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The beneficial effects of AMP kinase activation against oxidative stress are associated with prevention of PPARα-cyclophilin D interaction in cardiomyocytes

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Barreto-Torres G, Hernandez JS, Jang S, Rodríguez-Muñoz AR, Torres-Ramos CA, Basnakian AG, Javadov S. The beneficial effects of AMP kinase activation against oxidative stress are associated with prevention of PPARα-cyclophilin D interaction in cardiomyocytes. *Am J Physiol Heart Circ Physiol* 308: H749–H758, 2015. First published January 23, 2015; doi:10.1152/ajpheart.00414.2014.—AMP kinase (AMPK) plays an important role in the regulation of energy metabolism in cardiac cells. Furthermore, activation of AMPK protects the heart from myocardial infarction and heart failure. The present study examines whether or not AMPK affects the peroxisome proliferator-activated receptor-α (PPARα)/mitochondria pathway in response to acute oxidative stress in cultured cardiomyocytes. Cultured H9c2 rat embryonic cardioblasts were exposed to H_2O_2-induced acute oxidative stress in the presence or absence of metformin, compound C (AMPK inhibitor), GW6471 (PPARα inhibitor), or A-769662 (AMPK activator). Results showed that AMPK activation by metformin reverted oxidative stress-induced inactivation of AMPK and prevented oxidative stress-induced cell death. In addition, metformin attenuated reactive oxygen species generation and depolarization of the inner mitochondrial membrane. The antioxidative effects of metformin were associated with the prevention of mitochondrial DNA damage in cardiomyocytes. Coimmunoprecipitation studies revealed that metformin abolished oxidative stress-induced physical interactions between PPARα and cyclophilin D (CypD), and the abolishment of these interactions was associated with inhibition of permeability transition pore formation. The beneficial effects of metformin were not due to acetylation or phosphorylation of PPARα in response to oxidative stress. In conclusion, this study demonstrates that the protective effects of metformin-induced AMPK activation against oxidative stress converge on mitochondria and are mediated, at least in part, through the dissociation of PPARα-CypD interactions, independent of phosphorylation and acetylation of PPARα and CypD.

H9c2 cardiomyocytes; oxidative stress; mitochondria; metformin; AMPK; PPARα

THE ROLE OF MITOCHONDRIA in energy production is well established. Mitochondria are also involved in a range of other processes, such as reactive oxygen species (ROS) production, ion signaling, redox control, lipid metabolism, cell growth, and cell death through apoptosis, necrosis, and autophagy (16). Structural and functional abnormalities are implicated in the pathogenesis of cardiac diseases, including myocardial ischemia (infarction) and heart failure (37). Activation of numerous survival protein kinases, including the AMP-activated protein kinase (AMPK) (43), protein kinase C (28), phosphatidylinositol-4,5-bisphosphate 3-kinase (11), glycogen synthase kinase 3 (24), and cGMP-dependent protein kinase (27), has been demonstrated to protect cardiomyocytes against oxidative stress through a direct or indirect interaction with mitochondria. Specifically, AMPK activation is associated with a reduction in cardiac hypertrophy (49) and infarct size, preservation of cardiac energy sources, and reduction in both necrosis and apoptosis (26). AMPK has been widely accepted as the main cellular energy sensor that both initiates ATP-generating processes and blocks ATP-consuming processes. Furthermore, AMPK plays a central role in the regulation of mitochondrial metabolism and controls the redox state of the cell, though the underlying mechanisms of its action on mitochondria remain unclear. Previous studies demonstrated that pharmacological activation of AMPK stimulates fatty acid oxidation (FAO) through increased expression of nuclear receptor peroxisome proliferator-activated receptor-α (PPARα) target genes in skeletal muscle cells (47). We, along with other groups, demonstrated that PPARα was implicated in cardioprotective signaling in the heart (4, 7, 48). PPARα increases the expression of enzymes, transporters, and proteins that are involved in FAO, mitochondrial biogenesis, and the transport of fatty acids into the mitochondria. However, the role of the AMPK/PPARα pathway in the prevention of mitochondrial dysfunction in acute oxidative stress remains unknown.

The beneficial effects of AMPK activation are mediated through mitochondria, particularly the inhibition of mitochondrial permeability transition pore (PTP) opening (4, 19, 39). Although PTP opening is a well-known phenomenon that occurs in response to oxidative stress, the molecular identity of the pore complex is not yet clear. It has been well established that cyclophilin D (CypD) plays an important role in regulating of PTP opening (5, 23). In this study, we sought to determine whether or not the AMPK/PPARα pathway was involved in protecting H9c2 cells from oxidative stress-induced cell death via the inhibition of PTP opening. Our results showed that AMPK activation with metformin attenuated H_2O_2-induced cell death and depolarization of the inner mitochondrial membrane (IMM). The protective effects of metformin were associated with a reduction of both ROS generation and mitochondrial DNA (mtDNA) damage in the cultured cardiomyocytes. Coimmunoprecipitation studies showed that metformin abrogated oxidative stress-induced physical interactions between PPARα and CypD, and the abolishment of these interactions was associated with inhibition of PTP formation. The beneficial effects of metformin were not due to oxidative stress-induced acetylation or phosphorylation of PPARα and CypD.

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MATERIALS AND METHODS

Cell culture. H9c2 rat embryonic cardioblasts (American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's/Ham's F-12 medium (DMEM, Invitrogen, Carlsbad, CA) containing 5.5 mM glucose and supplemented with 10% fetal bovine serum, 10 μg/ml transferrin, 10 μg/ml insulin, 10 ng/ml selenium, 1% penicillin and streptomycin, 2 mg/ml BSA, 2.44 mg/ml $NaHCO_3$, 5 μg/ml linoleic acid, 3 mM pyruvic acid, 0.1 mM MEM non-essential amino acids, 10% MEM vitamin, 0.1 mM L-ascorbic acid, and 30 mM HEPES (pH 7.1) and maintained in 95% room air-5% CO_2 at 37°C. After starvation for 24 h, cells were pretreated with H_2O_2 in the presence or absence of metformin. The cells were serum starved for 24 h in DMEM with 5.5 mM glucose and 10 mg/ml BSA, 2.44 mg/ml $NaHCO_3$, 80 μg/ml $CaCl_2$, 0.25 mg/ml fetuin, 1% penicillin and streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin, 10 ng/ml selenium, 0.1 mM L-ascorbic acid, 0.1 mM MEM nonessential amino acids, 10% MEM vitamin, and 44 μM BSA-palmitate (pH 7.1 before treatments).

Cell viability. Cell viability was determined by the trypan blue exclusion assay. Cells were incubated with trypan blue, and dead cells (trypan blue positive) and live cells (trypan blue negative) were counted using the TC20 automated cell counter (Bio-Rad, Hercules, CA).

Mitochondrial membrane potential. To monitor mitochondrial membrane potential ($\Delta\Psi_m$), H9c2 cells (4×10^5 cells/well) plated in a 24-well culture plate were incubated with the membrane potential-sensitive dye 5,5′,6,6′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1, 10 μg/ml, Molecular Probes) for 30 min at 37°C. The intensity of fluorescence was measured using a Spectramax M3 microplate reader at 527 and 590 nm for emission and 488 nm for excitation. For confocal microscopy, the cells plated on glass-bottom dishes were loaded with JC-1 at the same concentration. The images were obtained using a Zeiss LSM510 META microscope (Carl Zeiss, Oberkochen, Germany).

