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Cdk1, PKCδ and calcineurin-mediated Drp1 pathway contributes to mitochondrial fission-induced cardiomyocyte death

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ABSTRACT

Myocardial ischemia–reperfusion (I/R) injury is one of the leading causes of death and disability worldwide. Mitochondrial fission has been shown to be involved in cardiomyocyte death. However, molecular machinery involved in mitochondrial fission during I/R injury has not yet been completely understood. In this study we aimed to investigate molecular mechanisms of controlling activation of dynamin-related protein 1 (Drp1, a key protein in mitochondrial fission) during anoxia-reoxygenation (A/R) injury of HL1 cardiomyocytes.

A/R injury induced cardiomyocyte death accompanied by the increases of mitochondrial fission, reactive oxygen species (ROS) production and activated Drp1 (pSer616 Drp1), and decrease of inactivated Drp1 (pSer637 Drp1) while mitochondrial fusion protein levels were not significantly changed. Blocking Drp1 activity with mitochondrial division inhibitor mdivi1 attenuated cell death, mitochondrial fission, and Drp1 activation after A/R. Trolox, a ROS scavenger, decreased pSer616 Drp1 level and mitochondrial fission after A/R. Immunoprecipitation assay further indicates that cyclin dependent kinase 1 (Cdk1) and protein kinase C isoform delta (PKCδ) bind Drp1, thus increasing mitochondrial fission. Inhibiting Cdk1 and PKCδ attenuated the increases in pSer616 Drp1, mitochondrial fission, and cardiomyocyte death. FK506, a calcineurin inhibitor, blocked the decrease in expression of inactivated pSer637 Drp1 and mitochondrial fission.

Our findings reveal the following novel molecular mechanisms controlling mitochondrial fission during A/R injury of cardiomyocytes: (1) ROS are upstream initiators of mitochondrial fission; and (2) the increased mitochondrial fission is resulted from both increased activation and decreased inactivation of Drp1 through Cdk1, PKCδ, and calcineurin-mediated pathways, respectively.

1. Introduction

Ischemic heart disease is one of the leading causes of death and disability worldwide. Patients with myocardial infarction are particularly at risk of developing ischemia–reperfusion (I/R) injury. Ischemia with ATP depletion and calcium overload, and reperfusion with excessive production of reactive oxygen species (ROS) leads to mitochondrial permeability transition pore opening and subsequent cardiomyocyte death [1,2]. Several recent studies indicate that increased mitochondrial fission was directly involved in cardiomyocyte death. Following an I/R injury, a significant increase of mitochondrial fission was observed in adult rodent cardiomyocytes, while inhibition of mitochondrial fission reduced cardiomyocyte death [3,4]. However, the underlying mechanisms remain unclear.

Mitochondria are dynamic organelles. Mitochondria continuously move, fuse, and divide. Mitochondrial fusion and fission result in the change of mitochondrial shape: either elongated, tubular, interconnected mitochondrial networks, or fragmented and discontinuous mitochondria, respectively [5]. Multiple proteins were found to participate in controlling the dynamics of mitochondrial fission and fusion. The most important fission protein was dynamin-related protein-1 (Drp1), which was mainly located in cytosol, but upon activation translocated to mitochondrial scission sites [6]. Two major phosphorylation sites were found in Drp1. When Drp1 was phosphorylated at Ser616 residue, mitochondrial fission occurred, whereas phosphorylation at Ser637 blocked fission and induced fusion and elongation of mitochondria [7]. Mitochondrial fusion as the opposite phenomena,

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involved several GTPase proteins, which were mainly localized at mitochondrial membrane. Mitofusin (Mfn)1, 2, and optic atrophy 1 (Opa1) were major mitochondrial fusion proteins [8].

Changes of mitochondrial morphology have been linked to cell death [9]. The fragmented mitochondria could not sustain sufficient energy requirements of the cell. Frank et al. showed that upon addition of staurosporine, an apoptosis inducer, mitochondria changed from interconnected networks to a fragmented discrete punctiforme structures, a process which was dependent on the activation and translocation of Drp1 in fibroblast-like cell lines [10]. Ong et al. showed that by over-expressing Drp1K38A, a dominant negative form of Drp1, cell death during I/R injury of HL-1 cardiac cell lines could be reduced. They have also showed that blocking Drp1 reduced infarct size in adult murine heart after I/R injury, suggesting that Drp1 is an upstream mediator of cardiomyocyte death [4].

However, the underlying upstream molecular mechanisms by which anoxia-reoxygenation (A/R) injury induced an increase of

