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Short Communication

ERK1/2 Inhibition Alleviates Diabetic Cardiomyopathy by Suppressing Fatty Acid Metabolism

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Abstract

Background: Diabetes mellitus is associated with morphological and functional impairment of the heart primarily due to lipid toxicity caused by increased fatty acid metabolism. Extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) have been implicated in the metabolism of fatty acids in the liver and skeletal muscles. However, their role in the heart in diabetes remains unclear. In this study, we tested our hypothesis that pharmacological inhibition of ERK1/2 alleviates cardiac remodeling in diabetic mice through a reduction in fatty acid metabolism. **Methods**: ERK1/2 phosphorylation in diabetes was determined both *in vitro* and *in vivo*. H9C2 cells were subjected to high glucose, high palmitic acid, or both high glucose and palmitic acid. *db/db* and streptozotocin (STZ)-induced diabetic mice were analyzed for ERK1/2 phosphorylation levels as well as the effects of U0126 treatment on cardiac remodeling. Administration of STZ and U0126 in mice was performed via intraperitoneal injection. Blood glucose levels in mice were measured using a glucometer. Mouse heart total RNAs were purified for reverse transcription. Real-time polymerase chain reaction (PCR) analysis of the messenger ribonucleic acid (mRNA) expression was performed for hypertrophy (*ANF*, *BNP*, and *βMHC*), fibrosis (*Col3α1*), and fatty acid metabolism genes (*PPARα*, *CPT1A*, and *FACS*). Interstitial fibrosis of the myocardium was analyzed using Masson's trichrome staining of the paraffin-embedded tissues. **Results**: ERK1/2 phosphorylation was significantly increased in diabetic conditions. Inhibition of ERK1/2 by U0126 in both streptozotocin-induced diabetic mice and *db/db* mice resulted in a significant reduction in the expression of genes associated with hypertrophy and fibrosis. In contrast, elevated phosphorylation of ERK1/2 in *Dusp6/8* knockout (DKO) mice resulted in fibrosis. Mechanistically, ERK1/2 activation enhanced the expression of fatty acid metabolism genes *PPARα*, *CPT1A*, and *FACS* in the heart, which was reversed by U0126 treatment. **Conclusion**: ERK1/2 are potential therapeutic targets for diabetic cardiomyopathy by modulating fatty acid metabolism in the heart.

Keywords: cardiac remodeling; diabetes mellitus; ERK1/2; fatty acid metabolism

1. Introduction

Diabetic cardiomyopathy is a pathologic condition of the heart with structural and functional changes that aren't caused by coronary artery disease, hypertension, and atherosclerosis [1]. Diabetic cardiomyopathy is characterized by cardiac hypertrophy, myocardial fibrosis, cardiac dysfunction, and eventual heart failure [2]. Complex cellular mechanisms have been implicated in the pathogenesis of diabetic cardiomyopathy including oxidative stress [3–5], inflammation [6,7], cell death [8,9], fibrosis [10,11], impaired calcium handling [7,12], accumulation of advanced glycation end products [13,14], and altered energy metabolism [15]. Various studies have indicated that reduced fuel flexibility and subsequent energetic deficits have been recognized as the main cause of diabetic cardiomyopathy [16,17]. Under normal conditions, the heart utilizes β-oxidation of free fatty acids to produce 50–70% of total energy and relies on glucose, amino acids, lactate, and ketone bodies for the remaining energy [18]. However, fatty acids are less efficient in ATP production with an ATP/oxygen atom of 2.3, whereas glucose has an ATP/oxygen atom of 5 [19,20]. In diabetes, the heart shifts to fatty acids to produce ATP as glucose utilization is impaired because of insulin resistance. This metabolic shift leads to increased mitochondrial oxygen consumption and intramyocardial overload of lipid metabolites including diacylglycerol and ceramide, which reduces cardiac efficiency and predisposes the heart to maladaptive remodeling and impaired ischemic tolerance [21–26].

Several studies on diabetic animal models and human patients have revealed increased myocardial triglyceride content [21,27,28]. Thus, one potential avenue of diabetic cardiomyopathy treatment could be through the reduction of fatty acid metabolism in the heart. Fatty acids enter the cardiomyocytes through fatty acid translocase (FAT)/CD36, a long-chain fatty acid transporter present in the sarcolemma [29]. Once inside the cytoplasm, fatty acids are esterified to form long-chain acyl CoAs through fatty acid CoA synthetase (FACS) [30]. Fatty acid CoAs are used to generate lipid intermediates such as diacylglycerol, triglyceride, and ceramide, which are associated with apoptosis and lipid toxicity in the diseased heart [23,31]. The acyl group of acyl CoA can also be transferred to carni-

tine to form long-chain acylcarnitine via carnitine palmitoyltransferase 1s (CPT1s) which enter the mitochondria for beta-oxidation to produce the energy. The expression of metabolic enzymes involved in fatty acid uptake and oxidation are tightly controlled by the nodal transcription factor peroxisome proliferator-activated receptor alpha (PPARα) [32,33]. PPARα expression was significantly elevated in the diabetic mice, and cardiac overexpression of PPARα resulted in enhanced expression of fatty acid oxidation genes and phenotypes like that of the diabetic heart [34]. Conversely, PPARα deficient mice demonstrated reduced fatty acid metabolism in the diabetic heart [35]. Thus, it is evident that targeting PPARα signaling could be a potential treatment strategy.