Complex I electron transport chain activity. The activity of the electron transport chain (ETC) complex I in cell lysates was determined by measuring the decrease in the concentration of NADH at 30°C (20). Cells were resuspended in hypotonic phosphate buffer containing 5 mM $MgCl_2$ and 0.55 mg/ml saponin and, then, freeze thawed three times to rupture the IMM completely. The assay was performed in phosphate buffer containing 1 mM KCN, 5 mM $MgCl_2$, 2.5 mg/ml BSA, 2 μM antimycin, 100 μM decylubiquinone, and 100 μM NADH (pH 7.4). Specific activities were determined by calculating the slope of the reaction in the linear range and normalizing per milligram of protein.

Total ROS levels. Cells were cultured in 3-cm dishes and administered corresponding treatments. Cells were then detached and centrifuged, and the pellet was resuspended in 200 μM 2′,7′-dichlorofluorescein diacetate (Life Technologies, Grand Island, NY) for 30 min. After incubation with the dye, the cells were washed, and the fluorescence was quantified using a Wallac 1420 VICTOR F microplate reader (PerkinElmer, San Diego, CA) with a 485-nm excitation filter and a 535-nm emission filter.

Mitochondrial PTP. To quantify mitochondrial PTP opening, cells were incubated with 5 μM calcein-acetoxymethyl ester (Molecular Probes, Eugene, OR) in the presence of 5 mM cobalt chloride, which quenched cytosolic and nuclear calcein. Cells were visualized with an Olympus IX73 inverted fluorescence microscope (Center Valley, PA), and fluorescence images were captured using an Olympus DP73 high-performance Peltier cooled digital color camera (Center Valley, PA). Olympus CellSens Dimension Imaging software (Center Valley, PA) was used to analyze calcein fluorescence in cells.

SDS-PAGE and Western blot analysis. Membranes were immunoblotted with acetyl-lysine, AMPK, phospho (P)-AMPKα1^{Thr172} (Cell Signaling, Boston, MA), CypD, andenine nucleotide translocator, PPARα, P-PPARα^{Ser21} (Santa Cruz Biotechnology, Santa Cruz, CA), PPARγ coactivator 1α (PGC-1α), nuclear respiratory factor (NRF) 1 and 2, mitochondrial transcription factor A (Abcam, Cambridge, MA), or actin (Sigma-Aldrich, St. Louis, MO). The chemiluminescence signals were visualized using Thermo Scientific Pierce ECL Western blotting detection reagents (Thermo Scientific, Rockford, IL) at the VersaDoc 3000 Gel Imaging System (Bio-Rad).

Coimmunoprecipitation. Protein samples were incubated with anti-PPARα or anti-CypD antibodies overnight at 4°C, and the immunoprecipitates were harvested by protein A/G-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoprecipitated complexes were washed and then subjected to SDS-PAGE, followed by immunoblotting using antibodies for CypD, andenine nucleotide translocator, voltage-dependent anion channel, and PPARα (Santa Cruz Biotechnology). For phosphorylated CypD and PGC-1α, samples were incubated with anti-phospho-threonine-proline antibodies (Abcam), as explained above. The complexes were then immunoblotted using antibodies for CypD (Santa Cruz Biotechnology) and PGC-1α (Abcam). Likewise, for acetylated CypD and PGC-1α, samples were incubated with acetyl-lysine (Cell Signaling), and the complexes were then immunoblotted using antibodies for CypD and PGC-1α.

Quantitative polymerase chain reaction analysis of mtDNA abundance and damage. The procedures for DNA isolation, quantification, mtDNA damage analysis, and mtDNA abundance determinations have been previously described (45). The quantitative polymerase

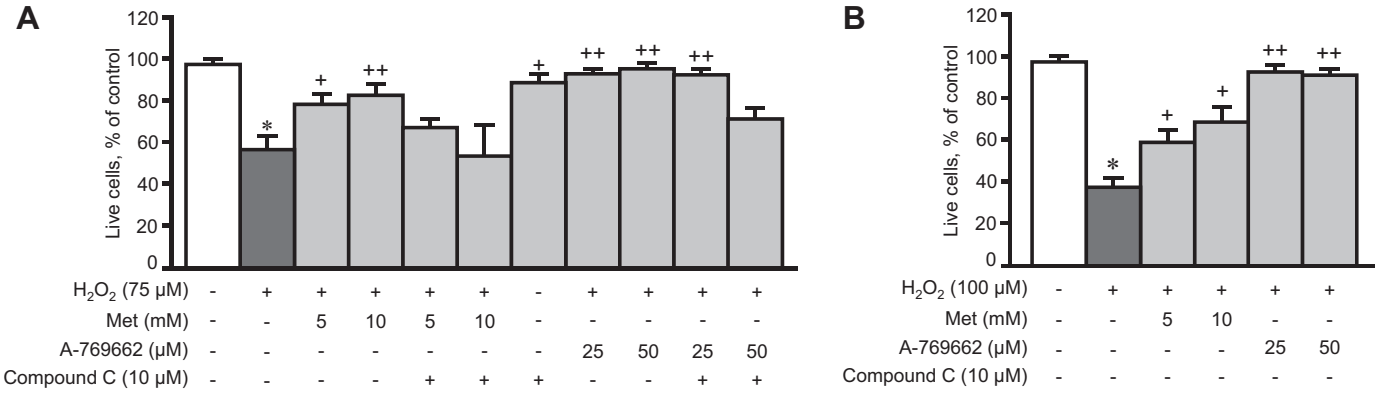

Fig. 1. The effects of metformin (Met) and A-769662 on cell death induced by 75 (*A*) and 100 (*B*) H_2O_2 in H9c2 cells. Cell death was assessed by the trypan blue exclusion test and shown as a percentage of live cells compared with the control group. The control group included cells that were treated with corresponding agonist/inhibitor in the absence of H_2O_2. *$P < 0.01$, H_2O_2 vs. control; +$P < 0.05$ and ++$P < 0.01$, H_2O_2 + Met or H_2O_2 + A-769662 vs. H_2O_2; $n = 3$–5 per each group.

chain reaction (PCR) assay is based on the ability of certain DNA lesions to block the movements of the PCR polymerase during PCR, thus allowing an estimation of the number of DNA lesions in a target sequence. DNA damage is inversely proportional to the relative amplification of the target sequence. To detect mtDNA damage in H9c2 cardioblasts, a 10-kb mtDNA fragment from the rat mitochondrial genome was amplified. This amplification was normalized to the amplification of a small fragment [113 base pair (bp)] of mtDNA to correct for possible changes in mtDNA abundance. Given the probability of the PCR encountering a lesion in such a small DNA target is very low, a small fragment can be amplified to provide a measure of relative mtDNA abundance. To ensure exponential amplification of the target sequences, preliminary experiments were performed to optimize the buffer (PreMixes, Epicentre), initial template concentration, and the number of PCR cycles (data not shown). Expected PCR products were verified by a polyacrylamide gel electrophoresis for the small amplicon and agarose gel electrophoresis for the large amplicon.

