPI; green, actinin; red, LPL; scale bar, 100 μm). (G) Left ventricular fractional shortening determined by echocardiogram before and at 1 day after coronary artery ligation ($n = 7$). (H) Cardiomyocyte apoptosis as determined by TUNEL assay of the heart ($n = 5$). ns, not significant; $^*p < .05$; $^{**}p < .01$; $^{***}p < .001$. Data are presented as the mean ± SEM and were analyzed with unpaired Student's *t*-test (B, D, and E) or two-way ANOVA followed by Tukey's post-hoc test (G and H)

assess cardiac functionality at 1 day after left coronary artery ligation (Figure 1G). In control mice, 1-day coronary artery ligation resulted in significantly decreased cardiac function, with cardiac LPL deficiency exacerbating the MI-induced cardiac dysfunction. To examine whether cardiac LPL deficiency affects cardiac apoptosis induced by ligation, apoptotic cardiomyocytes were assessed by TUNEL staining of heart sections at 1 day after the ligation (Figure 1H). LPL deficiency increased MI-induced apoptosis.

3.2 | Glycerol protects against hypoxia-induced cardiomyocyte apoptosis

As glycerol is produced when LPL converts triglycerides to FFA,[11] we next investigated whether the treatment with glycerol increased cardiomyocyte cell viability under hypoxic conditions. Isolated adult murine cardiomyocytes were incubated with glycerol and exposed to hypoxic conditions (Figure 2A). After 4-h of exposure to normoxia or hypoxia, we identified damaged cardiomyocytes using trypan blue vital staining. Glycerol treatment increased cardiomyocyte cell viability under normoxic conditions in a dose-dependent manner; however, this effect was enhanced under hypoxic conditions. In addition, we confirmed that glycerol concentration decreased in the heart of cmc-LPL KO mice (Figure S1D).

AQP7 is expressed in adipose tissues, skeletal muscles, and the heart, and serves as a glycerol channel.[14] To investigate whether the protective effect of glycerol under cardiac hypoxia requires AQP7, cardiomyocytes were isolated from $AQP7^{+/+}$ and $AQP7^{-/-}$ mice. No differences were observed in cell viability between $AQP7^{+/+}$ and $AQP7^{-/-}$ mice in the absence of glycerol (Figure S2); however, AQP7 deficiency decreased glycerol increased-related cardiomyocyte cell viability (Figure 2B). Moreover, AQP7 deficiency decreased glycerol concentration in the heart (Figure 2C). To examine whether AQP7 deficiency increased MI-induced cardiac apoptosis, apoptotic cardiomyocytes were identified by TUNEL staining of heart sections at 1 day after left coronary artery ligation (Figure 2D,E). No difference in apoptosis was observed between $AQP7^{+/+}$ and $AQP7^{-/-}$ mice, but AQP7 deficiency significantly increased MI-induced apoptosis. To determine whether glycerol attenuates the progression of MI-induced cardiac dysfunction in mice, infarct changes were ascertained in PicroSirius red-stained sections at 7 days after the ligation (Figure 2F,G). AQP7 deficiency increased infarct area in the heart. We also performed echocardiography to assess cardiac functionality (Figure 2H,I). In control mice, 7-day MI resulted in a significantly decreased fractional shortening, with the response enhanced in $AQP7^{-/-}$ mice. In addition, atrial natriuretic peptide (ANP) in the heart, which is increased upon cardiac dysfunction, was elevated in $AQP7^{-/-}$ mice at 7 days after ligation (Figure 2J).

3.3 | Glycerol is an alternate energy substrate for ATP production both in vitro in cardiomyocytes under hypoxia and in vivo in cardiac tissues in MI