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases that convert extracellular stimulation into sequential activation of intracellular signaling proteins, resulting in proliferation, differentiation, or death of the cell [36,37]. MAPKs include three major subfamilies: extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), p38, and c-Jun NH_2-terminal kinase 1 and 2 (JNK1/2). MAPKs have been involved in the pathogenesis of metabolic diseases [38,39]. For example, JNK1 knockout (*Jnk1*$^{-/-}$) mice demonstrated reduced adiposity and enhanced insulin sensitivity [40]. Mice with adipocyte-specific knockout of JNK1 were protected against diet-induced insulin resistance [41]. Mice with loss of p38α in the adipocytes showed resistance to diet-induced obesity with enhanced energy expenditure [42]. Lastly, several studies have suggested a direct association between ERK1/2 and energy metabolism [43–45]. ERK1/2 phosphorylation was increased in the livers of both high fat diet-induced and genetically obese mice, and activation of ERK1/2 in the liver by overexpressing the constitutively active mitogen-activated protein kinase kinase 1 (MEK1) led to reduced expression of fatty acid metabolism genes [45]. *Erk1*$^{-/-}$ mice developed diet-induced obesity and insulin resistance [43]. In addition, increased ERK1/2 signaling was associated with the upregulation of PPARα, suggesting a potential interaction between ERK1/2 and PPARα in energy metabolism in the diabetic heart disease [44,46].

This study investigated the role of ERK1/2 in the pathogenesis of diabetic cardiomyopathy in mice with either activation or inhibition of ERK1/2. We showed that ERK1/2 inhibition decreased gene expression for cardiac hypertrophy and fatty acid metabolism. ERK1/2 activation in diabetic mice developed cardiomyopathy. Thus, our study provided the first evidence that ERK1/2 could be potential therapeutic targets for treatment of diabetic cardiomyopathy.

2. Materials and Methods

2.1 Mice

The Institutional Animal Care and Use Committee at Grand Valley State University has approved this study (protocol number 23-05-A). 8-week-old C57BL/6J male mice (000664, The Jackson Laboratory, Bar Harbor, ME, USA) were utilized for diabetic induction by streptozotocin (STZ) (S0130, MilliporeSigma, Burlington, MA, USA) due to their high sensitivity compared to female mice [47,48]. 12-week-old male *db/db* mice (000697, The Jackson Laboratory, Bar Harbor, ME, USA) were utilized as an alternative diabetic mouse model. Upon arrival at the animal facility, mice were allowed one week to adapt to the new environment before any experiments. *Dusp6/8* double knockout (DKO) mice were generated for the loss of both *Dusp6* and *Dusp8* and had been characterized using gene-specific primers and antibodies [44]. Mice were kept in a room with a 12 h day/night cycle, at 21 °C and 50–70% humidity. Mouse anesthesia was performed through an anesthetic machine which delivers 1.5% isoflurane in oxygen at a flow rate of 0.8 L/min. Mice were monitored for their loss of righting reflex and slowed respiratory rate to determine their readiness for the drug injection procedures.

2.2 H9C2 Cell Culture

H9C2 cells were purchased from the American Type Culture Collection (ATCC) (CRL-1446, ATCC, Manassas, VA, USA) and tested for mycoplasma contamination using the universal mycoplasma detection kit (30-1012K, ATCC, Manassas, VA, USA) to ensure a negative result. And the cell lines were validated by short tandem repeat (STR) profiling. Cells were cultured in low glucose (1 g/L or 5.5 mM glucose) Dulbecco's Modified Eagle Medium (DMEM) (11885084, Thermo Fisher Scientific, Waltham, MA, USA) supplied with 10% fetal bovine serum (30-2020, ATCC, Manassas, VA, USA) and 5% CO_2 in a humidified incubator. 70% confluent cells were cultured in DMEM with either 5.5 mM glucose, 25 mM glucose, 150 µM palmitic acid, or both 25 mM glucose and 150 µM palmitic acid for 24 and 48 hours before harvest. In the U0126 treatment experiment, U0126 (J61246.MB, Thermo Fisher Scientific, Waltham, MA, USA) was solubilized in 6% DMSO. ~70% confluent cells were cultured in 5.5 mM glucose DMEM and pretreated with either 6% DMSO or 10 µM U0126 for 30 minutes before stimulation with 20% fetal bovine serum for 5 minutes. Cells were washed off residual medium with room temperature 1× phosphate-buffered saline (PBS) and lysed for biochemical analysis of ERK1/2 phosphorylation.

2.3 STZ Induction of Diabetes and U0126 Treatment

8-week-old C57BL/6J male mice were first fasted for four hours in the morning before baseline glucose measurement by a Contour Next glucometer (ASCENSIA Diabetes Care, Parsippany, NJ, USA) using blood from the tail vein. Mice were then subjected to intraperitoneal injections of either a sodium citrate solution (0.1 M, pH 4.5), or STZ (75 mg/kg/day) for five consecutive days. STZ is a compound that is selectively toxic to the β cells of the pancreatic islets. To ensure the effectiveness of STZ, the sodium citrate solution was freshly prepared to dissolve STZ prior to the daily

injection. 14 days post STZ injection, those with fasting glucose higher than 300 mg/dL (16.7 mmol/L) were considered diabetic [49,50]. Half of these diabetic mice were injected daily with 6% DMSO, while the other half were injected with 15 mg/kg of U0126. Similarly, *db/db* diabetic mice were injected daily with either 6% DMSO or 15 mg/kg of U0126. To ensure the development of cardiac abnormalities and the effectiveness of U0126, all mice were kept on U0126 for 6 weeks as suggested [34,51]. After U0126 treatment, mice were first tested for their fasting glucose levels and then euthanized with CO_2 inhalation and cervical dislocation. Full body weight was measured and then the mice were immediately dissected to retrieve the heart for heart weight, histological assessment, and RNA and protein analysis.