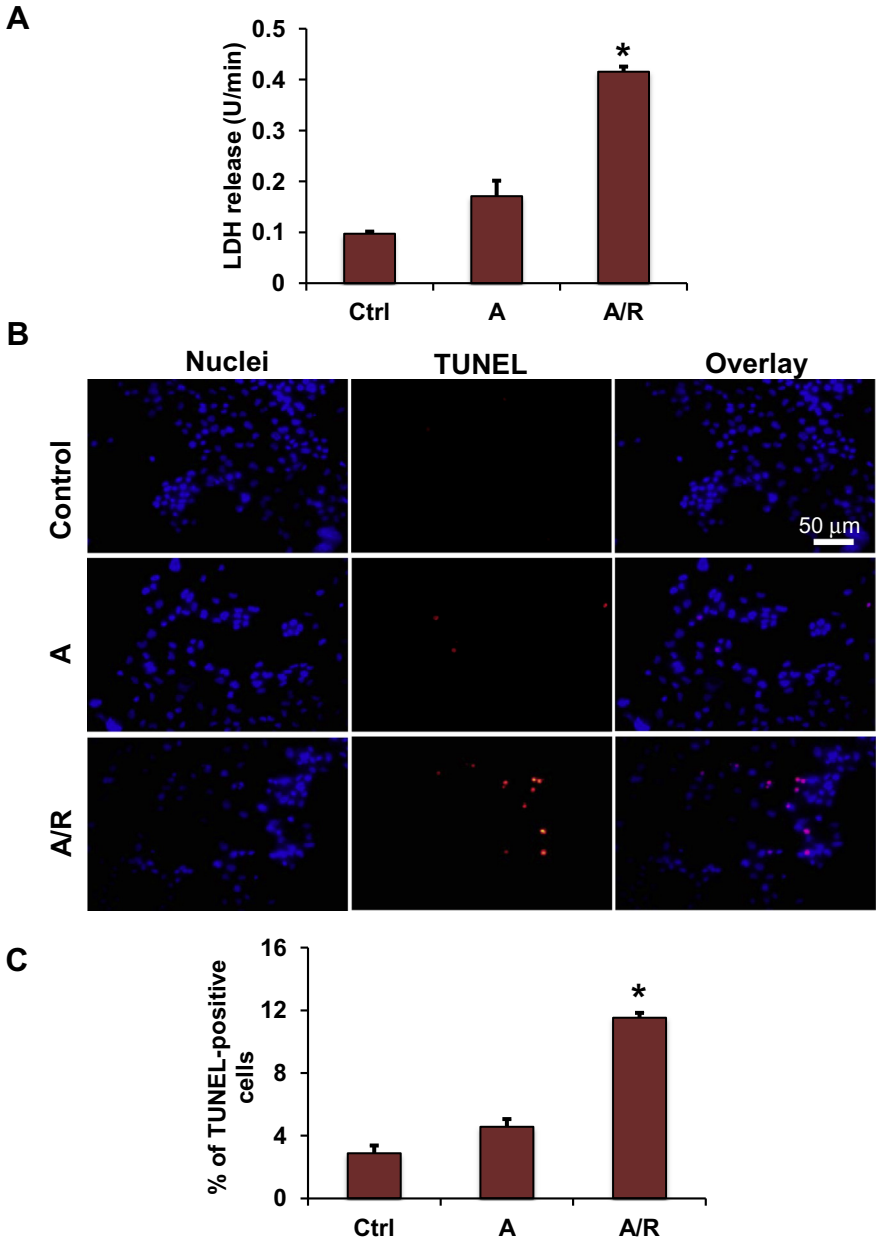


Fig. 1. A/R injury induces cardiomyocyte death and increases mitochondrial fission in cardiomyocytes. (A) A/R significantly induced cardiomyocyte death as evidenced by the increase of lactate-dehydrogenase (LDH) release from cells. (B and C) Confocal images of TUNEL staining (red) was used to identify DNA damage after A/R. Hoechst 33342 stains nuclei (blue). Overlaid images demonstrate that most of TUNEL signals were located in fragmented nuclei in A/R-treated cells. Significant increase in TUNEL-positive cells was observed in A/R-treated cells compared to the non-treated control (*$P < 0.001$ vs. Ctrl, $n = 3$). (D and E) Confocal images of mitochondria stained with TMRE show the elongated, branched, and interconnected mitochondrial networks in non-treated control compared to A/R-treated cells, which mostly displayed round, punctiforme and small mitochondria fragments. Quantification of mitochondria length and branching based on the confocal images of mitochondria showed that significant decreases of aspect ratio and form factor values were observed in A/R-treated cells (*$P < 0.001$ vs. Ctrl, $n = 4$). (F and G) Western blot assay demonstrates that a decrease in inactivated Drp1 (pSer637 Drp1) was observed in A and in A/R treated cells while an increase in activated Drp1 (pSer616 Drp1) levels was observed only in A/R-treated group (*$P < 0.05$ vs. Ctrl, $n = 4$). (H and I) Western blot analysis showed that there was no significant difference in fusion protein (MFN1, MFN2, and OPA1) levels between non-treated control, A and A/R-treated cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

activated Drp1 remain unclear. The aim of this study was to identify novel molecular mechanisms of mitochondrial fission-mediated cardiomyocyte death in order to better understand the importance of mitochondrial dynamics during reoxygenation injury in the ischemic heart. We hypothesized that A/R induces mitochondrial fission in cardiomyocytes through cyclin-dependent kinase 1 (Cdk1), protein kinase C isoform δ (PKCδ), and calcineurin-mediated Drp1 pathway.

2. Materials and methods

2.1. Cell culture

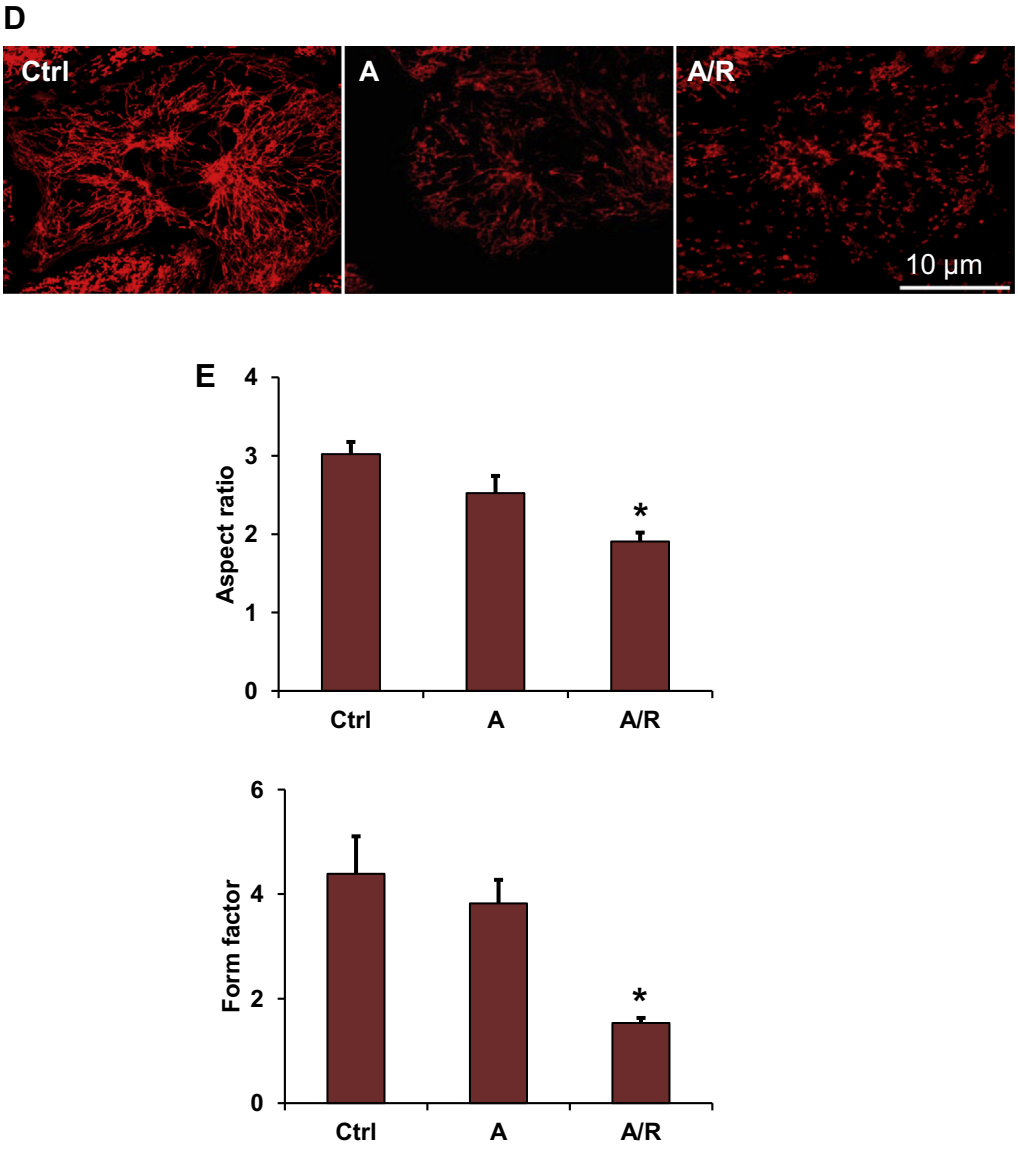
HL-1 cells, a cardiomyocyte line derived from the AT-1 mouse atrial myocyte tumor lineage were cultured in Claycomb media, supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin–streptomycin, 0.1 mM norepinephrine, 2 mM L-glutamine (Sigma–Aldrich, St. Louis, MO, USA) in humidified tissue culture incubator (20% O_2, 5% CO_2). Media was changed every 48 h and after reaching 100% confluency, cells were passaged with 0.05% trypsin–EDTA [11]. Passages 45–55 were used in this study.

2.2. A/R injury of cardiomyocytes in vitro

Prior to exposure to hypoxic chamber, all groups cardiomyocytes were switched to DMEM containing 10 mM glucose and 2% FBS for 1 h. Cardiomyocytes then underwent anoxia for 2 h by incubation in 5% CO_2 and 95% N_2 in an airtight chamber (Biospherix hypoxia chamber, Lacona, NY, USA) in the presence of FBS free DMEM containing 10 mM 2-deoxyglucose for inhibition of glycolysis. For reoxygenation, cells were switched to DMEM with 2% FBS and 10 mM glucose for 1 h. Roscovitine, FK506 (Sigma–Aldrich, St. Louis, MO, USA), mdivi1 (Drp1 inhibitor, Enzo Life Sciences, Farmingdale, NY, USA), and Trolox (a ROS scavenger, Sigma–Aldrich, St. Louis, MO, USA) were added prior to anoxia at the concentrations of 5, 1, 50, and 250 μM, respectively.

2.3. Lactate-dehydrogenase (LDH) release assay for cell viability

As an indicator of cellular membrane damage, LDH release in the medium was measured. Cardiomyocytes were plated in a 6-well plate (200,000 cells/well) and the A/R was performed after reaching 80% confluency. Following the reperfusion, LDH in the



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culture media was analyzed spectrophotometrically according to manufacturer instructions (Sekisui Diagnostics, San Diego, CA, USA) at a wavelength of 340 nm. LDH release was expressed as units per minute (U-min^{-1}).