To determine mtDNA abundance in rat cardiomyocytes, the 113-bp mtDNA fragment was amplified with the following primer sequences: 5′-ACGGGATTTCATGGCCTCCA-3′ (forward) and 5′-TGCGGCTTCAAATCCGAAATG-3′ (reverse). The PCR reactions (containing 7.5 ng of DNA in premix 3) were performed with an initial denaturation at 94°C for 45 s, followed by 22 cycles of denaturation at 94°C for 15 s, annealing/extension at 67°C for 45 s and 45 s at 72°C, and a final postextension for 10 min at 72°C. The 10-kb fragment was amplified with the following primer sequences: 5′-GCCGGAAACCTAGCCCATGC-3′ (forward) and 5′-ACGAGTGGGCGGGTTGTTGA-3′ (reverse). The PCR reactions (containing 7.5 ng of DNA in premix 6) were performed with an initial denaturation at 94°C for 45 s, 23 cycles of denaturation at 94°C for 15 s, annealing/extension at 68°C for 12 min, and a final extension at 72°C for 10 min.

Relative amplification was determined by quantifying the amount of DNA present in the PCR tube after the PCR reaction and then was expressed relatively to a reference DNA, which consisted of a pooled DNA (7.5 ng) from the cardiomyocytes grown under standard conditions. In all the experiments, a sample containing 3.75 ng of DNA was also included to ensure that reactions were in the exponential phase.

Statistical analysis. Data are presented as means ± SE. Differences among groups were compared by two-tailed Student's *t*-tests or one- or two-way factorial ANOVA. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Metformin attenuates oxidative stress-induced cell death associated with AMPK activation. To determine whether or not metformin protects H9c2 cardiomyocytes from oxidative stress-induced cell death, we examined the effects of 75 and 100 μM H_2O_2 on cell survival. It should be noted that H_2O_2 at these concentrations were used in further experiments. As shown in Fig. 1, cell survival was significantly reduced in cells treated with 75 and 100 μM H_2O_2. However, pretreatment with metformin or A-769662 significantly attenuated cell death in response to H_2O_2. The beneficial effects were observed at low and high concentrations of the AMPK agonists. Inhibition of AMPK by compound C prevented protective effects of metformin and A-769662 on cell survival, indicating that the effects are mediated through AMPK activation (Fig. 1, *A* and *B*).

In the following set of experiments, we examined the effect of metformin on AMPK activation in response to oxidative stress in cardiomyocytes. Phosphorylation of AMPK at Thr^{172} has been shown to be both necessary and sufficient to promote AMPK activation (41). Previous studies using pharmacological (39, 50) and genetic (29) inhibition of AMPK revealed that the beneficial effects of metformin are primarily associated with AMPK activation. As shown in Fig. 2*A*, H_2O_2 alone at concentrations of 50–300 μM reduced AMPK phosphorylation, with a maximum effect (40% of control, $P < 0.01$) at 300 μM H_2O_2. Treatment of control cells with metformin alone exerted the maximum effect on AMPK phosphorylation at 10 mM (Fig. 2*B*). Pretreatment with metformin at a concentration of 5 or 10 mM induced a 2.0- or 2.5-fold increase of AMPK

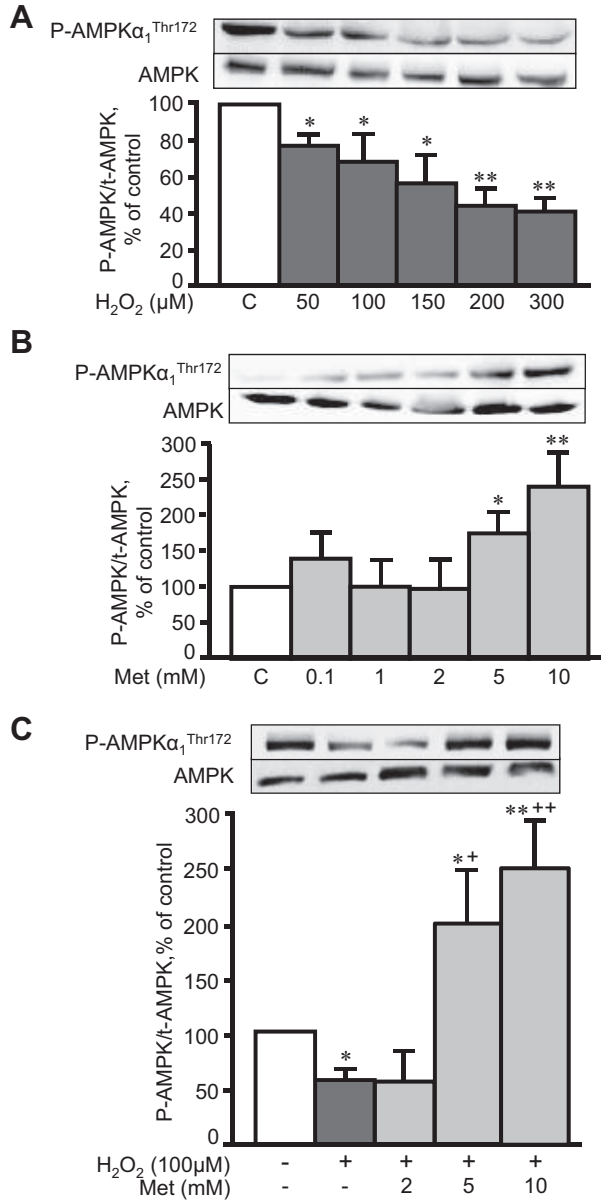

Fig. 2. The effects of H_2O_2 on AMP kinase (AMPK) activation in the presence or absence of Met. *A*: concentration-dependent effects of H_2O_2 on phospho (P)-AMPKα$_1^{Thr172}$ levels. *B*: concentration-dependent effects of Met on P-AMPKα$_1^{Thr172}$ levels. *C*: effects of oxidative stress on P-AMPK^{Thr172} in cardiomyocytes pretreated with 2, 5, and 10 mM Met. *A–C*, *top*: representative Western blot images of the phosphorylated and total levels of AMPKα. *A–C*, *bottom*: quantitative data of AMPKα phosphorylation were normalized to total (t)-AMPK and expressed as percent change relative to the control group (C). $^*P < 0.05$ and $^{**}P < 0.01$ vs. C; $^+P < 0.05$ and $^{++}P < 0.01$ vs. H_2O_2.; $n = 6–8$ per group.

phosphorylation, respectively, compared with that in control, in the presence of H_2O_2 (Fig. 2*C*).

Thus our data suggest that metformin may reduce oxidative stress-induced cell death at least in part via an increase in AMPK activation. This conclusion is supported by the fact that the beneficial effects of metformin and A-769662 on cell survival were eliminated in the presence of compound C.