2.4 RNA Purification and Real-Time PCR Analysis

Heart tissue (~30 mg) was minced by scissors followed by RNA extraction using a RNeasy Fibrous Tissue Kit (74704, Qiagen, Germantown, MD, USA). RNA concentration was determined by a nanodrop 2000 spectrophotometer (ND-2000, Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) synthesis of 1 µg RNA was performed using First-Strand Synthesis Kit (18080e051, Invitrogen, Waltham, MA, USA). Real-time polymerase chain reaction (RT-PCR) was performed with a SYBR green dye (172e5274, Bio-Rad, Hercules, CA, USA) in a final volume of 20 µL. The following gene-specific primers were used for RT-PCR: ribosomal protein 7 (*RPL7*), forward: 5′-gaagctcatctatgagaaggc-3′, reverse: 5′-aagacgaaggagctgcagaac-3′; peroxisome proliferator-activated receptor α (*PPAR*α), forward: 5′-atgccagtactgccgttttc-3′; reverse: 5′-ggccttgaccttgttcatgt-3′; peroxisome proliferator-activated receptor γ (*PPAR*γ), forward: 5′-ttttcaagggtgccagtttc-3′, reverse: 5′-aatccttggccctct gagat-3′; carnitine palmitoyltransferase 1A (*CPT1A*), forward: 5′-ccaggctacagtgggacatt-3′, reverse: 5′-gaacttgcccatgtccttgt-3′; fatty acid synthase (*FACS*), forward: 5′-ggctctatggattacccaa-3′, reverse: 5′-ccagtgttcgttcctcgga-3′; atrial natriuretic factor (*ANF*), forward: 5′-gccctgagcgagcagaccga-3′, reverse: 5′-cggaagctgttgcagccta-3′; B-type natriuretic peptide (*BNP*), forward: 5′-ctgctggagctgataagaga-3′, reverse: 5′-agtcagaaactggagtctcc-3′; β myosin heavy chain (β*MHC*), forward: 5′-acctaccagacagaggaaga-3′, reverse: 5′-ttgcaaagagtccaggtctgag-3′; collagen 3α1 (*Col3*α*1*), forward: 5′-tgaaggcgaattcaaggctgaagg-3′, reverse: 5′-agggccaatgtccacaccaaattc-3′. RT-PCR data was analyzed with a $2^{-\Delta\Delta CT}$ method and normalized using RPL7 as an internal control [44].

2.5 Western Blot

Confluent H9C2 cells from 60 mm culture dishes or ~40 mg of mouse heart tissues were solubilized into a buffer containing 10 mM Tris-HCl (pH 7.4), 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, proteinase inhibitors (05892970001, MilliporeSigma, Burlington, MA, USA), and phosphatase inhibitors (4906837001, MilliporeSigma, Burlington, MA, USA) [44]. Protein samples were harvested by centrifugation and quantified using a Pierce BCA Assay Kit (23227, Thermo Fisher Scientific, Waltham, MA, USA). 40 µg of proteins were separated by 10% SDS-PAGE, transferred onto a PVDF membrane (1620177, Bio-Rad, Hercules, CA, USA), blocked by 1% blocker bovine serum albumin (BSA) (37525, Thermo Fisher Scientific, Waltham, MA, USA), and incubated overnight with the following primary antibodies. ERK1/2 (9102S, Cell Signaling Biotechnology, Danvers, MA, USA) and phospho-ERK1/2 (9101S, Cell Signaling Biotechnology, Danvers, MA, USA) antibodies were used in 1:1000 dilution in 10 mL of 1% blocker BSA. IRDye@800CWgoat anti-rabbit secondary antibody (P/N:926-32211, Li-Cor Biosciences, Lincoln, NE, USA) was used in 1:10,000 dilution in 10 mL 1% blocker BSA. Visualization and quantification of Western blots were achieved by the Odyssey infrared system of Li-Cor Biosciences.

2.6 Histology

At the end of U0126 treatment, mice were euthanized for heart removal and cross sections of the hearts were cut. Heart tissues were fixed in 10% formalin (SF100-4, Thermo Fisher Scientific, Waltham, MA, USA) for 24 h, dehydrated in concentrated ethanol solutions, and embedded in paraffin. 5 µm thick sections were cut with a rotary microtome (90-520-0STS, Thermo Fisher Scientific, Waltham, MA, USA) and stained with Masson's trichrome (HT15-1KT, MilliporeSigma, Burlington, MA, USA) for fibrosis detection. At least three images per section were taken using a Nikon A1 microscope (Nikon Instruments Inc, Melville, NY, USA).

2.7 Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 10 (GraphPad Software, La Jolla, CA, USA) and all the data was shown as the mean $\pm$ standard error of mean. The normality of data was analyzed using the Shapiro-Wilk test. Student's *t*-test was used to compare only two groups, and a *p* value smaller than 0.05 was considered significant. For analysis of multiple groups with one variable, one-way ANOVA was used to analyze the variance followed by a Bonferroni post hoc test for comparison of differences across multiple groups. An adjusted *p* value of <0.05 was considered significant.

3. Results

3.1 ERK1/2 Phosphorylation is Upregulated in Diabetic Conditions

To assess whether ERK1/2 are key signaling molecules in diabetic cardiomyopathy, we first analyzed ERK1/2 phosphorylation in H9C2 cells in medium containing high glucose or palmitic acid, which mimics the hyperglycemia and hyperlipidemia conditions in diabetes.