After cells capture glycerol, glycerol kinase catalyzes the phosphorylation of glycerol to yield glycerol-3-phosphate (G3P).[22] Glycerol kinase expression is considered to be restricted to the liver, kidney, and skeletal muscle. To examine *Gk* gene expression in the heart, we performed qPCR analysis of various murine tissues (Figure 3A), which revealed *Gk* was also expressed in the heart. In turn, glycerol-3-phosphate dehydrogenase 2 (GPD2), which is anchored to the mitochondrial membrane, oxidizes G3P to dihydroxyacetone phosphate (DHAP) in the cytoplasm.[23] To examine GPD2 enzymatic activity on cardiac mitochondria, mitochondria were isolated from the heart; they exhibited GPD2 activity in vitro (Figure 3B). Next, as Ca^{2+} increases GPD2 activity on the mitochondria in the liver, but not in the brain of rats,[24] cardiac mitochondria were incubated with a high concentration of Ca^{2+}. This increased GPD2 activity, whereas treatment with EDTA suppressed Ca^{2+}-induced GPD2 activation, suggesting that the high Ca^{2+} concentration enhances GPD2 activity in the heart. To examine whether MI affects GPD2 enzymatic activity in the heart, mitochondria were isolated from the heart at 1 h after sham surgery or coronary artery ligation (Figure 3C). Ischemic conditions significantly increased cardiac GPD2 enzymatic activity. Next, we examined whether glycerol is involved in energy metabolism in cardiomyocytes. As 4-h hypoxia was shown to decrease cardiomyocyte cell viability (Figure 2A), we examined intracellular ATP concentrations in isolated adult murine cardiomyocytes after 1-h of culture under hypoxic condition to avoid the effects of cell viability on ATP production (Figure 3D). Notably, 1-h hypoxia did not affect cell viability, and no difference between control and glycerol treatment was observed. Conversely, 1-h hypoxia significantly decreased ATP production in cardiomyocytes (Figure 3E). Although glycerol treatment did not increase ATP production under normoxic conditions, glycerol-mediated ATP production was significantly increased under hypoxic conditions. In comparison, glycerol-dependent ATP production under early hypoxic conditions was suppressed by the GPD2 inhibitor in a dose-dependent manner, whereas significant effects on ATP production under normoxic conditions were not detected. The GPD2 inhibitor did not