2.4. TUNEL assay

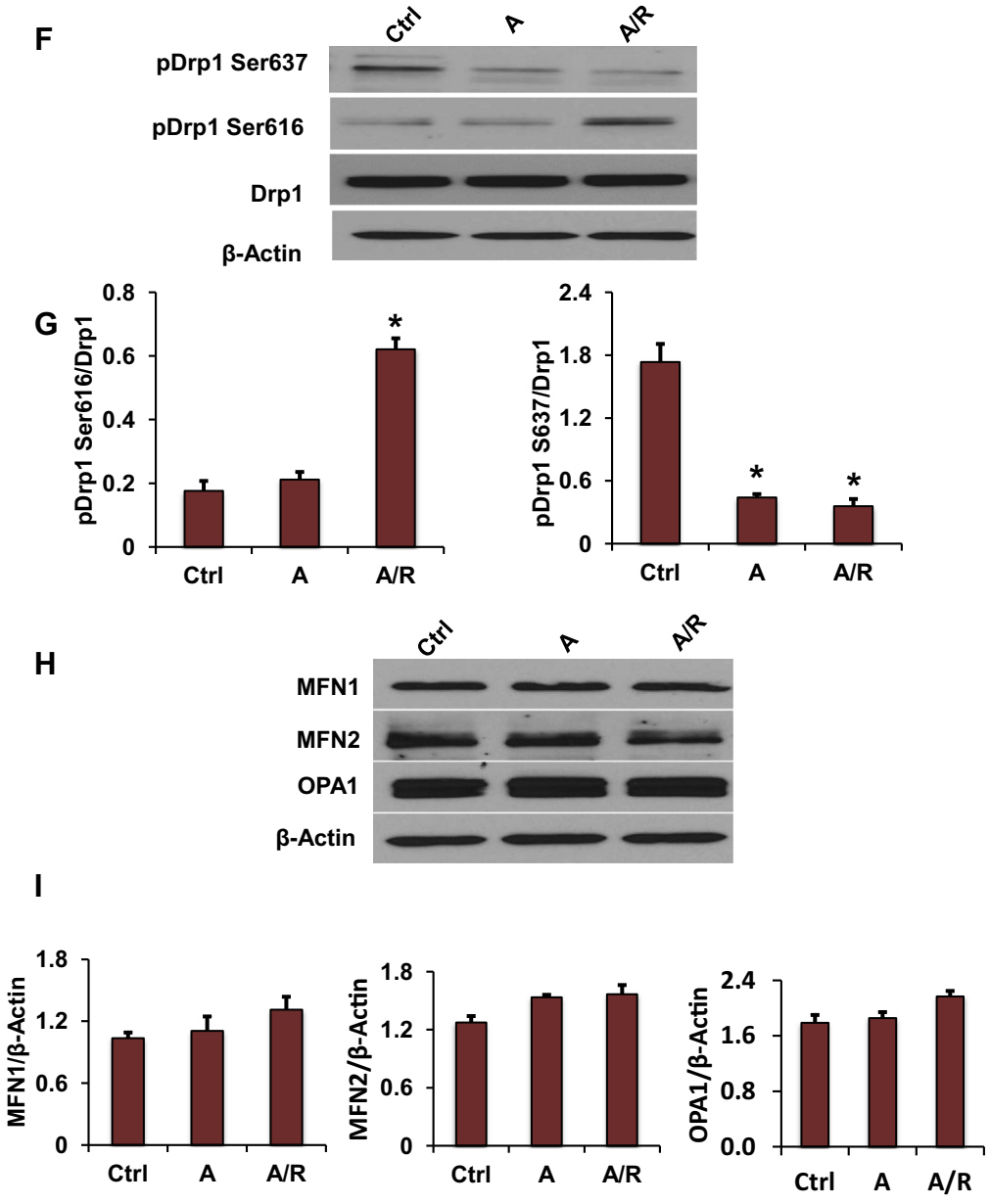
DNA fragmentation as a marker for apoptosis and to some extent necrosis was measured using TUNEL assay (Cell Death Detection Kit, Roche, Indianapolis, IN, USA) following the manufacturer's instructions. Coverslips were counterstained with Hoechst 33342 dye (Molecular Probes, Eugene, OR, USA) for nuclei labeling. For each coverslip five random fields of view were taken in three independent experiments. Apoptotic index was calculated as a percentage following formula: TUNEL-positive cells/number of total cells.

2.5. Small interfering RNA (siRNA) transfection

We used small interfering RNA (siRNA) targeted to PKCδ (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cardiomyocytes were transfected with siRNA or scrambled control using transfection reagent (Santa Cruz). After 72 h cell lysates were collected and efficiency of PKCδ knockdown was confirmed by Western blotting (S1). Knockdown efficiency was approximately 80%.

2.6. Mitochondrial morphology and network analysis

4×10^4 cells were plated on coverslips coated with Matrigel (BD Biosciences, San Jose, CA, USA). To visualize mitochondrial networks, cells were incubated in 30 nM tetramethylrhodamine ethyl ester (TMRE; Molecular Probes, Eugene, OR, USA), as previously described [12]. TMRE selectively accumulates inside of mitochondria. At the end of each treatment, images were captured with



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Nikon TE2000 inverted microscope (Nikon, Melville, NY, USA) and analyzed with ImageJ [12]. The morphology of each mitochondrion was described by two factors: aspect ratio (AR) and form factor (FF). Both factors have minimal value of 1, which represents a perfect circle. Higher values of AR and FF represent longer and more branched mitochondrial structures [13]. For each group five cells in the four independent experiments were analyzed.

2.7. ROS production measurement in cardiomyocytes

Cells were incubated in 2 μM 5-(and 6)-chloromethyl-2, 7-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA; Molecular Probes, Eugene, OR, USA) dissolved in DMEM for 30 min. Upon cleavage of the acetate groups by intracellular esterases and oxidation, nonfluorescent H_2DCFDA is converted to highly fluorescent 2′,7′-dichlorofluorescein (DCF). Images were analyzed using ImageJ. Fluorescence intensities were shown in arbitrary units (a.u).

2.8. Western blot

At the end of the each treatment cultured cells were washed with PBS and lysed using RIPA lysis buffer (Cell Signaling, Danvers, MA). Lysates were then centrifuged for 20 min at 14,000*g* and supernatant was collected. Proteins (30 μg/lane) were separated on SDS/PAGE gel and then transferred onto nitrocellulose membrane. Membranes were probed overnight with primary antibodies against phosphoSer616 Drp1 (pSer616 Drp1) and pSer637 Drp1 (Cell Signaling, Danvers, MA, USA), Drp1 (BD Transduction Laboratories, Lexington, KY, USA), β-Actin, MFN1, MFN2, OPA1 (Abcam,