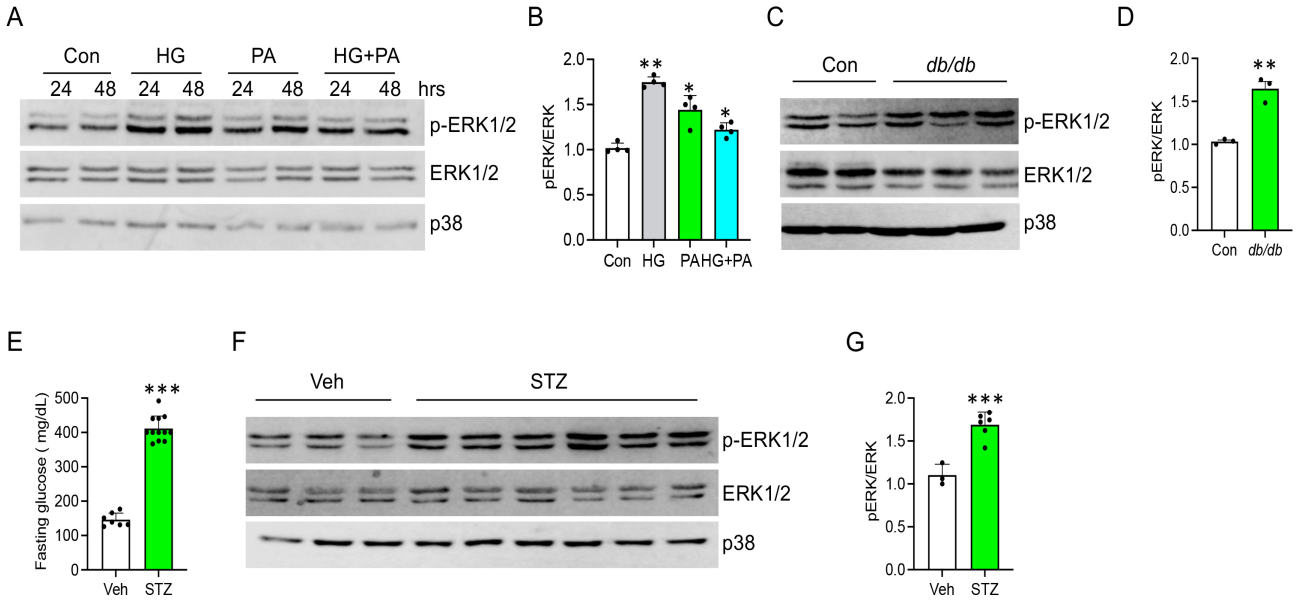

Fig. 1. ERK1/2 phosphorylation is elevated in diabetic conditions. (A) Western blot analysis of ERK1/2 phosphorylation as well as their total levels in H9C2 cells subjected to high glucose (HG), palmitic acid (PA), or both HG and PA (HG+PA) for 24 and 48 hours. Control (con) were H9C2 cells cultured in DMEM that had 5.5 mM glucose. p38 was used as a loading control. (B) Quantification of ERK1/2 phosphorylation based on Fig. 1A. * $p < 0.05$ vs. con, and ** $p < 0.01$ vs. con. (C) Analysis of phosphorylation and total protein levels of ERK1/2 in *db/db* mouse hearts by a Western blot approach. p38 was used as a loading control. (D) Quantification of ERK1/2 phosphorylation levels based on Fig. 1C. ** $p < 0.01$ vs. con. (E) Fasting serum glucose level in mice 7 days after receiving 5 consecutive days of either a citrate solution (veh) or STZ. *** $p < 0.001$ vs. veh. (F) Western blot analysis of phosphorylation and total protein levels of ERK1/2 in the hearts of control (veh) and STZ-treated mice. p38 was used as a loading control. (G) Western blot quantification based on Fig. 1F. *** $p < 0.001$. All the samples in Fig. 1 were biological replicates. ERK1/2, extracellular signal-regulated protein kinases 1 and 2; STZ, streptozotocin.

We chose palmitic acid (PA) to stimulate the cells as PA is a saturated fatty acid commonly found in the human body [52], and PA stimulation of liver HepG2 cells has been shown to activate ERK1/2 [53]. H9C2 cells cultured in 25 mM of glucose (HG) demonstrated a substantial increase in ERK1/2 phosphorylation (75% increase, $p = 0.008$) (Fig. 1A,B). Similarly, PA elevated ERK1/2 phosphorylation (39% increase, $p = 0.016$). Combination of both high glucose and palmitic acid did not induce an additional increase in ERK1/2 phosphorylation. Consistent with these *in vitro* observations, ERK1/2 phosphorylation was also found to be increased in the myocardium of 12-week-old *db/db* mice (68% increase, $p = 0.0019$), although the total ERK1/2 levels were lower as reported in another study (Fig. 1C,D) [54].

STZ has been commonly used to generate animal models of diabetes, reliably resulting in hyperglycemia and hyperlipidemia [48]. STZ-treated animals demonstrate increased production of reactive oxygen species, inflammation, apoptosis, and fibrosis which are all features of late-stage diabetic hearts [1,47]. Moreover, STZ treatment didn't cause any mortality and has been shown to enhance serum lipid level [53]. We successfully established a protocol to generate diabetic mice by administering STZ injection to 8-week-old male C57Bl/6J mice for 5 consecutive days. Fasting glucose was significantly elevated in the STZ-treated animals 1 week post STZ administration (Fig. 1E). More than 90% of STZ-treated mice in our study developed diabetes with a glucose level higher than 300 mg/dL (16.7 mM), which is considered the threshold for diabetes in mice [49,50]. Again, ERK1/2 phosphorylation in the mouse hearts was significantly higher in the STZ-treated group (68% increase, $p = 0.00068$) (Fig. 1F,G). Taken together, ERK1/2 phosphorylation is elevated in diabetic conditions, suggesting their potential role in the diabetic hearts.