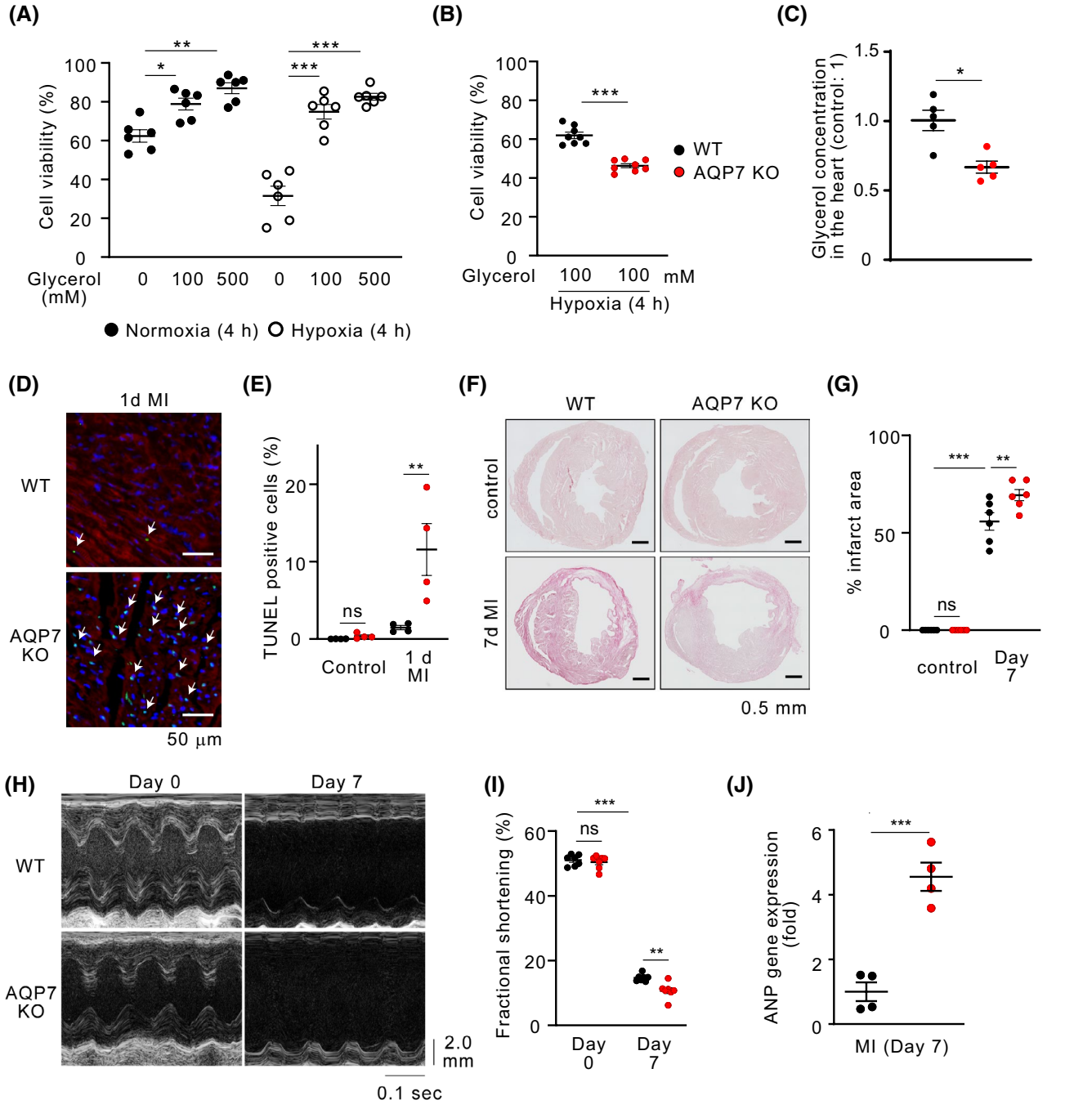

FIGURE 2 AQP7 deficiency exacerbates myocardial infarction (MI)-associated damage in mice. (A) Cardiomyocyte cell viability in response to hypoxia and glycerol treatment as determined by trypan blue dye exclusion test ($n = 4$). (B) Effect of AQP7 deficiency on cardiomyocyte cell viability ($n = 8$). (C) Glycerol concentration in the heart ($n = 5$). (D, E) Cardiomyocyte apoptosis (determined by TUNEL assay) in the left ventricle was measured at 1 day after coronary artery ligation ($n = 4$) (red, actin; green, TUNEL; blue, DAPI; scale bar, 20 μm). (F, G) Infarct area in the left ventricles at 7 days after MI, as determined by PicroSirius red staining ($n = 6$). (H, I) Left ventricular fractional shortening as determined by echocardiogram before and at 7 days after coronary artery ligation ($n = 7$). (J) *Anp* gene expression at 7 days after coronary artery ligation, as determined by qRT-PCR analysis of the heart ($n = 4$). ns, not significant; $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$. Data are presented as mean ± SEM and were analyzed by unpaired Student's *t*-test (B, C and J), one-way ANOVA (A), or two-way ANOVA followed by the Tukey's post-hoc test (E, G, and I)

significantly decrease ATP production in the absence of glycerol (Figure S3). To further evaluate the role of GPD2 in vivo, we performed coronary artery ligation in $GPD2^{+/+}$ and $GPD2^{-/-}$ mice. In control mice, cardiac dysfunction was observed at 2 and 4 weeks after ligation (Figure 3F). GPD2 deficiency significantly exacerbated MI-induced cardiac dysfunction and increased infarct area in the heart (Figure 3G).