Metformin attenuates mitochondrial dysfunction and ROS production in H_2O_2-treated cardiomyocytes. Mitochondrial membrane potential is a marker of the structural and functional integrity of mitochondria. It is linked to mitochondrial metabolism including ATP synthesis, maintenance of ion homeostasis, and transport of solutes and proteins to and from the mitochondria. To determine whether the cardioprotective effects of metformin against oxidative stress are mediated through the protection of mitochondrial function, we measured the $\Delta\Psi_m$ in cardiomyocytes. Results showed that H_2O_2 induced depolarization of the IMM by 76% ($P < 0.01$). Pretreatment with metformin at both 5 and 10 mM attenuated these effects and significantly improved the $\Delta\Psi_m$ (Fig. 3, *A* and *B*). The effects of metformin were abolished by compound C (AMPK inhibitor) and GW6471 (PPARα inhibitor), indicating that both AMPK and PPARα were involved in the action of metformin on mitochondria (Fig. 3*C*). Furthermore, GW6471 eliminated beneficial effects of A-769662 on the complex I ETC in H_2O_2-treated cells (Fig. 3*D*). Altogether, these data demonstrate that PPARα is involved in the AMPK-mitochondria pathway under H_2O_2-induced acute oxidative stress.

Loss of $\Delta\Psi_m$ stimulates mitochondrial ROS production, which, in turn, enhances total ROS levels through the ROS-induced ROS release mode (51). Therefore, we measured total ROS levels in H9c2 cells subjected to H_2O_2 in the presence and absence of metformin. This experiment showed a twofold increase ($P < 0.01$) in intracellular ROS levels after H_2O_2 exposure for 1 h at increasing concentrations (100–300 μM) (Fig. 3*E*). As expected, pretreatment of cells with metformin at 5 and 10 mM blocked the increase of H_2O_2-induced ROS production (Fig. 3*F*).

Since it is well established that high ROS levels can cause PTP opening and lead to cell death, we sought to determine whether PTP opens in response to H_2O_2 treatment and whether metformin mediates its beneficial effects through inhibition of PTP opening. Exposure of H9c2 cardiomyocytes to 5 mM metformin alone induced a 12% ($P < 0.05$) decrease in PTP formation. In contrast, 100 μM H_2O_2 for 1 h resulted in a 34% ($P < 0.001$) increase in PTP opening, as measured with a decreased mitochondrial calcein fluorescence (Fig. 4, *A* and *B*). Even though the levels of calcein fluorescence in mitochondria

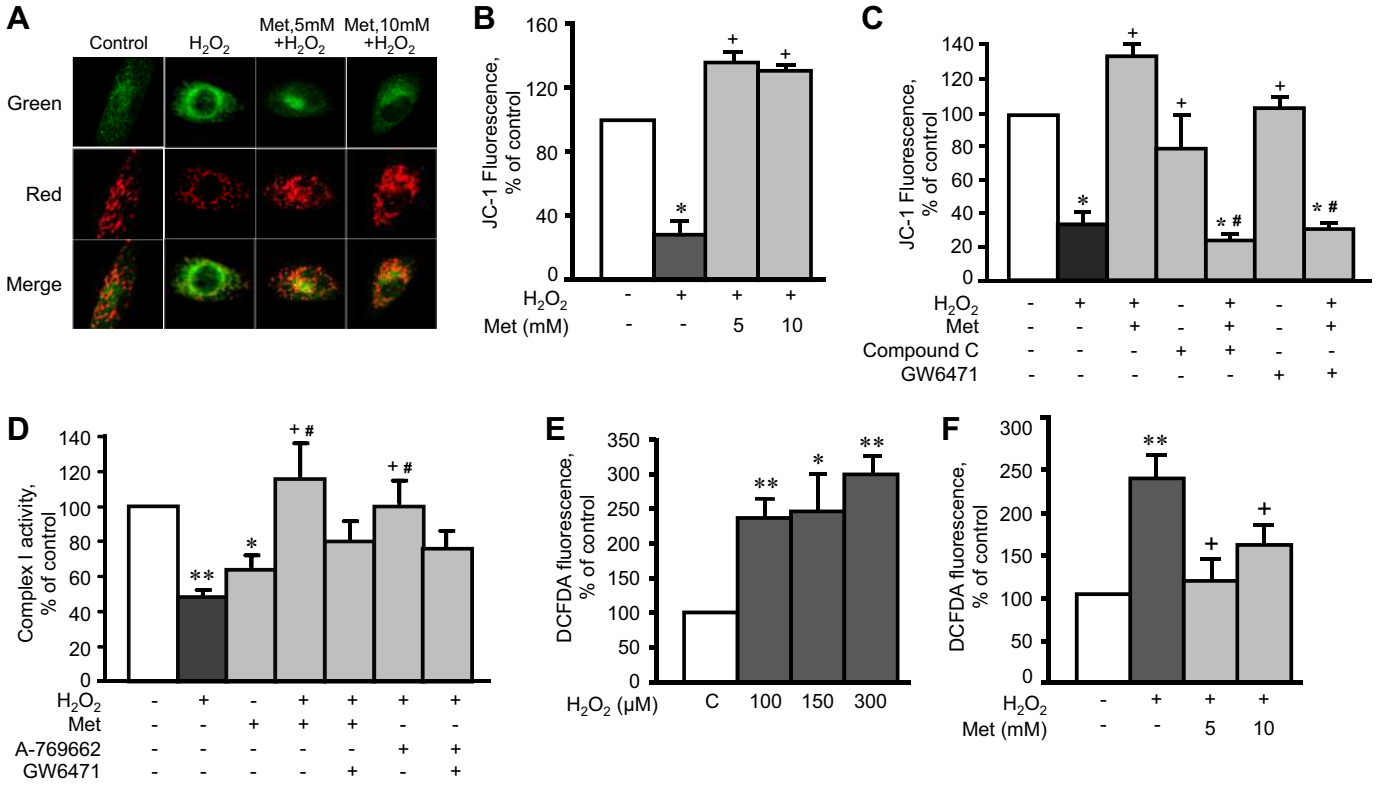

Fig. 3. Effects of H_2O_2 on total reactive oxygen species (ROS) levels and mitochondrial membrane potential ($\Delta\Psi_m$) in Met-treated cells. *A*: representative images of 5,5′,6,6′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) fluorescence. The images were obtained using a Zeiss LSM510 META (Carl Zeiss) microscope from H9c2 cells after incubation with JC-1. The cells pretreated with Met were subjected to 100 μM H_2O_2. *B*: quantitative results of JC-1 fluorescence intensity, measured using a Spectramax M3 microplate reader (Molecular Devices, Sunnyvale, CA). Data were normalized as the ratio of red fluorescence of dye aggregates (J-aggregates, high $\Delta\Psi_m$) to green fluorescence of dye monomers (JC-1 monomers, low $\Delta\Psi_m$) and expressed as a percentage of the control group (C). *C*: effects of 100 μM H_2O_2 on JC-1 fluorescence in cardiomyocytes pretreated with Met, 10 μM compound C, and/or 10 μM GW6471 (GW). *D*: electron transport chain complex I activity. Cells were pretreated with 5 mM Met, 10 μM GW, and/or 25 μM A-769662 before 100 μM H_2O_2 exposure. *E*: concentration-dependent effects of H_2O_2 on total ROS levels. *F*: effects of 100 μM H_2O_2 on total ROS levels in the cells pretreated with Met. ROS levels were determined by incubation of the cells with the ROS-specific probe 2′, 7′-dichlorofluorescein diacetate (DCFDA). Data are expressed as percent change compared with control. $*P < 0.05$ and $**P < 0.01$ vs. C; $+P < 0.05$ vs. H_2O_2; $\#P < 0.05$ vs. Met; $n = 8$–14 per each group.