3.2 Inhibition of ERK1/2 Activity by U0126 Attenuates Diabetic Cardiomyopathy in Mice

To further determine the potential role of ERK1/2 in diabetic cardiomyopathy, we sought to inhibit ERK1/2 phosphorylation *in vivo* and analyze the effect on the heart. We first determined the effect of U0126 on ERK1/2 phosphorylation *in vitro*. We found U0126 pretreatment of H9C2 cells for 30 minutes completely abolished ERK1/2 phosphorylation at both baseline as well as FBS stimulation conditions (Fig. 2A,B). We then tested the effective U0126 dose for ERK1/2 inhibition *in vivo*. Mice were subjected to 7 consecutive days of intraperitoneal injections of either a vehicle (6% DMSO), low (1 mg/kg) or high dose (15 mg/kg) of U0126 as previous studies suggested

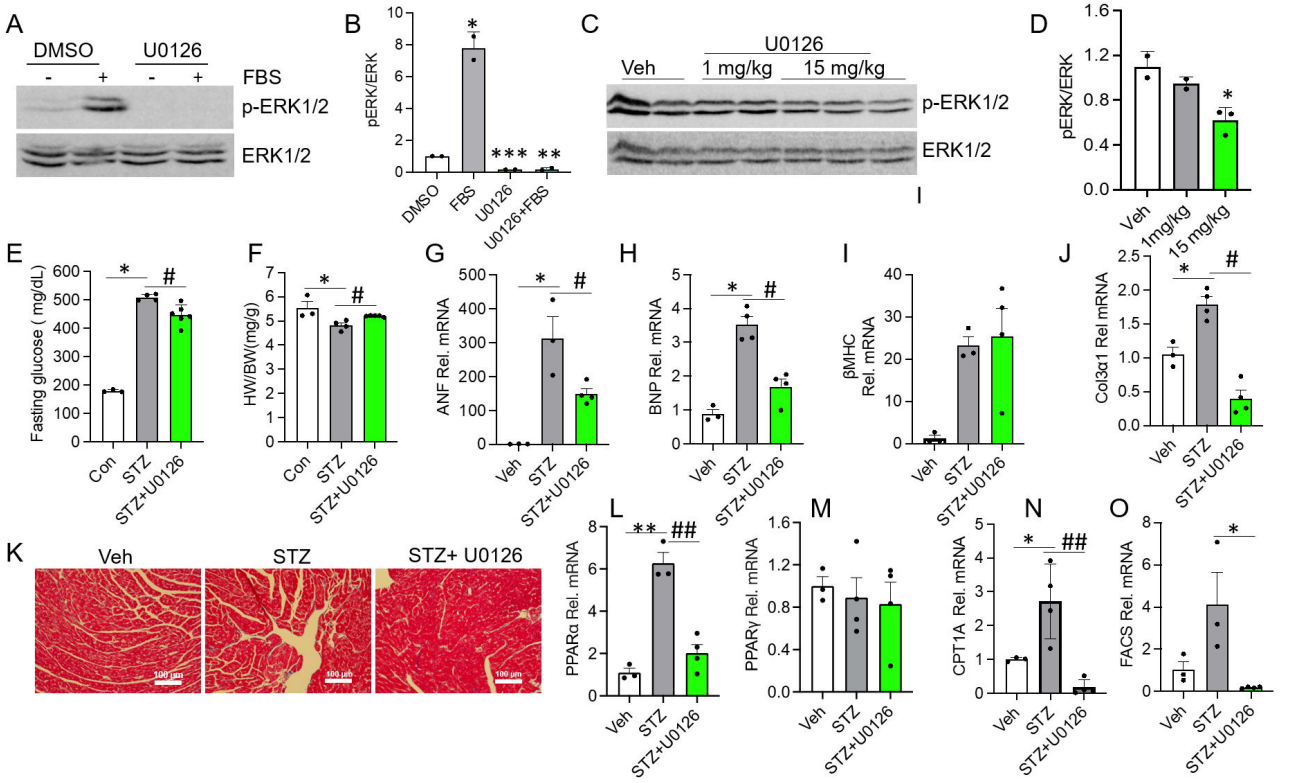

Fig. 2. U0126 inhibition attenuates the diabetic cardiomyopathy. (A) Effect of U0126 on ERK1/2 phosphorylation in H9C2 cells at baseline and 20% FBS stimulation conditions. (B) Quantification of ERK1/2 phosphorylation based on Fig. 2A. * $p < 0.05$ vs. DMSO; ** $p < 0.01$ vs. DMSO/FBS; *** $p < 0.001$ vs. DMSO/FBS. (C) Determining ERK1/2 phosphorylation levels in the hearts of mice treated with either 6% DMSO (veh) or U0126 at different doses for one week. (D) Quantification of ERK1/2 phosphorylation in Fig. 2C. * $p < 0.05$ vs. veh. (E,F) Fasting glucose measurement and heart/body weight ratios in mice 6 weeks post U0126 treatment. * and # $p < 0.05$. (G–J) RT-PCR analysis of genes in cardiac hypertrophy (G–I) and fibrosis (J). * and # $p < 0.05$. (K) Representative Masson's trichrome images for fibrosis analysis in the mouse heart. Scale bar, 100 μm. (L–O) RT-PCR analysis of genes involved in fatty acid uptake (*FACS*), fatty acid oxidation (*CPT1A*), fatty acid metabolism (*PPARα*), and fatty acid synthesis and storage (*PPARγ*). * $p < 0.05$; ** and ## $p < 0.01$. All the samples in Fig. 2 were biological replicates. RT-PCR, real-time polymerase chain reaction.