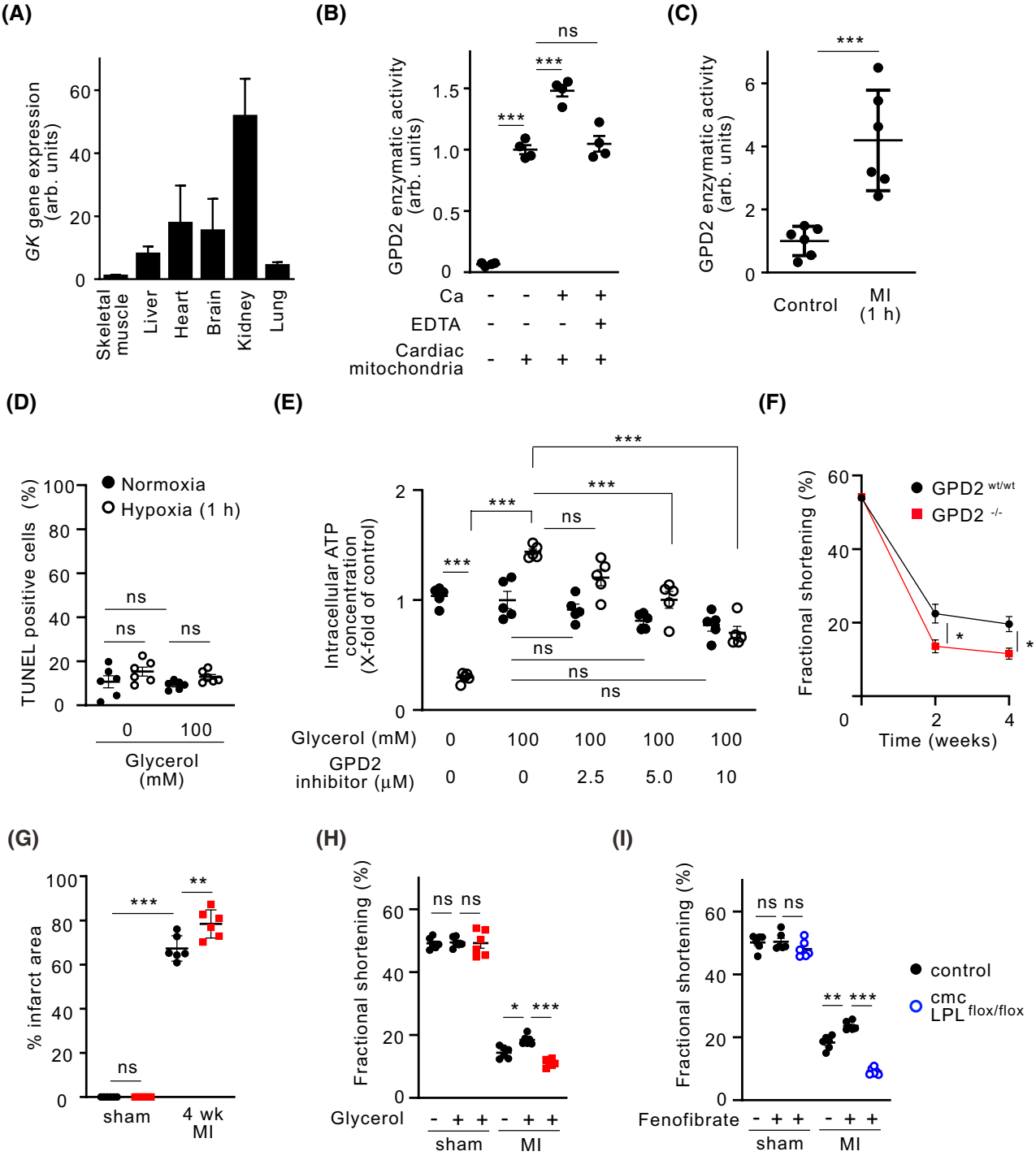

FIGURE 3 GPD2 mediates cardiomyocyte ATP production in response to hypoxia. (A) *Gk* tissue distribution as assessed by qRT-PCR analysis ($n = 4$). (B) GPD2 enzymatic activity in isolated murine cardiomyocytes ($n = 4$). (C) GPD2 enzymatic activity in the heart at 1 h after coronary ligation ($n = 6$). (D) Cardiomyocyte apoptosis (determined by TUNEL assay) after 1-h hypoxia ($n = 6$). (E) Intracellular ATP production in isolated murine cardiomyocytes treated with glycerol and a GPD2 inhibitor ($n = 5$). (F) Echocardiogram analysis results of left ventricular fractional shortening performed before, as well as at 2 and 4 weeks after coronary artery ligation ($n = 9$). (G) Infarct area in the left ventricles at 7 days after coronary artery ligation, as assessed by PicroSirius red staining ($n = 6$). (H, I) Echocardiogram analysis results of left ventricular fractional shortening performed after coronary artery ligation with glycerol or fenofibrate treatment ($n = 6$). ns, not significant; $^*p < .05$; $^{**}p < .01$; $^{***}p < .001$. Data are represented as mean ± SEM and were analyzed by unpaired Student's *t*-test (C), with two-way ANOVA followed by the Tukey's post-hoc test (B, D, E, H, and I), or two-way repeated-measures ANOVA followed by Bonferroni's post-hoc test (F and G)