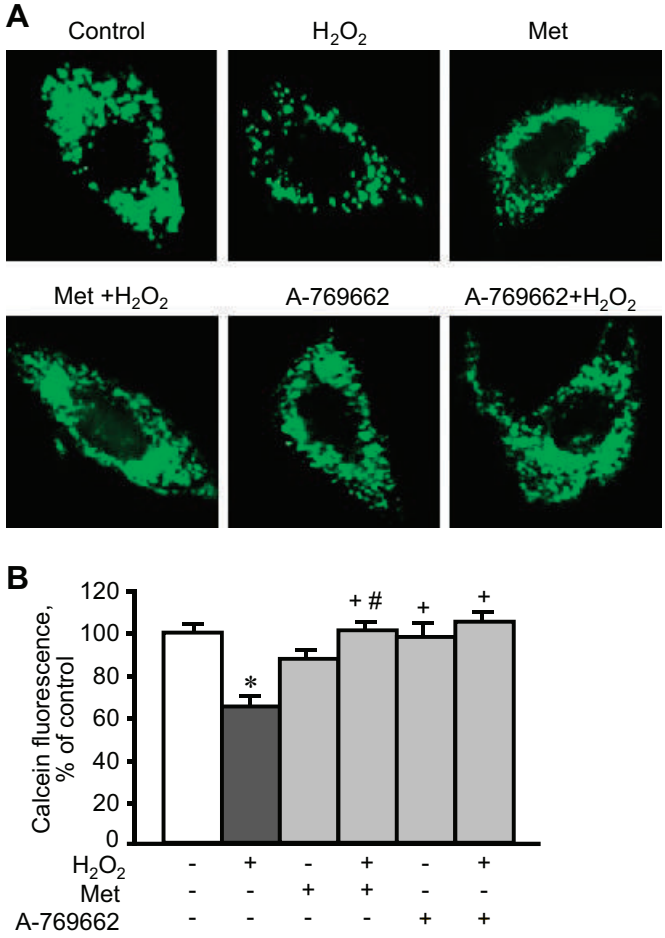

Fig. 4. Effects of H_2O_2 on permeability transition pore opening in cells pretreated with Met or A-769662. *A*: representative images of calcein fluorescence. Cells treated with 100 μM H_2O_2 in the presence or absence of Met (5 mM) or A-769662 (25 μM) were coloaded with cobalt chloride and calcein-AM and then imaged using an Olympus IX73 (Center Valley, PA) inverted fluorescence microscope. *B*: quantitative results of calcein fluorescence normalized to individual cells and expressed as a percentage of the control group (C). *$P < 0.01$ vs. C; +$P < 0.01$ vs. H_2O_2; #$P < 0.01$ vs. Met; $n = 3$ per each group.

varied from cell to cell, the overall average value of calcein staining was reduced in cells subjected to oxidative stress. Pretreatment of the cells with both metformin and A-769662 preserved the mitochondrial calcein fluorescence.

These data demonstrate that AMPK activation decreases ROS production and enhances resistance to oxidative stress by preventing $\Delta\Psi_m$ loss and inhibiting mitochondrial PTP opening in H9c2 cells.

AMPK activation in H_2O_2-exposed cells prevents CypD-PPARα interaction. CypD is a main regulator of PTP formation (22), and the cytoplasmic proteins including heat shock protein 90, Bcl-2, and p53 have been shown to interact with CypD and modulate the PTP (5). We tested whether oxidative stress can potentiate PPARα interaction with CypD. Oxidative stress significantly increased the interaction between PPAR and CypD, which was inhibited by pretreatment with metformin (Fig. 5, *A* and *B*).

Physical interaction between PPARα and CypD might be due to post-translational modification(s) of one or both proteins. Therefore, we next determined if H_2O_2 induces acetylation or phosphorylation of PPARα and CypD in H9c2 cells. The cells were subjected to H_2O_2, and the levels of acetylated or phosphorylated PPARα and CypD were monitored at 10, 20, 30, and 60 min after the treatment. As shown in Fig. 6, *A* and *B*, H_2O_2 had no effect on acetylation of PPARα; however, it increased PPARα phosphorylation at 10 and 20 min. The same trends were observed for acetylation and phosphorylation of PGC-1α, a coactivator of PPARs (Fig. 6, *A* and *B*). Interestingly, pretreatment with metformin did not affect H_2O_2-induced PPARα phosphorylation (Fig. 6*C*). It should be noted that acute oxidative stress had no effect on expression of the proteins involved in the mitochondrial transcriptional network, PGC-1α, NRF1, NRF2, and mitochondrial transcription factor A (Fig. 6*D*). Analysis of P-CypD and acetyl-CypD protein levels revealed no changes in CypD acetylation and phosphorylation within 30 min of H_2O_2 exposure. Acetylation and phosphorylation of CypD were 32 ($P < 0.05$) and 34% ($P < 0.05$) less than control at 60 min of oxidative stress, respectively (Fig. 7, *A* and *B*). Overall, these results suggest that metformin abrogated oxidative stress-induced physical interactions between PPARα and CypD likely via a mechanism that is independent of acetylation and phosphorylation of PPARα.

Metformin reduces oxidative stress-induced mtDNA damage. In addition to opening the PTP, ROS also causes mtDNA damage. To determine the effect of H_2O_2 on mtDNA abundance and damage in cultured cardiomyocytes, the cells were exposed to varying concentrations of H_2O_2 (0–200 μM) for 1 h, followed by DNA isolation and quantitative PCR analysis. Analysis of a small mtDNA amplicon (113 bp), which provides information about relative mtDNA abundance, showed a trend toward increased mtDNA abundance after H_2O_2 treatment. Maximum mtDNA abundance was reached at 75 μM H_2O_2, which was 1.88 fold higher ($P < 0.01$) than nontreated cells (Fig. 8*A*). The effect of H_2O_2 was even more robust inducing mtDNA lesions. The amplification of the large (10 kb) mtDNA amplicon, an indicator of mtDNA lesions, was 40% ($P < 0.05$), 79% ($P < 0.01$), and 90% ($P < 0.01$) less after exposure with 75, 100, and 200 μM of H_2O_2, respectively, compared with untreated control cells (Fig. 8*B*).

To determine the effect of metformin on H_2O_2-induced mtDNA lesions, we exposed cells with 75 μM H_2O_2 in the presence or absence of metformin. The results showed that H_2O_2 induced a 43% decrease in amplification ($P < 0.05$ vs. control), whereas no effect was seen in cells treated with metformin alone. However, metformin prevented the mtDNA damage caused by H_2O_2 (Fig. 8*C*). Overall, these results indicate that metformin can prevent H_2O_2-induced mtDNA lesions in cultured cardiomyocytes.