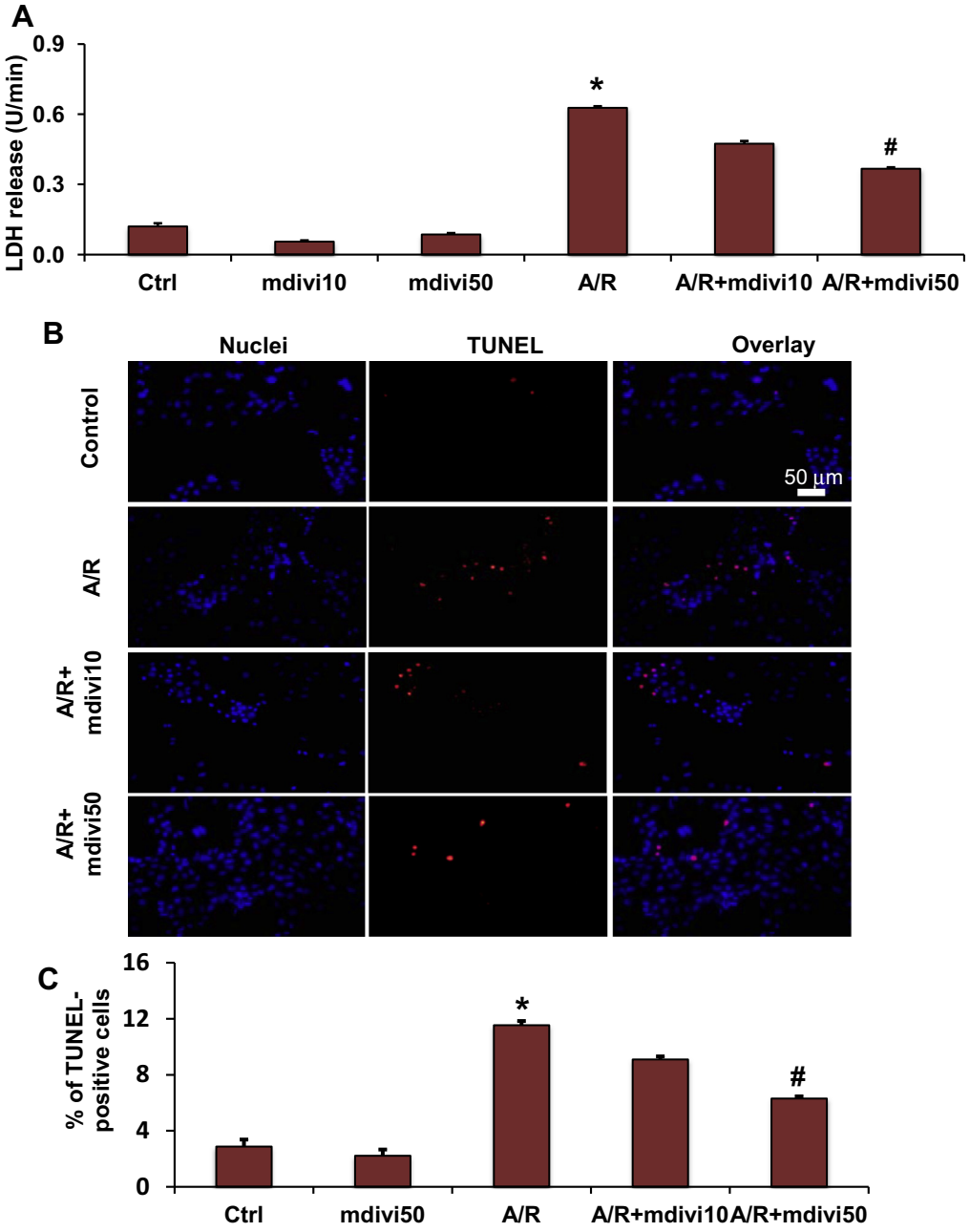


Fig. 2. Inhibiting mitochondrial fission reduces cardiomyocyte death after A/R. (A) Mdivi1, a Drp1 inhibitor, dose-dependently attenuated A/R-induced LDH release. (B and C) A significant increase in TUNEL-positive cells after A/R was attenuated with 50 μM mdivi1 treatment ($^{*}P < 0.001$ vs. Ctrl, $^{\#}P < 0.05$ vs. A/R, $n = 3$).

Boston, MA, USA), and PKCδ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by appropriate secondary antibodies conjugated to horseradish peroxidase. Bands were visualized using enhanced chemiluminescence (Pierce, Rockford, IL, USA) and intensities were analyzed using ImageJ 1.41. Data are shown in a.u.

2.9. Immunoprecipitation

Cells were washed in ice-cold PBS and lysed in RIPA buffer. Lysates were centrifuged for 20 min at 14,000*g*. Proteins (200 μg) in the supernatant from the cell lysates were incubated with Cdk1 or PKCδ antibodies (Santa Cruz) for 3 h at 4 °C followed by the incubation with protein A/G beads (Santa Cruz) overnight at 4 °C. Non-specific IgG (Santa Cruz) was used as control in the immunoprecipitation. Samples were then centrifuged for 5 min at 500*g*, supernatant was removed and pellet was heated for 5 min at 95 °C to break the bonds between beads and immunocomplexes. Input lysates, immunoprecipitation with IgG, Cdk1, or PKCδ were analysed by western blotting using antibodies specific to Drp1.