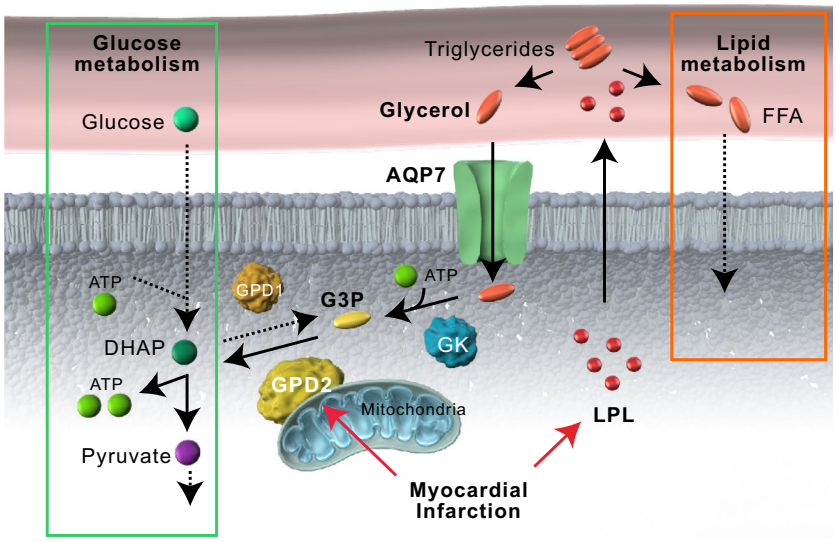

FIGURE 4 Schematic diagram showing LPL-, AQP7-, and GPD2-mediated glycerol metabolism activated in response to myocardial infarction. AQP7, aquaporin 7; ATP, adenosine triphosphate; DHAP, dihydroxyacetone phosphate; FFA, free fatty acids; GK, glycerol kinase; G3P, glycerol-3-phosphate; GPD2, glycerol-3-phosphate dehydrogenase 2; LPL, lipoprotein lipase

We next investigated whether the activation of LPL/AQP7/GPD2-mediated glycerol metabolism would attenuate the progression of MI-induced cardiac dysfunction. For this, GPD2$^{+/+}$ and GPD2$^{-/-}$ mice were orally administered glycerol at 0, 8, and 16 h after coronary artery ligation (Figure 3H). In control mice, glycerol treatment significantly suppressed MI-induced cardiac dysfunction. GPD2 deficiency resulted in reduced therapeutic response. Finally, to examine whether peroxisome proliferator-activated receptor alpha (PPARα), which increases the hydrolysis of plasma triglycerides due to induction of LPL expression in the liver and adipose tissues,[25] mediated cardiac glycerol metabolism under ischemic conditions, oral treatment with fenofibrate, a PPARα agonist, was initiated 5 days before coronary artery ligation in cmc-LPL WT and cmc-LPL KO mice (Figure 3I). Treatment with the PPARα agonist protected mice from MI-induced cardiac dysfunction, and cardiomyocyte-specific LPL deficiency suppressed the therapeutic effect of the PPARα agonist. These findings suggest that LPL/AQP7/GPD2-mediated glycerol metabolism in the heart attenuates cardiac dysfunction during acute MI.

4 | DISCUSSION

In this study, we showed that glycerol is a substrate for energy production in cardiomyocytes under hypoxic conditions (Figure 4). Glycerol plays a biochemically important role as the backbone of glyceride lipids.[26] Once glycerol enters the major pathways of carbohydrate metabolism, such as glucose metabolism, it also functions as an energy substrate. For example, intracellular G3P and pyruvate are synthesized upon culturing of neonatal rat cardiomyocytes with glycerol.[27] In vivo, the use of ^{14}C-labeled glycerol reveals that an increase in rat heart rate induces a concomitant increase in glycerol uptake in the heart.[28]

In particular, the use of glycerol as fuel is mediated via AQP7 in the heart under pathological situations, such as MI and heart failure. The expression of AQP7, which is the most prominent aquaglyceroporin in the heart, increases under conditions of altered energy supply, such as diabetes mellitus, fasting, exercise, and high-protein diets.[13] Immunohistochemical staining revealed that AQP7 is localized in the capillaries of the heart,[29] whereas in vivo experiments using AQP7$^{-/-}$ mice indicated that AQP7 acts as a glycerol channel in cardiomyocytes. Specifically, although AQP7$^{-/-}$ mice exhibit normal cardiac histology and morphology under basal conditions, AQP7 deficiency exacerbates pressure overload-induced cardiac hypertrophy and isoproterenol-induced cardiac dysfunction as well as increases mortality following pressure overload-induced heart failure.[14,30]