DISCUSSION

This study demonstrates that AMPK activation, in response to H_2O_2-induced oxidative stress in H9c2 cardiomyocytes, *1*) ameliorated cell death and preserved the activity of AMPK, *2*) attenuated depolarization of the IMM and prevented ROS generation, *3*) reduced mtDNA damage, *4*) abrogated oxidative stress-induced physical interaction between PPARα and CypD that was associated with the inhibition of PTP formation, and *5*) beneficial effects of AMPK activation were not associated with acetylation or phosphorylation of PPARα and CypD.

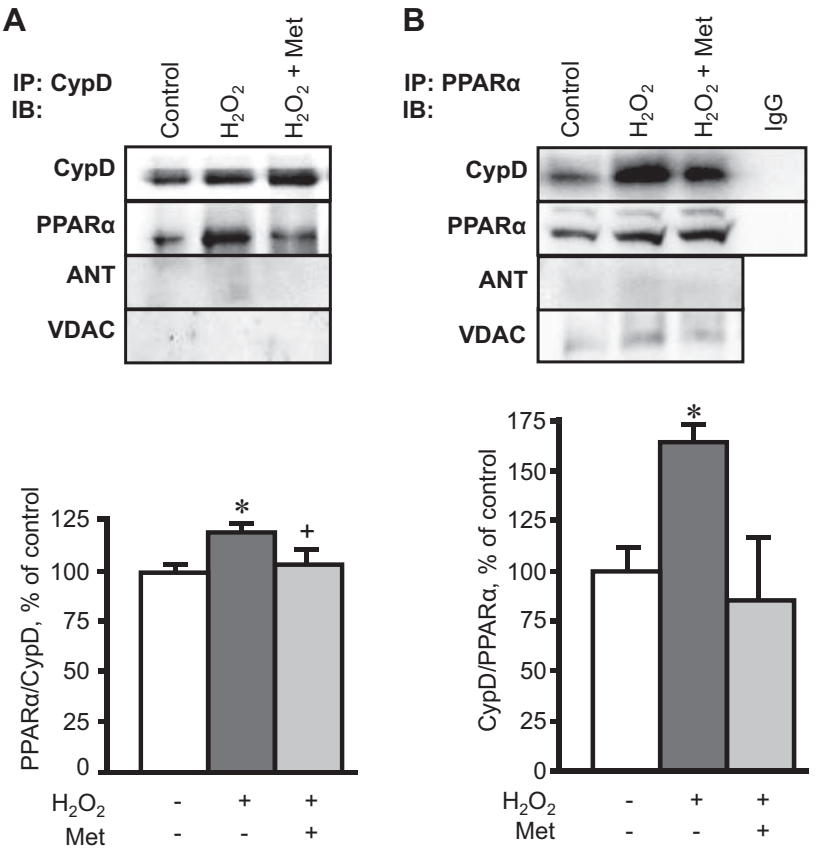

Fig. 5. Effects of Met on H_2O_2-induced cyclophilin D (CypD)-peroxisome proliferator-activated receptor-α (PPARα) interaction. Cell lysates from each group were immunoprecipitated (IP) with anti-CypD (*A*) or anti-PPARα (*B*) antibodies. The complexes were subjected to SDS-PAGE followed by immunoblotting (IB) with CypD, PPARα, adenine nucleotide translocator (ANT), and voltage-dependent anion channel (VDAC). Representative immunoblots (*A* and *B*, *top*) show the effects of oxidative stress on the interaction between PPARα and CypD in the presence and absence of Met. Quantitative results (*A* and *B*, *bottom*) were expressed as percent change compared with control. $*P < 0.05$ vs. C; $+P < 0.05$ vs. H_2O_2; $n = 3$ to 4 per group.

AMPK activation during oxidative stress induced by cardiac ischemia-reperfusion or heart failure may have both beneficial and harmful effects on the heart. Stimulation of glucose metabolism and FAO by AMPK can promote cell death due to accumulation of lipid intermediates and metabolic acidosis. On the other hand, AMPK can restore ATP to maintain cell metabolism (13). Notably, activation of AMPK depends on severity and durability of oxidative stress. In our studies, H9c2 cells exposed to 50–300 μM H_2O_2 for 60 min revealed gradual dephosphorylation of AMPK (Fig. 2). In contrast, H_2O_2 at a high concentration (1 mM) increased AMPK phosphorylation in H9c2 cells (18) and adult rat ventricular cardiomyocytes (14).

Beneficial effects of AMPK activation by various pharmacological agents are mostly mediated through mitochondrial mechanisms, in particular, the inhibition of PTP opening (4, 6, 10). Several mechanisms can mediate prosurvival effects of AMPK activation on mitochondria. One of the downstream targets of AMPK is GSK-3β, which upon phosphorylation translocates to mitochondria and inhibits PTP opening (9). Recent studies demonstrated that AMPK activation by A-769662 was associated with phosphorylation of GSK-3β and inhibition of PTP during cardiac ischemia-reperfusion in rats (35). In addition, AMPK-induced phosphorylation of GSK-3β reduced ROS production in response to oxidative stress (8). We showed that the beneficial effects of metformin on mitochondria are mediated through PPARα, since the PPARα inhibitor GW6471 prevented the cardioprotective effects of metformin against ischemia-reperfusion in rat hearts (4). In the present study, H_2O_2-induced oxidative stress promoted a protein-protein interaction between PPARα and CypD that was associated with PTP opening. Conversely, metformin prevented this interaction, suggesting that PPARα was involved in regulating pore formation indirectly through interaction with CypD. Interestingly, the effects of metformin were not associated with acetylation or phosphorylation of PPARα and CypD, indicating that other types of post-translational protein modifications might induce their interaction. In addition, post-translational modifications of CypD including acetylation (36, 40) can also initiate its interaction with other proteins and promote PTP formation (23). Previous studies provided contradictory data on the post-translational modifications of PPARα. Protein kinase A (32) and p38 (3) phosphorylated PPARα, leading to a ligand-dependent increase of PPARα activity in neonatal rat cardiomyocytes and HEK-293 cells. On the other hand, activation of ERK1/2 in cardiomyocyte hypertrophy induced downregulation of PPARα (2).

The beneficial effects of metformin on mitochondria in cultured cardiomyocytes was associated with the attenuation of ROS, an inducer of PTP opening, as well as reduction of mtDNA damage. Notably, the inhibition of PTP opening might be due to a direct effect of metformin on complex I of the ETC, which, in turn, could block pore formation (34). Inhibition of complex I by rotenone or metformin and displacement of CypD by cyclosporin A have been proposed to affect the PTP through a common mechanism (33). However, a direct inhibitory effect of 10 mM metformin on complex I was observed only several hours after incubation with mitochondria because of low uptake rate of the drug (34). In our studies, metformin reduced the complex I activity in control cells but recovered it