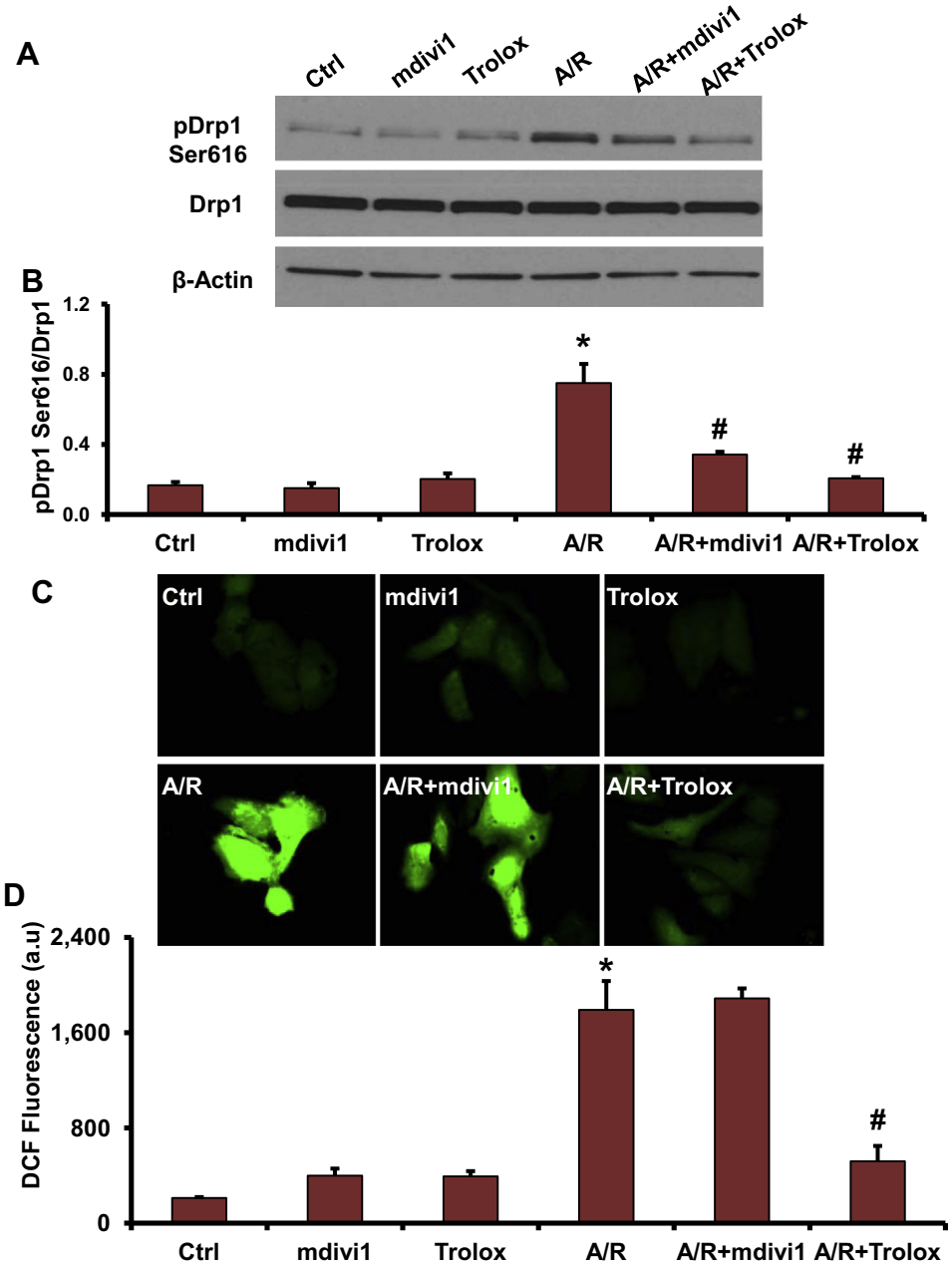


Fig. 3. An increase in reactive oxygen species (ROS) production during reoxygenation causes activation of Drp1. (A and B) An increase of pSer616 Drp1 after A/R was observed compared to non-treated control analyzed using Western blot. Mdivi1 partially and Trolox completely abolished the increase in the level of activated pSer616 Drp1 after A/R ($^{*}P < 0.001$ vs. Ctrl, $^{\#}P < 0.05$ vs. A/R, $n = 3$). (C and D) ROS production was analyzed through confocal images of the cells stained with ROS-sensitive dye CM-H2DCFDA. A significant increase in DCF fluorescence (indicating ROS production) in A/R-treated group was abolished if cells were treated with ROS scavenger Trolox ($^{*}P < 0.001$ vs. Ctrl, $^{\#}P < 0.001$ vs. A/R, $n = 3$). (E–H) pSer637 Drp1 expression was decreased after A/R compared to non-treated control. Pretreatment with calcineurin inhibitor FK506 during A/R attenuated the decrease of pSer637 Drp1 after A/R while Trolox did not influence pSer637 Drp1 expression ($n = 3$). (I and J) Confocal images of mitochondria stained with TMRE show that that pretreatment of the cells with mdivi1, Trolox, or FK506 attenuated A/R-induced mitochondrial fission as evidenced by the increased aspect ratio and form factor values compared to A/R alone group ($^{*}P < 0.05$ vs. Ctrl, $^{\#}P < 0.05$ vs. A/R, $n = 4$).

2.10. Statistical analysis

Data are presented as mean ± SEM; *n* indicates number of independent experiments. Statistical comparison was performed using Student's *t*-test or one-way analysis of variance with Tukey's post hoc test where appropriate. *P* values <0.05 were considered as statistically significant.

3. Results

3.1. A/R injury induces cardiomyocyte death

We exposed cells to 2 h of anoxia followed by 1 h of reoxygenation. A/R injury significantly increased cardiomyocyte death evidenced by the increase in LDH release and TUNEL-positive cells. Anoxia alone did not induce cardiomyocyte death compared to non-treated control (Fig. 1A–C).

3.2. A/R injury induces mitochondrial fission

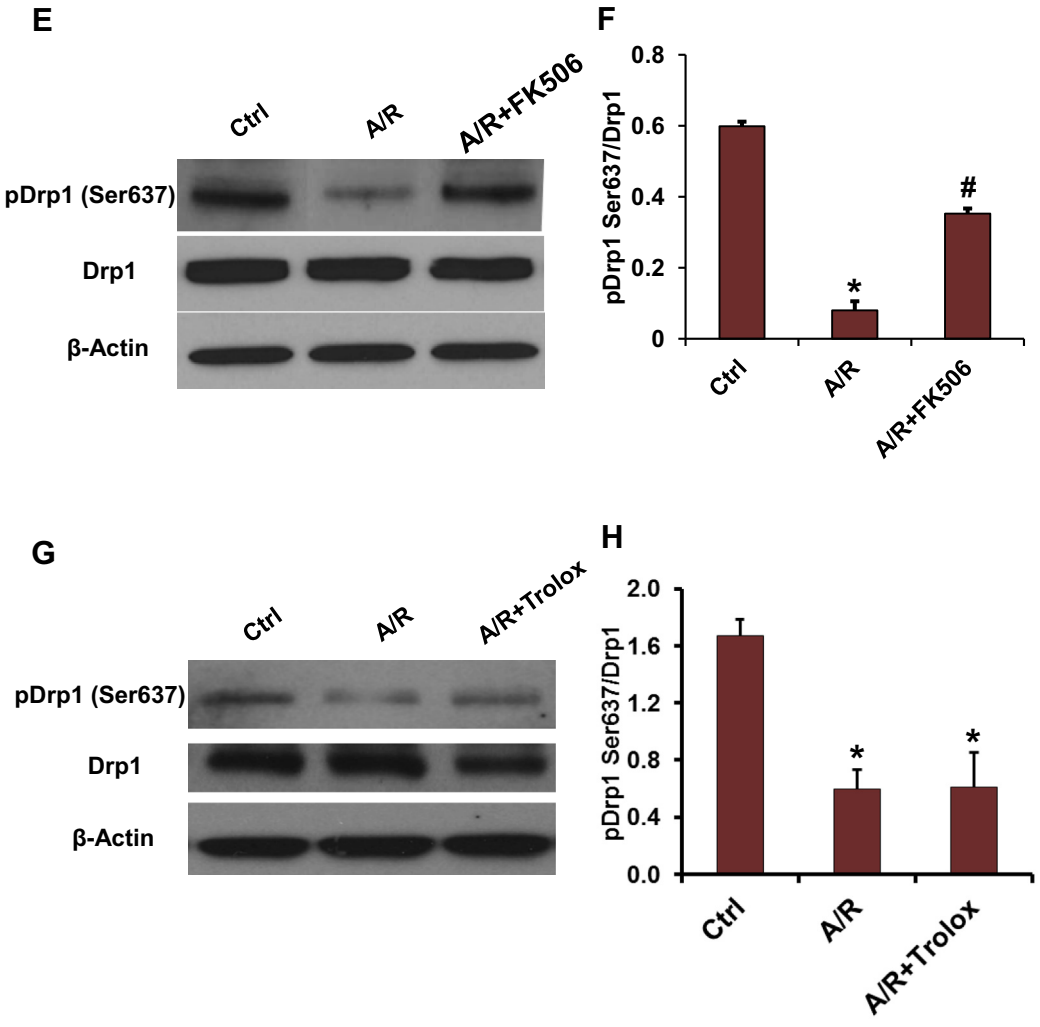
In order to visualize changes in morphology of mitochondria during A/R, cells were stained with TMRE. Confocal images showed the elongated, branched, and interconnected mitochondrial structures in the non-treated control cardiomyocytes. During anoxia there was no significant increase in mitochondrial fission observed, however, small, round, punctiforme mitochondria were dominated in the A/R cardiomyocytes. To quantify structural changes of mitochondria, two factors were used; AR and FF. A/R injury significantly decreased AR and FF values (Fig. 1D and E), suggesting that A/R induces shift of fusion-fission balance and mitochondrial fission becomes a predominant process.

3.3. A/R injury induces an increase in mitochondrial fission through Drp1 activation but does not alter the mitochondrial fusion-related protein expression

Western blotting revealed that A/R injury induced Drp1 activation measured both as increase in activated pSer616 Drp1 and decrease in inactivated pSer637 Drp1 (Fig. 1F and G). Significant increase of pSer616 Drp1 was observed at the end of reoxygenation, while decrease in expression levels of inactivated pSer637 was observed at the end of anoxia and reoxygenation period. Mitochondrial fusion involves several large GTPase proteins located on the mitochondrial outer and inner membrane (MFN1, MFN2, and OPA1). The expression of these proteins did not significantly change during A/R (Fig. 1H and I).

3.4. Blocking mitochondrial fission attenuates the A/R injury-induced cardiomyocyte death

Cells were pretreated with different concentrations of Drp1-specific inhibitor, mitochondrial division inhibitor, or mdivi1,



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for 1 h and then exposed to A/R injury. Significant decreases in LDH release and in number of TUNEL-positive cells were observed in 50 μM mdivi1 group (Fig. 2), suggesting that Drp1-involved mitochondrial fission contributes to A/R-induced cardiomyocyte death.