The heart avidly acquires lipids both from circulating FFA and esterified fatty acids bound to lipoproteins,[31] with esterified FFA constituting a major source of cardiac lipids and circulating FFA bearing minor importance as a fuel for the heart. Because the water solubility of FFA is limited, they must undergo esterification with glycerol to form triglycerides, which make up a significant portion of lipoprotein triglycerides in circulation.[32] LPL is synthesized in parenchymal cells, such as adipocytes and cardiomyocytes. GPI-anchored LPL transgenic mice, in which LPL is anchored to cardiomyocytes, cannot transport LPL from cardiomyocytes to the vascular lumen, suggesting that LPL is produced

in cardiomyocytes and is transferred to the apical side of endothelial cells, where the enzyme functions in the heart.[33] Moreover, tamoxifen-inducible cardiac LPL deficient mice (MerCreMer LPL$^{flox/flox}$) exhibited increased plasma triglyceride concentration and decreased left ventricular fractional shortening at 4 weeks after tamoxifen treatment.[19] Thus, in the present study, we ligated the coronary artery of cmc-LPL WT and cmc-LPL KO mice at 5 days after tamoxifen treatment to avoid basal cardiac dysfunction caused by LPL deficiency (Figure S1C). Our in vivo investigation indicated that MI induced LPL expression on cardiomyocytes, which in turn prevented MI-induced cardiac dysfunction. Alternatively, under ischemic conditions, fatty acid translocase translocates away from the sarcolemma in the heart to limit fatty acid uptake.[34]

Although GPD2 plays an important role in physiological and pathological situations, such as t insulin release in pancreatic islets β cells and macrophage inflammatory responses,[17,35] the role of GPD2 in the heart remains unclear. Here, we found that GPD2 deficiency did not affect basal cardiac function, and that GPD2 played an important regulatory role bridging glucose and lipid metabolism in MI. In glycerol metabolism, the conversion of DHAP to pyruvate, which is oxygen-independent, assists in ATP production under anaerobic conditions. These findings indicated that the use of glycerol as an energy source by GPD2-mediated myocardial metabolism is suitable under hypoxic conditions during MI.

Furthermore, the correlation between LPL activity and phosphorylation of AMP-activated protein kinase (AMPK), which is an essential component of the adaptive response to cardiomyocyte stress during MI, has been revealed using fasted rats.[36] The intracellular Ca^{2+} concentration in cardiomyocytes increases under MI; additionally, Ca^{2+} is a modulator of GPD2 isolated from rat liver in vitro.[24] We found that Ca^{2+} activated GPD2 on mitochondria isolated from the heart and that coronary artery ligation increased GPD2 enzymatic activity in vivo. These findings suggest that AMPK and Ca^{2+} may mediate ATP production from glycerol in MI via LPL and GPD2 activation; however, the mechanism through which glycerol metabolism is activated under ischemic conditions requires further investigation.

In conclusion, our findings revealed that MI-induced an increase in LPL expression in the heart, preventing myocardial ischemia. AQP7 acts as a glycerol channel in cardiomyocytes during MI. GPD2 increases ATP synthesis from glycerol under hypoxic conditions. The discovery that LPL/AQP7/GPD2-mediated glycerol metabolism plays an important role in preventing myocardial ischemia-related damage makes this glycerol pathway a promising target for therapeutic intervention in patients with acute MI.

ACKNOWLEDGMENTS

The authors thank the staff from the Division of Experimental Animals at the Nagoya University School of Medicine for assisting with the animal experiments. The AQP7 KO mice (RIKEN, RBRC06294) were obtained from Dr. Shimomura (Osaka University). The LPL flox mice generated by Dr. Goldberg (Columbia University) were provided by Dr. Ishibashi and Dr. Takahashi (Jichi Medical University). This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

DISCLOSURES

The authors declared no competing interests.