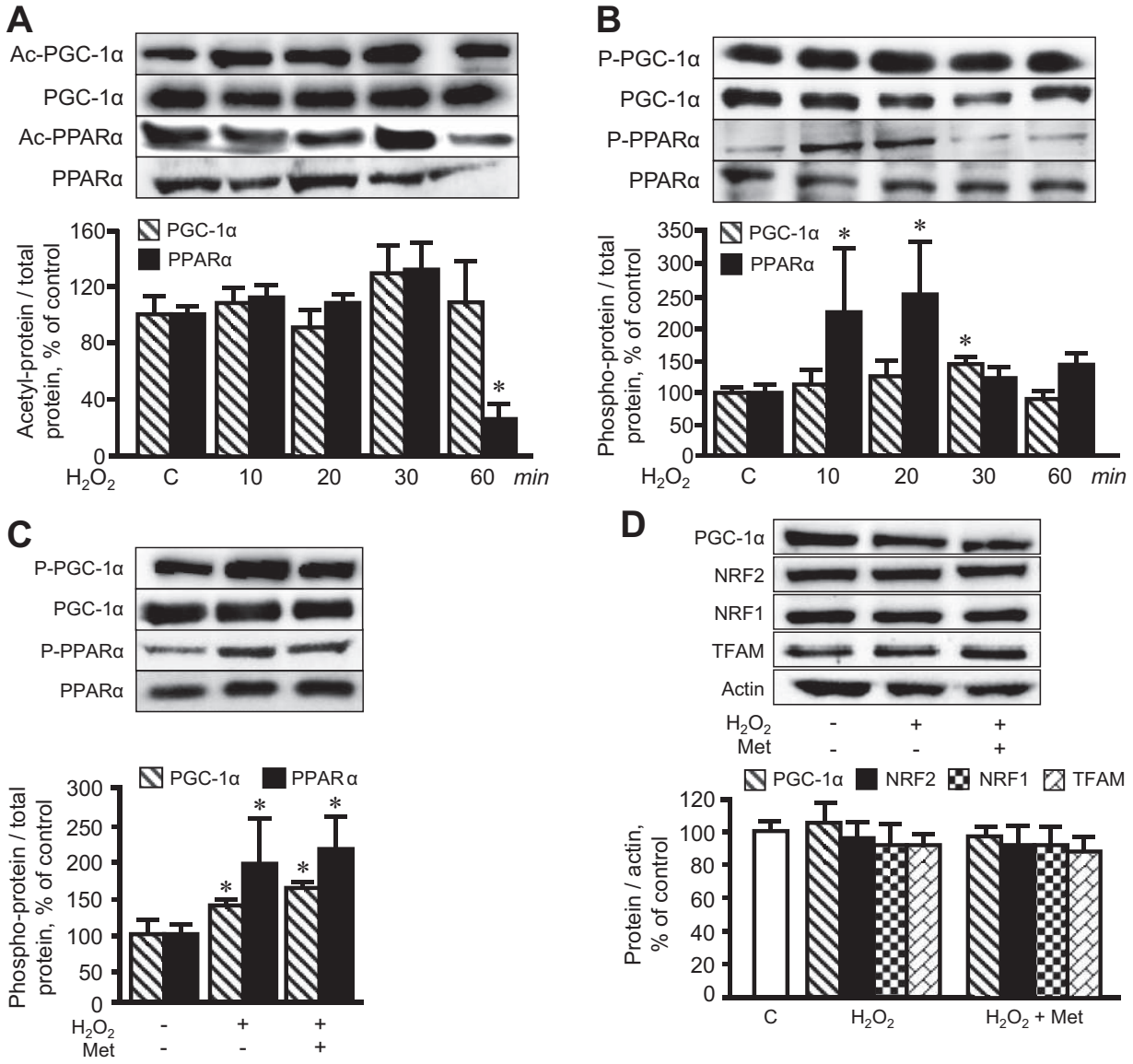

Fig. 6. Expression of mitochondrial transcriptional network proteins and time-dependent changes of phosphorylation and acetylation (Ac-PPARα) of PPARγ coactivator 1-α (PGC-1α) and PPARα in response to H_2O_2. *A*: PGC-1α and PPARα acetylation (Ac-PGC-1α and Ac-PPARα). *B*: PGC-1α and PPARα phosphorylation (P-PGC-1α and P-PPARα^{Ser21}). *C*: effects of H_2O_2 on PGC-1α and PPARα phosphorylation in the presence of Met. *D*: effects of H_2O_2 on protein expression of PGC-1α, nuclear respiratory factor (NRF) 1 and 2, and mitochondrial transcription factor A (TFAM). Protein levels were normalized to total PGC-1α or PPARα (*A*–*C*) and actin (*D*) and expressed as a percentage of the control (untreated) group. *$P < 0.05$ vs. C; $n = 3$–6 per group.

in response to oxidative stress (Fig. 3*D*). Although metformin has been shown to exert various AMPK-independent effects on cell signaling (25, 38), the cardioprotective effects of this compound are apparently mediated through activation of AMPK. H9c2 cells pretreated with compound C markedly reduced beneficial effects of metformin and A-769662 on cell survival (Fig. 1) and mitochondria (Fig. 3*C*). Our data are consistent with previous studies that showed that pretreatment of primary cardiomyocytes exposed to H_2O_2 with compound C similarly inhibited the effects of both metformin and AICAR to prevent apoptosis (39).

The AMPK-PGC-1α relationship was mostly investigated with chronic activation of AMPK in various animal models (17, 46). Metformin exerted beneficial effects on cardiac function and survival in postinfarction heart failure via AMPK activation and increased PGC-1α expression associated with improved mitochondrial respiration and ATP (19). Likewise, chronic AMPK activation by metformin improved mitochondrial biogenesis by inducing PGC-1α expression (42) or direct activation of PGC-1α by phosphorylation in skeletal muscle (21). PGC-1α affects mitochondrial metabolism through PPARs: PPARα, PPARβ/δ, and PPARγ. PPARα is highly expressed in the heart, where it upregulates genes involved in transport, activation, and oxidation of fatty acids (15). To our knowledge, there are no data on the effect of PPARα on mitochondrial metabolism in acute oxidative stress. The effects of PPARα on mitochondria may differ in acute stress since the short duration of the stress is not sufficient to affect gene expression. In addition to regulation of CypD, PPARα can improve mitochondrial function through upregulation of nitric

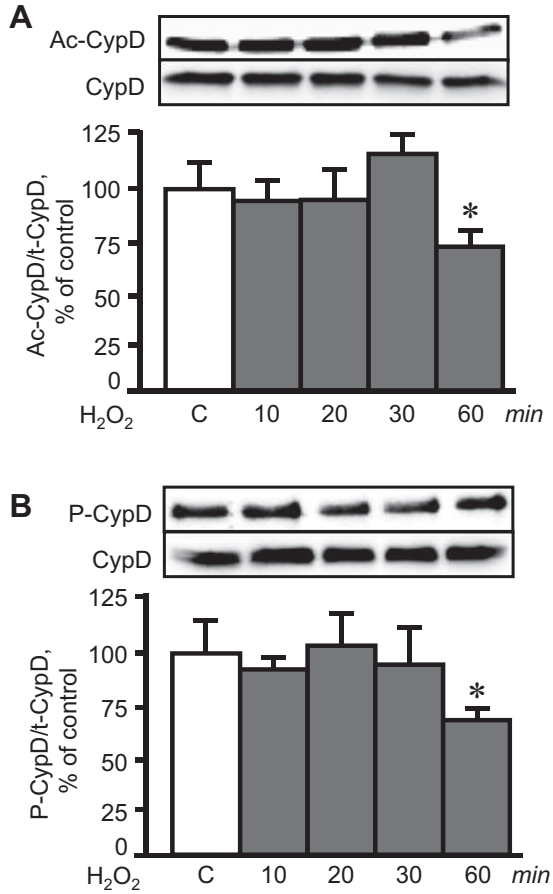

Fig. 7. Time-dependent changes of acetylation and phosphorylation of CypD in response to oxidative stress. *A*: CypD acetylation. *B*: CypD phosphorylation. Cell were treated with 100 μM H_2O_2 and taken for protein analysis at indicated time points. *$P < 0.05$ vs. C; $n = 3$ to 4 per group.

oxide synthase activity (7). Metformin promoted phosphorylation of nitric oxide synthase through AMPK activation in cultured cardiomyocytes (20, 39). Nitric oxide can improve mitochondrial function by opening mitochondrial ATP-sensitive K^+ channels via the cGMP-protein kinase G pathway and inhibiting complex I.