3.5. Increased production of ROS causes Drp1 activation while activation of calcineurin induces decreases of inactivated Drp1 during reoxygenation

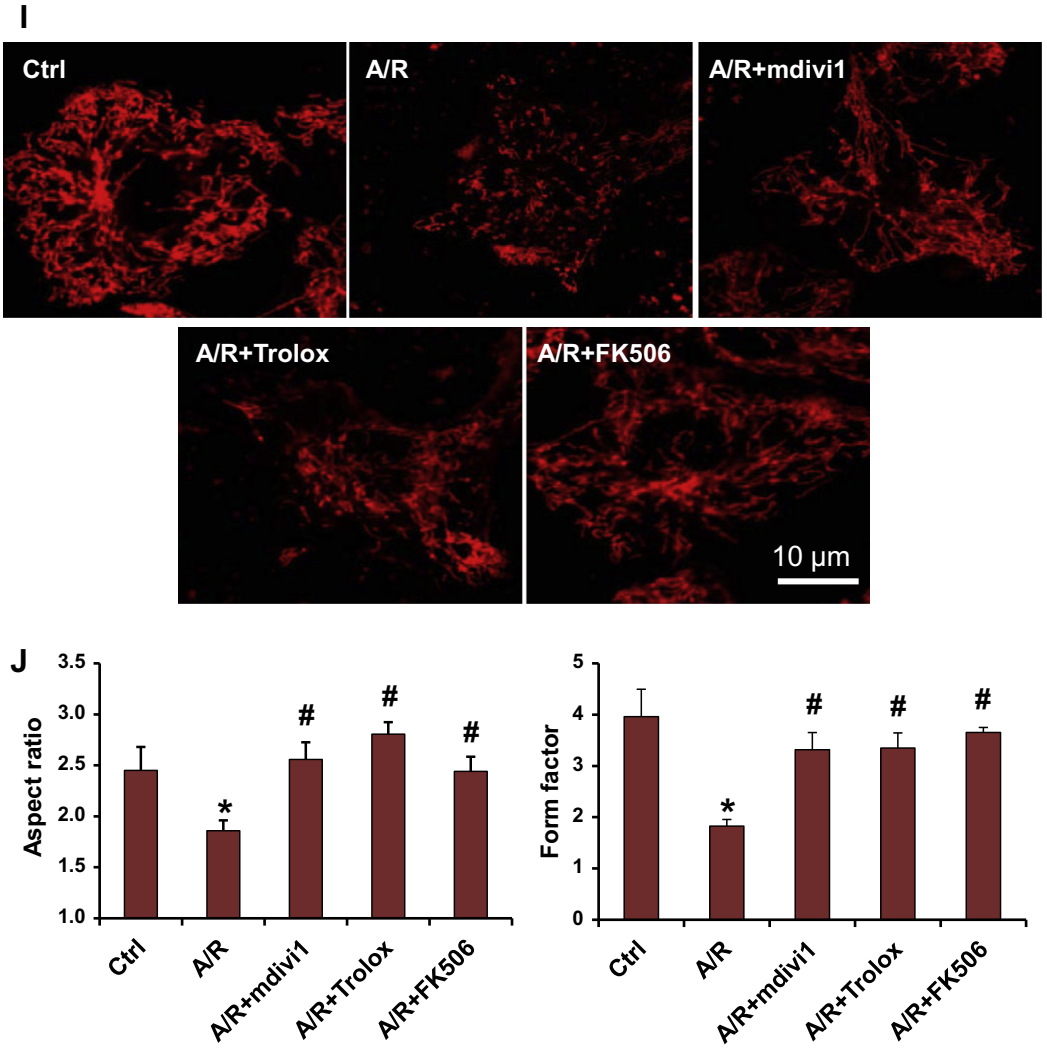
During reoxygenation, ROS overproduction occurs. In order to dissect the event order of ROS production and mitochondrial fission during A/R injury, cardiomyocytes were pretreated with mdivi1 and ROS scavenger, Trolox. Both mdivi1 and Trolox reduced pSer616 Drp1 (Fig. 3A and B). In addition, Trolox decreased ROS production during reoxygenation, while mdivi1 did not have any effect on ROS production (Fig. 3C and D). To determine the upstream signals that decreased the inactivation of pSer637 Drp1, the cardiomyocytes were pretreated with FK506, a calcineurin blocker. FK506 but not Trolox significantly attenuated the A/R-induced decrease in pSer637 Drp1 (Fig. 3E–H). Confocal images showed that in the mdivi1-, Trolox-, and FK506-treated cultures, the mitochondrial shape and branches were similar to those in no-treatment control compared with A/R group. In addition, the A/R-induced decrease of AR and FF values was attenuated with mdivi1, Trolox, and FK506 pretreatment (Fig. 3I and J). These data suggest that these blockers prevent the increased mitochondrial fission conferred by A/R and further confirm that ROS and calcineurin are the upstream initiators of Drp1 activation (pSer616 Drp1) and Drp1 inactivation (pSer637 Drp1), respectively.

3.6. PKCδ and Cdk1 mediate an increase in Drp1, mitochondrial fission, and cardiomyocyte death conferred by A/R injury

Our immunoprecipitation results showed that both Cdk1 and PKCδ interacted with Drp1 and that interaction was increased during A/R injury (Fig. 4A). Blocking PKCδ with PKCδ siRNA (Fig. 4B) or Cdk1 with roscovitine partially reduced increase in pSer616 Drp1 during A/R injury. Blocking both PKCδ and Cdk1 during A/R abolished an increase in pSer616 Drp1 and mitochondrial fission, and attenuated cardiomyocyte death (Fig. 4C–G), confirming that PKCδ and Cdk1 are major kinases responsible for Drp1 activation.

4. Discussion

In the present study, we have demonstrated that A/R injury induced mitochondrial fission in cardiomyocytes. In particular, we confirmed that blocking Drp1 with mdivi1 reduced A/R injury-induced cardiomyocyte death. In addition, we presented