AUTHOR CONTRIBUTIONS

S.I., S.Y., and T.Y. performed most of the in vitro and in vivo experiments, analyzed, and discussed the data, and commented on the manuscript. Y.M., S.E., T.S., T.T., K.K., and Y.S. performed the in vivo experiments and mouse generation. H.Y. generated the LPL flox mice. N.W. and S.O. discussed the data and generated the αMHC-CreERT2 mice. N.K. and T.K. commented on the manuscript and generated the GPD2 KO mice. Y.B., K.O., N.O., and T.M. supervised the study and commented on the manuscript. M.T. initiated the study, performed the in vitro and in vivo experiments, analyzed and discussed the data, and wrote the manuscript.

ORCID

Mikito Takefuji https://orcid.org/0000-0003-4870-7913

REFERENCES

1. Abdurrachim D, Luiken JJ, Nicolay K, Glatz JF, Prompers JJ, Nabben M. Good and bad consequences of altered fatty acid metabolism in heart failure: evidence from mouse models. *Cardiovasc Res*. 2015;106:194-205.
2. Kolwicz SC Jr, Purohit S, Tian R. Cardiac metabolism and its interactions with contraction, growth, and survival of cardiomyocytes. *Circ Res*. 2013;113:603-616.
3. Lopaschuk GD, Ussher JR, Folmes CD, Jaswal JS, Stanley WC. Myocardial fatty acid metabolism in health and disease. *Physiol Rev*. 2010;90:207-258.
4. Doenst T, Nguyen TD, Abel ED. Cardiac metabolism in heart failure: implications beyond ATP production. *Circ Res*. 2013;113:709-724.
5. Yeh RW, Sidney S, Chandra M, Sorel M, Selby JV, Go AS. Population trends in the incidence and outcomes of acute myocardial infarction. *N Engl J Med*. 2010;362:2155-2165.
6. Fillmore N, Mori J, Lopaschuk GD. Mitochondrial fatty acid oxidation alterations in heart failure, ischaemic heart disease and diabetic cardiomyopathy. *Br J Pharmacol*. 2014;171:2080-2090.
7. Gupta A, Houston B. A comprehensive review of the bioenergetics of fatty acid and glucose metabolism in the healthy