[49,55]. Both concentrations of U0126 decreased the phosphorylation of ERK1/2 in the mouse heart (Fig. 2C,D). 15 mg/kg of U0126 significantly reduced it by ~40%, a reduction sufficient to impact ERK1/2-regulated physiology without causing observable changes in the physical wellbeing of the mice [49]. U0126 treatment notably decreased the serum glucose level in the STZ-treated mice (446.5 mg/dL vs. 508.2 mg/dL in STZ mice, $p = 0.01$) (Fig. 2E). However, this reduction might be a systemic effect. U0126 treatment also preserved the cardiac muscle mass (hw/bw: 5.2 in STZ-U0126 mice vs. 4.8 in STZ mice, $p = 0.01$), suggesting that ERK1/2 inhibition could be beneficial against cell death in STZ hearts (Fig. 2F) [56]. Similarly, the expression for cardiac hypertrophy marker genes *ANF*, *BNP*, and interstitial fibrosis gene collagen 3α1 were significantly attenuated in U0126 treated mice (Fig. 2G–J). Masson's trichrome staining of mouse heart tissues demonstrated moderate increase of fibrosis in STZ-treated mouse hearts, which was attenuated by U0126 treatment (Fig. 2K and **Supplementary Fig. 1**). Moreover, the messenger ribonucleic acid (mRNA) levels for fatty acid metabolism genes (*FACS*, *CPT1A*, and *PPARα*) were all downregulated (Fig. 2L–O). This gene expression alteration is consistent with our hypothesis that increased fatty acid metabolism is detrimental to the heart.

We also used *db/db* mice to further investigate the role of ERK1/2 in diabetic cardiomyopathy. At 12 weeks of age, these mutant mice already demonstrated a significantly high level of plasma insulin (9.2 ng/mL vs. 0.4 ng/mL in control mice, data not shown). Consistent with STZ-treated mice (Fig. 2), 6 weeks of U0126 administration significantly reduced the plasma glucose level (Fig. 3A). Neither the heart/body weight ratios, nor the fibrosis levels, were altered by U0126 (Fig. 3B,F,G and **Supplementary Fig. 2**). This inconsistency with STZ mice could be due to the difference in mouse models and sensitivity to develop heart disease [57]. Nevertheless, we did observe the attenuation in the mRNA expression for cardiac hypertrophic markers (*ANF*, *BNP*, *βMHC*) (Fig. 3C–E) as well as fatty acid metabolism (*PPARα*, *CPT1A*, and *FACS*) (Fig. 3H–K).

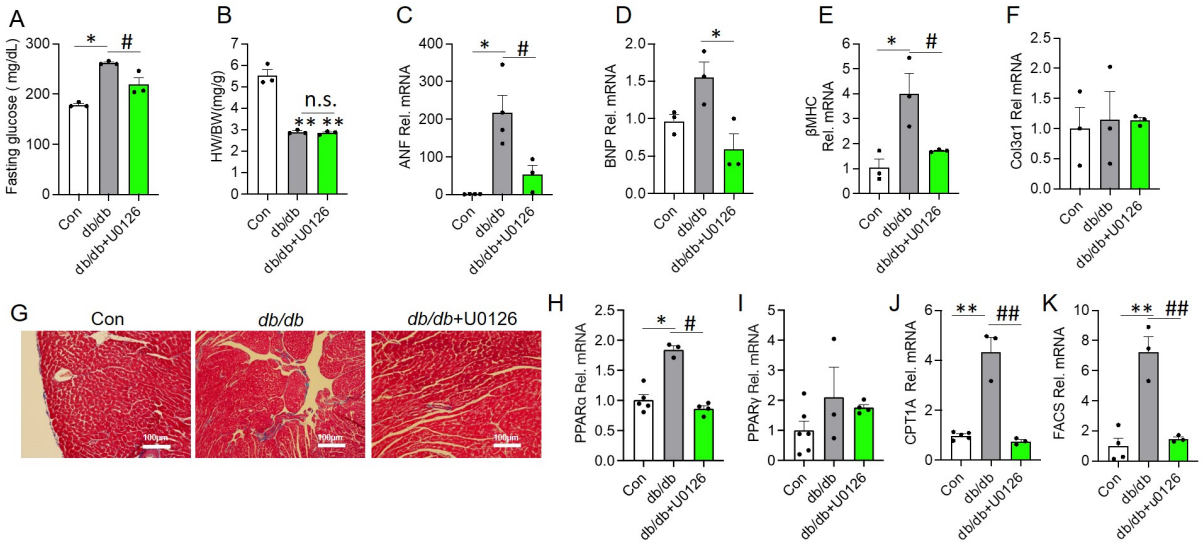

Fig. 3. U0126 inhibition in *db/db* mice alleviates the diabetic cardiomyopathy. (A) Fasting glucose levels in indicated groups of mice. * and # $p < 0.05$. (B) Comparison of heart/body weight ratios in all three groups of mice. ** $p < 0.05$ vs. con. n.s., not significant. (C–F) RT-PCR analysis of marker genes for cardiac hypertrophy and interstitial fibrosis. * and # $p < 0.05$. (G) Fibrosis comparison among three groups of mouse hearts by Masson's trichrome staining. Scale bar, 100 μm. (H–K) RT-PCR analysis of fatty acid metabolism genes. * and # $p < 0.05$; ** and ## $p < 0.01$. All the samples in Fig. 3 were biological replicates.

PPARγ expression was not significantly influenced by either STZ or U0126 treatment in both diabetic mouse models (Fig. 3I and Fig. 2M), consistent with distinct functions of PPARα and PPARγ in fatty acid metabolism [33,58]. Together, these results suggest that increased ERK1/2 activity induced by hyperglycemia or hyperlipidemia causes cardiomyopathy in mice.