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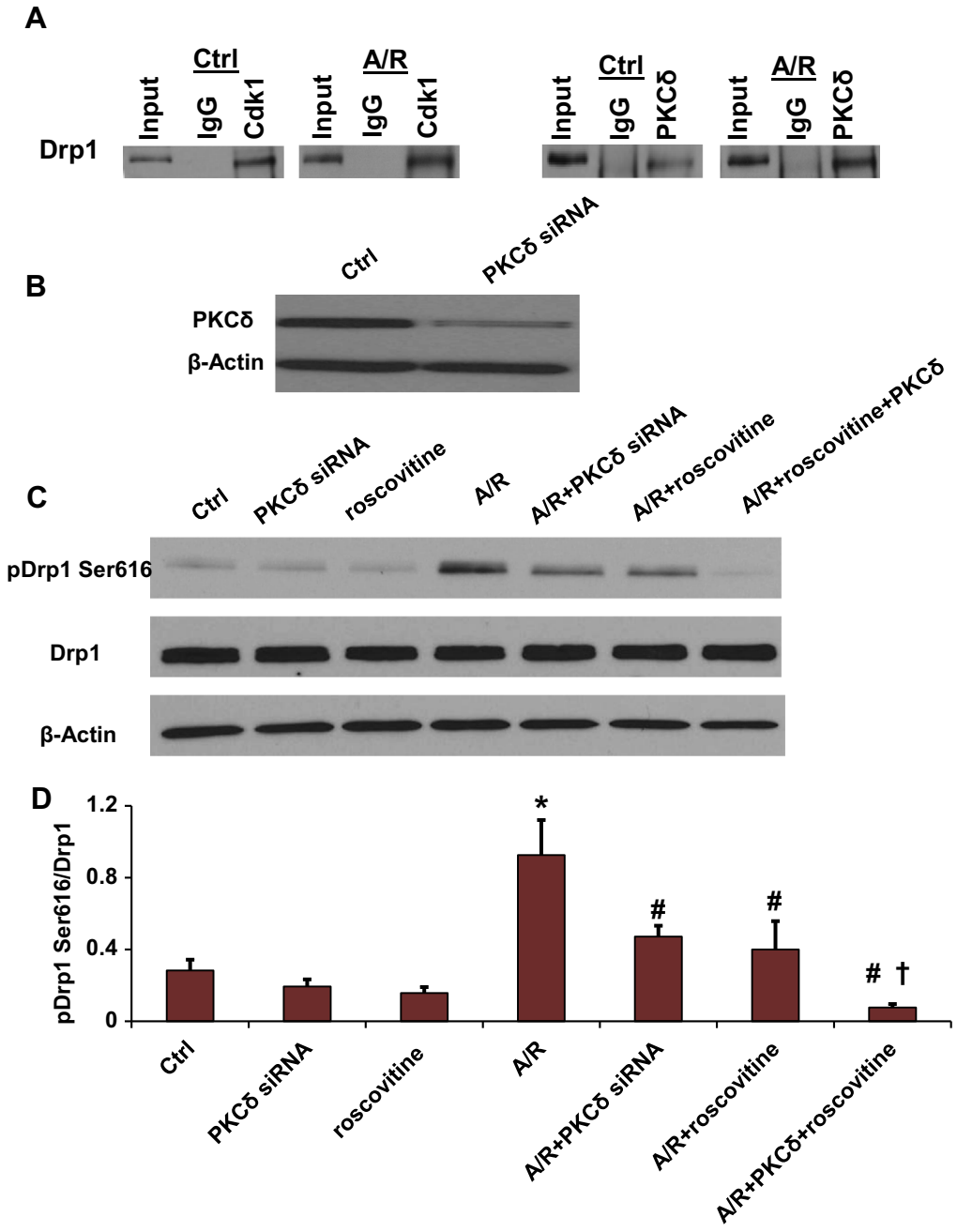
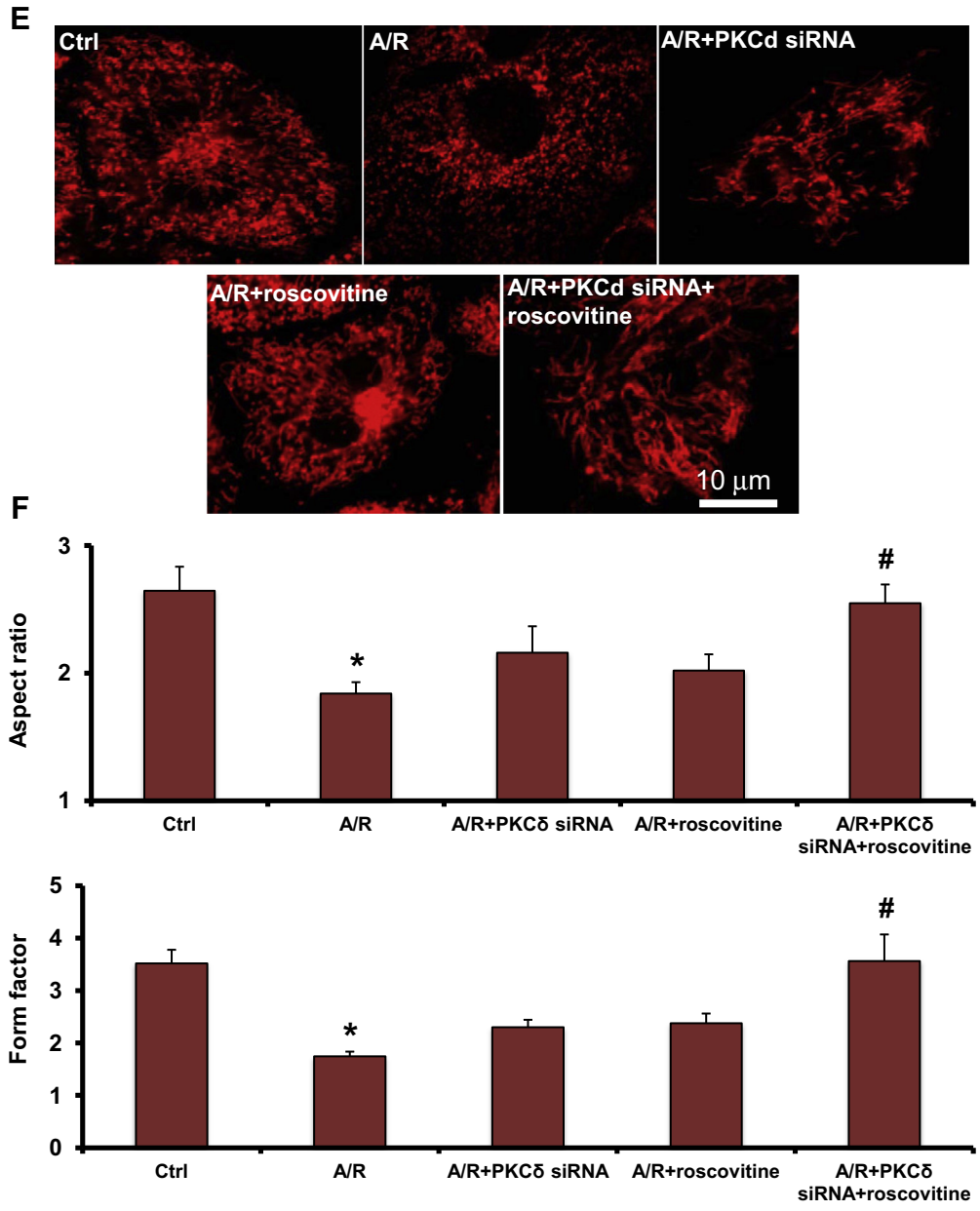


Fig. 4. A/R injury induces an increase in the expression of pSer616 Drp1 through PKCδ- and Cdk1-mediated pathway in cardiomyocytes. (A) Input lysates, nonspecific IgG, co-immunoprecipitated PKCδ or Cdk1 were detected by western blotting using an anti-Drp1. Both Cdk1 and PKCδ interacted with Drp1 and these interactions were increased in A/R injury. (B) PKCδ siRNA knockdown efficiency in the cardiomyocytes. (C and D) There was a significant increase of pSer616 Drp1 after A/R compared to non-treated control analyzed using Western blot. When added separately, PKCδ siRNA or roscovitine, partially reduced the increase in expression levels of pSer616 Drp1. Adding both PKCδ siRNA and roscovitine during A/R completely abolished the increase of pSer616 Drp1 ($^{*}P < 0.001$ vs. Ctrl, $^{\#}P < 0.05$ vs. A/R, $^{\dagger}P < 0.05$ vs. A/R + PKCδ siRNA and A/R + roscovitine, $n = 5$). (E) Representative confocal images of mitochondria stained with TMRE. (F) Mitochondria fission was evaluated through the analysis of confocal images of mitochondria stained with TMRE. Significant decreases of aspect ratio and form factor values were observed in A/R-treated cells, indicating the increased mitochondrial fission. Treatment of the cells with PKCδ siRNA, roscovitine or both attenuated A/R-induced increases of mitochondria fission and increased aspect ratio and form factor values compared to A/R alone group ($^{*}P < 0.05$ vs. Ctrl, $^{\#}P < 0.05$ vs. A/R, $n = 3$). (G) PKCδ siRNA or Cdk1 inhibitor roscovitine significantly attenuated LDH release in cardiomyocytes during A/R ($^{*}P < 0.001$ vs. Ctrl, $^{\#}P < 0.05$ vs. A/R, $n = 3$).

the first experimental evidence showing that (1) A/R injury-induced mitochondria fission that was associated with Drp1 activation measured as both increased expression of activated pDrp1 Ser616 and decreased expression of inactivated pDrp1 Ser637 levels. The activation of Drp1 was partially blocked with mdivi1 and completely with Trolox; and (2) the increase in pDrp1 Ser616 after A/R was regulated by two major serine-threonine kinases: Cdk1 and PKCδ. Similarly, dephosphorylation at Ser637 of Drp1 during A/R could be blocked with calcineurin inhibitor FK506.