and failing heart in nondiabetic condition. *Heart Fail Rev*. 2017;22:825-842.
8. Young SG, Fong LG, Beigneux AP, et al. GPIHBP1 and lipoprotein lipase, partners in plasma triglyceride metabolism. *Cell Metab*. 2019;30:51-65.
9. Kersten S. Physiological regulation of lipoprotein lipase. *Biochim Biophys Acta*. 2014;1841:919-933.
10. Khera AV, Won HH, Peloso GM, et al, Myocardial Infarction Genetics Consortium, DiscovEHR Study Group, CARDIoGRAM Exome Consortium, and Global Lipids Genetics Consortium. Association of rare and common variation in the lipoprotein lipase gene with coronary artery disease. *JAMA*. 2017;317:937-946.
11. Miles JM, Park YS, Walewicz D, et al. Systemic and forearm triglyceride metabolism: fate of lipoprotein lipase-generated glycerol and free fatty acids. *Diabetes*. 2004;53:521-527.
12. Rojek A, Praetorius J, Frøkiaer J, Nielsen S, Fenton RA. A current view of the mammalian aquaglyceroporins. *Annu Rev Physiol*. 2008;70:301-327.
13. Verkerk AO, Lodder EM, Wilders R. Aquaporin channels in the heart-physiology and pathophysiology. *Int J Mol Sci*. 2019;20:2039.
14. Hibuse T, Maeda N, Nakatsuji H, et al. The heart requires glycerol as an energy substrate through aquaporin 7, a glycerol facilitator. *Cardiovasc Res*. 2009;83:34-41.
15. Ackers-Johnson M, Li PY, Holmes AP, O'Brien SM, Pavlovic D, Foo RS. A simplified, Langendorff-Free method for concomitant isolation of viable cardiac myocytes and nonmyocytes from the adult mouse heart. *Circ Res*. 2016;119:909-920.
16. Eguchi S, Takefuji M, Sakaguchi T, et al. Cardiomyocytes capture stem cell-derived, anti-apoptotic microRNA-214 via clathrin-mediated endocytosis in acute myocardial infarction. *J Biol Chem*. 2019;294:11665-11674.
17. Eto K, Tsubamoto Y, Terauchi Y, et al. Role of NADH shuttle system in glucose-induced activation of mitochondrial metabolism and insulin secretion. *Science*. 1999;283:981-985.
18. Maeda N, Funahashi T, Hibuse T, et al. Adaptation to fasting by glycerol transport through aquaporin 7 in adipose tissue. *Proc Natl Acad Sci U S A*. 2004;101:17801-17806.
19. Augustus A, Yagyu H, Haemmerle G, et al. Cardiac-specific knock-out of lipoprotein lipase alters plasma lipoprotein triglyceride metabolism and cardiac gene expression. *J Biol Chem*. 2004;279:25050-25057.
20. Takefuji M, Wirth A, Lukasova M, et al. G(13)-mediated signaling pathway is required for pressure overload-induced cardiac remodeling and heart failure. *Circulation*. 2012;126:1972-1982.
21. Takagawa J, Zhang Y, Wong ML, et al. Myocardial infarct size measurement in the mouse chronic infarction model: comparison of area- and length-based approaches. *J Appl Physiol*. 1985;2007(102):2104-2111.
22. Brisson D, Vohl MC, St-Pierre J, Hudson TJ, Gaudet D. Glycerol: a neglected variable in metabolic processes? *BioEssays*. 2001;23:534-542.
23. Mráček T, Drahota Z, Houštěk J. The function and the role of the mitochondrial glycerol-3-phosphate dehydrogenase in mammalian tissues. *Biochim Biophys Acta*. 2013;1827:401-410.
24. Beleznai Z, Szalay L, Jancsik V. Ca^{2+} and Mg^{2+} as modulators of mitochondrial L-glycerol-3-phosphate dehydrogenase. *Eur J Biochem*. 1988;170:631-636.
25. Schoonjans K, Staels B, Auwerx J. Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. *J Lipid Res*. 1996;37:907-925.
26. Lin EC. Glycerol utilization and its regulation in mammals. *Annu Rev Biochem*. 1977;46:765-795.
27. Gambert S, Helies-Toussaint C, Grynberg A. Regulation of intermediary metabolism in rat cardiac myocyte by extracellular glycerol. *Biochim Biophys Acta*. 2007;1736:152-162.
28. Gambert S, Héliès-Toussaint C, Grynberg A. Extracellular glycerol regulates the cardiac energy balance in a working rat heart model. *Am J Physiol Heart Circ Physiol*. 2007;292: H1600-H1606.
29. Skowronski MT, Lebeck J, Rojek A, et al. AQP7 is localized in capillaries of adipose tissue, cardiac and striated muscle: implications in glycerol metabolism. *Am J Physiol Renal Physiol*. 2007;292:F956-F965.
30. Gerdes AM, Capasso JM. Structural remodeling and mechanical dysfunction of cardiac myocytes in heart failure. *J Mol Cell Cardiol*. 1995;27:849-856.
31. Goldberg IJ, Trent CM, Schulze PC. Lipid metabolism and toxicity in the heart. *Cell Metab*. 2012;15:805-812.
32. Chiu AP, Wan A, Rodrigues B. Cardiomyocyte-endothelial cell control of lipoprotein lipase. *Biochim Biophys Acta*. 2016;1861: 1434-1441.
33. Yagyu H, Chen G, Yokoyama M, et al. Lipoprotein lipase (LpL) on the surface of cardiomyocytes increases lipid uptake and produces a cardiomyopathy. *J Clin Invest*. 2003;111:419-426.
34. Heather LC, Pates KM, Atherton HJ, et al. Differential translocation of the fatty acid transporter, FAT/CD36, and the glucose transporter, GLUT4, coordinates changes in cardiac substrate metabolism during ischemia and reperfusion. *Circ Heart Fail*. 2013;6:1058-1066.
35. Langston PK, Nambu A, Jung J, et al. Glycerol phosphate shuttle enzyme GPD2 regulates macrophage inflammatory responses. *Nat Immunol*. 2019;20:1186-1195.
36. Qi D, Young LH. AMPK: energy sensor and survival mechanism in the ischemic heart. *Trends Endocrinol Metab*. 2015;26: 422-429.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Ishihama S, Yoshida S, Yoshida T, et al. LPL/AQP7/GPD2 promotes glycerol metabolism under hypoxia and prevents cardiac dysfunction during ischemia. *FASEB J*. 2021;35:e22048. doi:10.1096/fj.202100882R