We observed a small, but statistically significant, increase in mtDNA abundance in cells exposed to H_2O_2. This increase in mtDNA abundance could represent a compensatory mechanism in response to damaged mtDNA. Previous studies revealed a PGC-1α-dependent increase of mtDNA abundance in response to aging (12). A similar mechanism may exist in response to the acute changes induced during H_2O_2 treatment. However, at higher H_2O_2 doses, we observed no increase in mtDNA abundance. This could be due to the deleterious effects of high H_2O_2 concentrations to inactivate the above proposed mechanism.

H_2O_2 treatment also induced extensive mtDNA damage in a concentration-dependent manner, and metformin abolished this effect (Fig. 8). Our DNA damage detection method does not allow the identification of the specific mtDNA lesions induced by the H_2O_2 treatment, but recent studies in H9c2 cells exposed to oxidative stress revealed high levels of 8-hydroxy 2′-deoxyguanine and apurinic/apyrimidinic sites in the mitochondrial genome (44). Furthermore, this study underlines the importance of mtDNA repair since mitochondrial overexpression of the base excision repair enzyme 8-oxoguanosine DNA glycosylase-1 decreased mtDNA lesions and improved mitochondrial function. The protective effect of metformin suggests that mtDNA damage is an important hallmark in the cascade of events induced by the acute oxidative stress treatment, which eventually culminates in cell death. Previous studies showed that metformin can reduce oxidative stress and DNA damage induced by paraquat in fibroblasts (1). Interestingly, it did not protect against H_2O_2-induced DNA damage in these cells. The discrepancy can be explained by differences in DNA damage detection methods employed; our method is focused exclusively on mtDNA damage versus the histone γH2AX phosphorylation method employed by the previous study, which detects exclusively nuclear DNA damage.

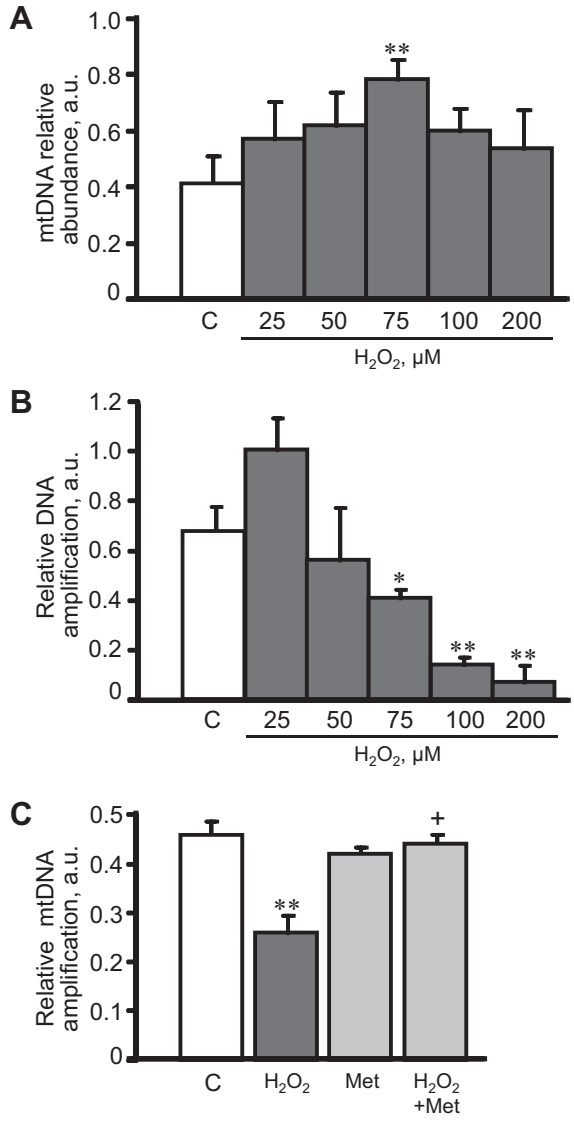

Fig. 8. Effects of oxidative stress on mtDNA abundance and damage in H9c2 cardiomyocytes in the presence or absence of Met. Cells were treated with varying concentrations of H_2O_2 for 1 h followed by DNA isolation and quantitative PCR analysis. *A*: mtDNA abundance. *B*: mtDNA damage. *C*: effect of Met on H_2O_2-induced mtDNA damage. Graphs represent the average of two PCR reactions performed in duplicate. AU, arbitrary units. *$P < 0.05$ and **$P < 0.01$ vs. C; +$P < 0.05$ vs. H_2O_2.

Overall, based on the present study, we propose the following mechanism to explain the protective effects of metformin-induced AMPK activation on mitochondria with acute oxidative stress. Metformin prevents oxidative stress-induced interactions between PPARα and CypD, thus blocking formation of the PTP. In addition to membrane potential, AMPK may reduce ROS production by increasing expression of mitochondrial SOD, although this is unlikely in acute oxidative stress (30). Further studies are required to clarify a cause-effect relationship between PPARα-CypD interaction and PTP opening under acute oxidative stress by using hypoxia-reoxygenation in primary cardiomyocytes and ischemia-reperfusion in intact hearts. Also, a cause-effect relationship between PPARα-CypD interaction and PTP opening should be addressed in future studies. It should be noted that we employed H9c2 cells which are undifferentiated myoblasts derived from the embryonic rat heart. They do not contract and exhibit several metabolic and functional properties of skeletal muscle. Although we have recently shown that H9c2 cells are similar to primary cardiomyocytes on energy metabolism features and can be successfully used as an in vitro model to study oxidative stress (31), some caution should be taken into consideration during the interpretation of present results.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

G.B.-T., J.S.H., S. Jang, and A.R.R.-M. performed experiments; G.B.-T., J.S.H., A.R.R.-M., C.A.T.-R., and A.G.B. analyzed data; G.B.-T. drafted manuscript; G.B.-T., J.S.H., S. Jang, A.R.R.-M., C.A.T.-R., and S. Javadov approved final version of manuscript; J.S.H., S. Jang, and C.A.T.-R. prepared figures; C.A.T.-R., A.G.B., and S. Javadov interpreted results of experiments; A.G.B. and S. Javadov edited and revised manuscript; S. Javadov conception and design of research.

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