3.3 ERK1/2 Activation Exacerbates Diabetes-Induced Cardiac Remodeling

Next, we used a different mouse model with knockout of both dual-specificity phosphatase 6 and 8 (DKO), two dual phosphatases for ERK1/2 inactivation [44]. ERK1/2 phosphorylation was significantly elevated in these DKO mouse hearts (Fig. 4A,B), consistent with previous findings [44]. Upon diabetic induction by STZ, both wild type (WT) and DKO mice developed similar levels of hyperglycemia within a week (Fig. 4C). 6 weeks after STZ injection, there was no statistical difference in cardiac hypertrophy in DKO-STZ hearts compared to WT-STZ (Fig. 4D). Again, we observed elevated but moderate interstitial fibrosis in the hearts of STZ-treated DKO mice (Fig. 4E and **Supplementary Fig. 3**). An upregulation of gene expression for hypertrophy (*ANF*, *BNP*, and *βMHC*), fibrosis (*Col3α1*), and fatty acid metabolism (*PPARα*, *CPT1A*, and *FACS*) was found in STZ-treated DKO mouse hearts, suggesting the association between ERK1/2 activation and development of diabetic cardiomyopathy (Fig. 4F,G).

4. Discussion

ERK1/2 play a crucial role in regulating cardiomyocyte growth [59–61]. Due to their emerging role in fatty acid metabolism, ERK1/2 have been implicated as potential therapeutic targets for improvement of insulin sensitivity in diabetes [62,63]. For instance, administration of the MEK1/2 inhibitor PD184352 to target the ERK1/2 pathway reversed the diabetic conditions in the *db/db* mice [64]. Hepatic ERK1/2 activation was associated with impaired insulin sensitivity, and short hairpin interfering RNA-mediated knockdown of ERK1/2 in the liver restored the insulin sensitivity [45]. However, the effect of ERK1/2 inhibition on diabetic cardiomyopathy hasn't been investigated. The strength of this study is that we show the ERK/PPARα axis is involved in diabetic cardiomyopathy by promoting fatty acid metabolism. We observed 6 weeks of ERK1/2 inhibition by daily U0126 administration significantly reduced the mRNA expression for *PPARα* and its target genes (*CPT1A* and *FACS*), which alleviated fibrosis and hypertrophy in the diabetic mouse hearts (Figs. 2,3). In contrast, elevated ERK1/2 activity in DUSP6/8KO mice enhanced the expression of fatty acid metabolism genes, which was associated with fibrosis and cardiac dysfunction (Fig. 4). Ultimately, our study suggests ERK1/2 as therapeutic targets against lipotoxicity in the diabetic heart.

Diabetic cardiomyopathy is a multifaceted disease characterized by hyperglycemia, hyperlipidemia, and insulin resistance. Numerous studies have been conducted to ameliorate one aspect of its pathogenesis such as inflammation [65–67]. For example, Li *et al.* [65] showed that administration of alpha lipoic acid, a naturally occurring antioxidant, into the STZ-induced diabetic rats significantly reduced the level of cardiac collagens and fibrotic gene expression such as transforming growth factor β (TGFβ) and α smooth muscle actin. Interestingly, the protective ef-