Recent studies have shown that the increase in mitochondrial fission was associated with various pathological processes, including ischemic heart disease [4] and Drp1 played a pivotal role in cardiomyocyte damage. For instance Cribbs and Stack et al. showed that β-adrenergic stimulation by isoproterenol caused activation



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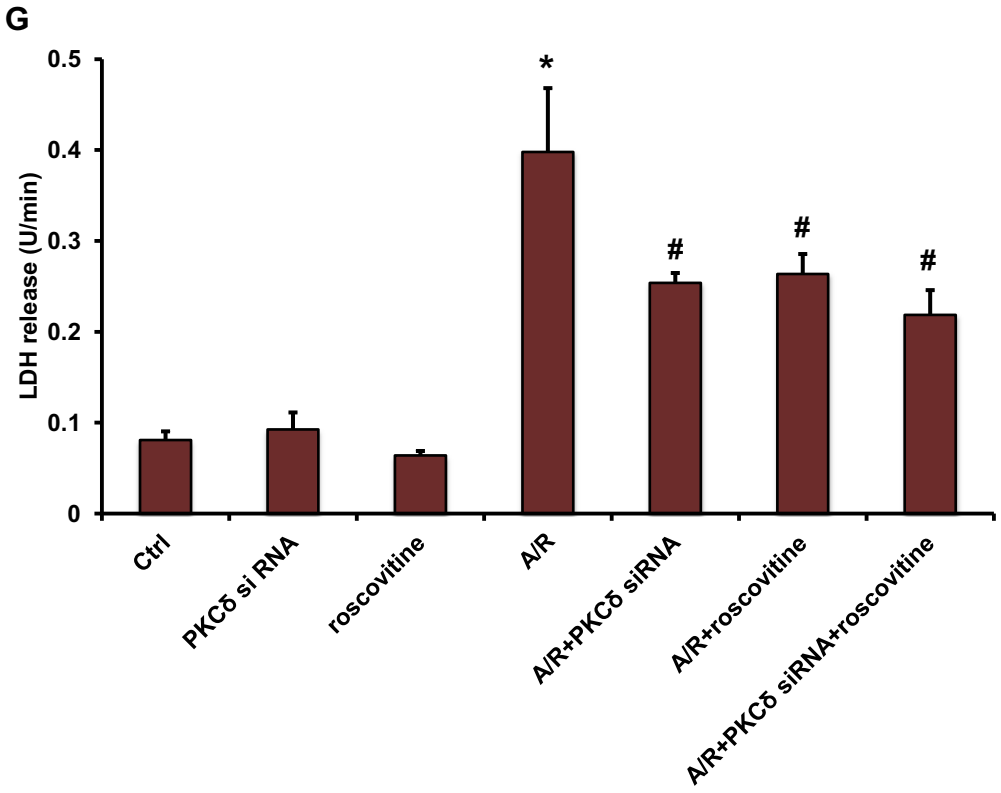
of mitochondrial fission through changes in phosphorylation status of Drp1 in adult murine heart [7]. In the present study, we had similar observation showing that the A/R-induced cardiomyocyte was accompanied with the increased mitochondrial fission (Fig. 1). The increased mitochondria fission during reoxygenation was well correlated with an increase of activated Drp1 pSer616 and a decrease of inactivated Drp1 pSer637. Both the increase in pDrp1 Ser616 and the decrease of pDrp1 Ser637 have been shown to induce Drp1 translocation to scission sites of mitochondrial outer membrane in HeLa cells [14]. Thus increased pDrp1 Ser616 and decreased pDrp1 Ser637 may be involved in the A/R-induced cardiomyocyte death.

Although overexpression of mitochondrial fusion proteins has been shown to increase the resistance to I/R injury-induced stress [4], our study also showed that expression levels of major proteins involved in this process MFN1, MFN2 and OPA1 did not significantly change after A/R (Fig. 1), indicating that these fusion-related proteins are unlikely to play significant roles during cardiomyocyte damage. However, blocking activation of the major mitochondrial fission protein Drp1 with mitochondrial division inhibitor mdivi1 attenuated cardiomyocyte death (Fig. 2), further confirming the importance of the increased mitochondrial fission in A/R injury of cardiomyocytes.

Accumulating evidence has shown that reperfusion after prolonged ischemia is necessary to salvage the ischemic myocardium, but paradoxically triggers the immediate overproduction of ROS due to increased activity of mitochondrial electron transport chain and disrupted intracellular Ca^{2+} gradients [15]. Our data showed the importance of ROS in A/R-induced cardiomyocyte death. Significant increase in ROS production was observed in the cells at reperfusion stage (Fig. 3). Trolox, a vitamin E analog and antioxidant, completely reduced ROS overproduction, pDrp1 Ser616 expression levels, and mitochondrial fission. Mdivi1 was ineffective in reducing ROS overproduction during reoxygenation, but dose dependently reduced mitochondrial fission and cell death (Fig. 3), suggesting that ROS mediates the increased mitochondrial fission



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through Drp1. Similar signaling pathways were also discovered in endothelial cells, ROS scavengers blocked Drp1-dependant mitochondrial fission and conferred protection against I/R-induced endothelial cell injury, and laser irradiation-induced Drp1 translocation to mitochondria [16].

Previous studies suggest that δ isoform of PKC, a novel calcium-insensitive PKC isoenzyme is activated at onset of reperfusion and that ROS precede PKCδ activation [17,18]. Cdk, a key protein kinase regulating cell cycle progression and apoptosis, is influenced by ROS and activated during the period of ischemia [19]. Activation of PKCδ and Cdk resulted in an increase in phosphorylation of downstream effectors raising the possibility of the association with Drp1 activation and subsequent mitochondrial fragmentation. Recently, PKCδ has been implicated to phosphorylate Drp1 at Ser616 residue during oxidative stress in neurons [20]. However, there is no report of the interplay between these two kinases and Drp1 in the cardiomyocytes. In the current study, we found the direct interactions between PKCδ and Drp1, as well as Cdk1 and Drp1, in the HL1 cardiomyocytes confirmed by immunoprecipitation (Fig. 4). In addition, PKCδ siRNA and Cdk inhibitor roscovitine attenuated the increase in phosphorylation of Drp1 at Ser616, Drp1-mediated mitochondrial fission, and cardiomyocyte death (Fig. 4), suggesting that following reperfusion, ROS induces an increase in pDrp1 Ser616 through regulation of two upstream serine-threonine kinases: Cdk1 and PKCδ.

Decrease in pDrp1 Ser637 was mediated by calcineurin but not ROS (Fig. 3). Recent study showed the importance of calcineurin-mediated mitochondrial fission in rat tubular kidney cell death. Phosphorylation at Ser637 residue by protein kinase A caused Drp1 inactivation and blocked translocation of Drp1 to mitochondrial scission sites, whereas, dephosphorylation at Ser637 by calcineurin induced Drp1 mitochondrial fission. Blocking calcineurin reduced the dephosphorylation of Drp1 at Ser637 during ATP depletion in renal rat tubular cells [21], conferred powerful protection and reduced cell death.

In summary, mitochondrial structural integrity plays an important role in cardiomyocyte death during A/R injury. We presented for the first time that A/R injury induced mitochondrial fission through ROS signaling pathway, which is involved in the activation of PKCδ, Cdk1, and calcineurin, followed by the increased levels of activated Drp1 and the decreased levels of inactivated Drp1. These findings contribute to a better understanding of the underlying molecular mechanisms of the increased mitochondrial fission-mediated cardiomyocyte death, further providing a useful strategy to interfere with the activation of Drp1 to protect cardiomyocytes against A/R-induced cell death.

Acknowledgments

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