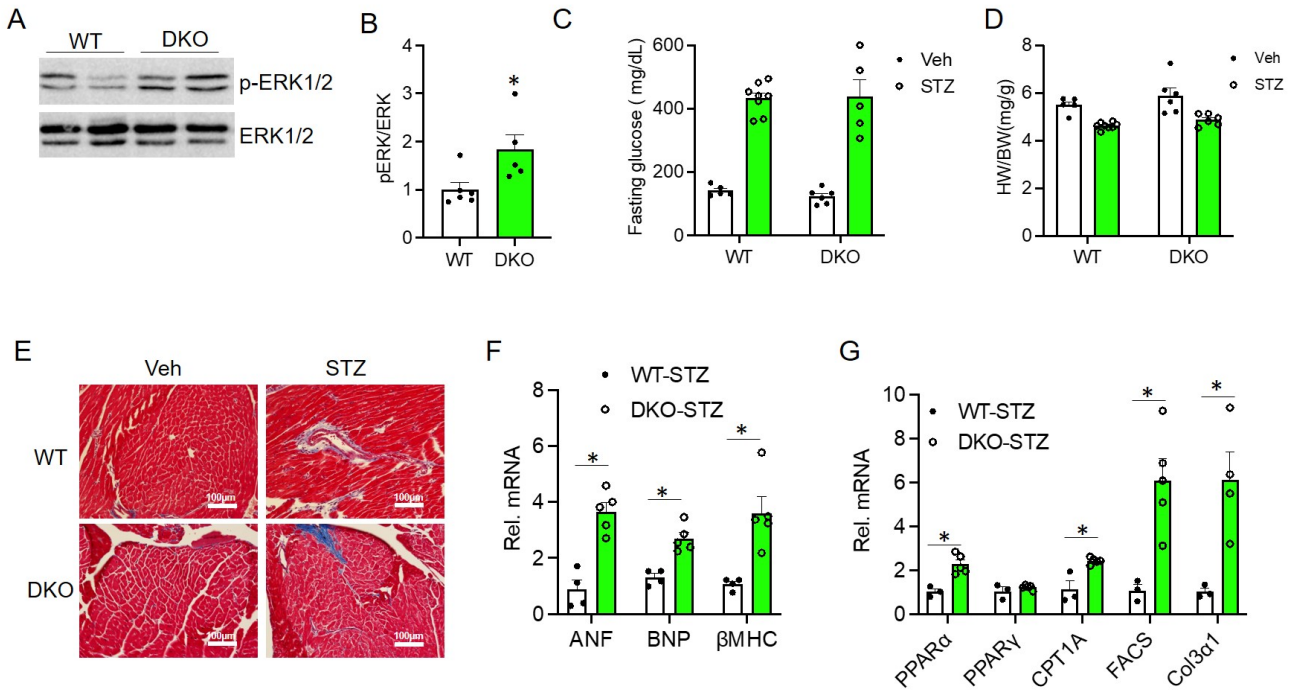

Fig. 4. Activation of ERK1/2 exacerbates diabetic cardiomyopathy. (A) Comparison of ERK1/2 phosphorylation in the hearts of wild type (WT) and DUSP6/8 double knockout mice (DKO). (B) ERK1/2 phosphorylation quantification based on Fig. 4A. * $p < 0.05$ vs. WT. (C) Fasting glucose levels in indicated groups of mice 1 week after STZ administration. (D) Comparison of heart/body weight ratios in all four groups of mice. (E) Representative Masson's trichrome images for fibrosis analysis in the mouse hearts. Scale bar, 100 µm. (F,G) Gene expression analysis for cardiac hypertrophy, fibrosis, and fatty acid metabolism. * $p < 0.05$. All the samples in Fig. 4 were biological replicates.

fect of alpha lipoic acid was partially achieved through reduction in JNK and p38 activity, further highlighting the critical role of MAPKs in energy metabolism. Similarly, high pericardial adiposity is strongly associated with diabetes [66]. Administration of hemin, a heme oxygenase inducer, potentiated the insulin signaling and suppressed the cardiac inflammation responses [67]. However, controlling diabetes through reduction in fatty acid oxidation and heart damage may still be the best option [19]. Medications like Trimetazidine, Perhexiline, and Etomoxir, have been shown to target mitochondrial oxidative metabolism and improve cardiac function [19,68–70]. Consistent with this notion, administration of β adrenergic receptor agonist isoprenaline into a pancreatectomy-induced diabetic rat model restored the metabolic flexibility through enhancing glycolysis [71].

We acknowledge that our study provides limited functional and mechanistic characterization of diabetic mice regarding ERK1/2 inhibition. Since diabetic cardiomyopathy is associated with left ventricular chamber dilation and contractile dysfunction [72,73], further analysis by Doppler echocardiography will help determine whether ERK1/2 inhibition restores the cardiac function in the diabetic mice. In addition, STZ-induced diabetic mice demonstrate decreased ventricular muscle mass [73]. Our study could be further strengthened by determining whether ERK1/2 inhibition reduces lipotoxicity and cell death in the diabetic hearts by analyzing the lipid contents and apoptotic protein levels in the myocardium. Moreover, it is known that the expression of glucose transporter 4 (GLUT4) and glucose transporter 1 (GLUT1) in diabetic mouse hearts is decreased [74]. We demonstrate that ERK1/2 inhibition reduces the serum glucose in STZ-induced diabetic mice (Fig. 2E and Fig. 3A). It is possible that reduction of fatty acid metabolism in the hearts of U0126-treated mice is compensated by an increase in GLUT4 expression, as demonstrated by a histone deacetylase (HDAC) inhibition study in restoring the GLUT4 level [74]. Lastly, our study is limited by the lack of direct molecular mechanism for the association between ERK1/2 activation and elevation in lipid metabolism gene expression [74]. As numerous cytoplasmic and nuclear proteins have been reported as the targets of ERK1/2 [62], it is possible that in the diabetic hearts increased fatty acid uptake activates ERK1/2, which then translocate to the nucleus to phosphorylate PPARα at serine 12 and 2 to initiate the transcriptional activity of PPARα [75].

Activation of PPARα and its target genes increase fatty acid metabolism, subsequently resulting in lipotoxicity in the heart. Targeting PPARα signaling through regulating its cofactors could be another avenue for the treatment of diabetic cardiomyopathy. Interestingly, ubiquitin-

specific protease 7 (USP7), a deubiquitinase, was found to bind to the PPARα coactivator 1β (PGC1β), leading to the deubiquitination and stabilization of PGC1β. Conditional gene knockout or chemical inhibition of USP7 reversed the cardiac phenotype in the diabetic mice, further supporting the notion that activation of PPARα signaling leads to lipotoxicity and the development of diabetic cardiomyopathy [76]. Of note, although increased fatty acid uptake into the myocardium leads to lipotoxicity, activation of mitochondrial protein kinase B (PKB/AKT1) signaling enhances the whole-body energy expenditure which protects the mice from diabetic cardiomyopathy [77]. Thus, further studies are needed to elucidate the balance between fatty acid metabolism and energy expenditure in the context of diabetic heart disease.

5. Conclusion

ERK1/2 phosphorylation is increased in the diabetic heart which is associated with elevated gene expression for hypertrophy and fatty acid metabolism. U0126 administration into the diabetic mice blocks the ERK1/2 activity, decreases the fatty acid metabolism, and reverses the pathological cardiac remodeling. ERK1/2 are promising targets against diabetic heart disease.

Availability of Data and Materials

All the data and materials are available from the corresponding author RL upon request.

Author Contributions

All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work. MC tested the optimal dose of STZ for diabetic induction in mice. RL and MC initiated the study. EM and CDR performed STZ and U0126 injection, measured blood glucose levels, and analyzed body and heart weights of the mice, as well as acquired images of Masson's trichrome staining. AM performed all RT-PCR analysis. JC performed H9C2 culture and evaluated ERK1/2 phosphorylation. RL analyzed all the data and wrote the manuscript. All authors contributed to the editorial changes in the manuscript and approved the final manuscript.

Ethics Approval and Consent to Participate

The animal experiments in this study have been approved by the Grand Valley State University Ethics Committee (approval number: 23-05-A).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL26700.

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