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Exogenous Ubiquitin Attenuates Hypoxia/Reoxygenation-Induced Cardiac Myocyte Apoptosis via the Involvement of CXCR4 and Modulation of Mitochondrial Homeostasis

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Abstract

Exogenous ubiquitin (UB) plays a protective role in β-AR-stimulated and ischemia/reperfusion (I/R)-induced myocardial remodeling. Here, we report that UB treatment inhibits hypoxia/reoxygenation (H/R)-induced apoptosis in adult rat ventricular myocytes (ARVMs). Activation of Akt was higher, while activation of GSK-3β was lower in UB-treated cells post-H/R. Oxidative stress was lower, while the number of ARVMs with polarized mitochondria was significantly greater in UB-treated samples. ARVMs express CXCR4 with majority of CXCR4 localized in the membrane fraction. CXCR4 antagonism using AMD3100, and siRNA-mediated knockdown of CXCR4 negated the protective effects of UB. Two mutated UB proteins (unable to bind CXCR4) had no effect on H/R-induced apoptosis, activation of Akt and GSK-3β or oxidative stress. UB treatment enhanced mitochondrial biogenesis, and inhibition of mitochondrial fission using mDivi1 inhibited H/R-induced apoptosis. *Ex vivo*, UB treatment significantly decreased infarct size and improved functional recovery of the heart following global I/R. Activation of caspase-9, key player of mitochondrial death pathway, was significantly lower in UB-treated hearts post-I/R. UB, most likely acting via CXCR4, plays a protective role in H/R-induced myocyte apoptosis and myocardial I/R injury via the modulation of mitochondrial homeostasis and mitochondrial death pathway of apoptosis.

Keywords

Apoptosis; CXCR4; Heart; Myocytes; Ubiquitin

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Disclosures

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Introduction

Coronary heart disease is a leading cause of death worldwide. The effects of coronary heart disease are generally correlated with myocardial ischemia/reperfusion (I/R) injury (Powers et al. 2007; Hausenloy and Yellon, 2013). I/R injury induces oxidative stress and cardiac myocyte apoptosis (Tanaka et al. 1994; Kang et al. 2000; Kim et al. 2006). Mitochondria are considered as a major source of reactive oxygen species (ROS) and an increase in ROS causes mitochondrial dysfunction including a loss in mitochondrial membrane potential (Kalogeris et al. 2014; Zorov et al. 2014). Despite advances in understanding the molecular and cellular mechanisms that regulate the damage caused by I/R injury, alleviating myocardial I/R injury remains a challenge.

Ubiquitin (UB), a highly conserved protein of ~8.5 kDa, is found in all eukaryotic cells. The most important intracellular function of UB is to regulate protein turnover by the UB-proteasome pathway (Goldberg, 2003). The UB-proteasome pathway is suggested to modulate hypertrophic response, apoptosis, and tolerance to I/R injury in cardiac myocytes (Zolk et al. 2006). UB is a normal constituent of plasma. Elevated levels of UB are described in the serum or plasma of patients with a variety of diseases, and extracellular UB is proposed to play an important role in modulation of inflammatory response, neuroprotective activity and apoptosis (Majetschak, 2011; Scofield et al. 2015). C-X-C chemokine receptor (CXCR4) plays an important role in a variety of biological processes, and is suggested to be involved in the pathophysiology of various disease processes, such as cancer, HIV, ischemic myocardial injury, and angiogenesis (Tsou et al. 2018). Stromal derived factor-1α (SDF-1α) is the cognate ligand for CXCR4 which is implicated in homing of hematopoietic stem cells during wound repair (Döring et al. 2014). Interestingly, CXCR4 is identified as a receptor for UB in THP1 leukemia cell line (Saini et al. 2010). Evidence has been provided that UB-CXCR4 interaction follows a two-site binding mechanism in which the hydrophobic surfaces surrounding Phe-4 and Val-70 are important for receptor binding (Saini et al. 2011).

Previous work from our lab has shown that stimulation of β-adrenergic receptor (β-AR) increases extracellular levels of UB, and treatment of adult rat ventricular myocytes (ARVMs) with UB inhibits β-AR-stimulated apoptosis (Singh et al. 2010). *In vivo*, exogenous UB decreased β-AR-stimulated increases in cardiac myocyte apoptosis and myocardial fibrosis (Daniels et al. 2012). It also decreased inflammatory response and preserved heart function 3 days post-I/R injury (Scofield et al. 2019). In cardiac microvascular endothelial cells, UB treatment enhanced expression of angiogenic molecules and angiogenesis (Steagall et al. 2014). UB/CXCR4 axis also modulated phenotype and function of cardiac fibroblasts (Scofield et al. 2018). Cardiac myocyte apoptosis is a considered as a pivotal form of cell death in I/R injury (Xia et al. 2016). Hypoxia/reoxygenation (H/R) is generally used as an *in vitro* model to simulate myocardial I/R injury. It is shown to induce apoptosis in myocytes (Yang et al. 1999; Song et al. 2008). A major objective of this study was to investigate the role of UB/CXCR4 axis in cardiac myocyte apoptosis following H/R injury. It was hypothesized that exogenous UB plays a protective role in H/R-induced myocyte apoptosis and myocardial I/R-induced injury, and anti-apoptotic effects of UB occur via the involvement of CXCR4 and mitochondrial death pathway. The data presented here suggest that UB/CXCR4 axis activates Akt (anti-apoptotic

kinase), decreases oxidative stress, helps maintain mitochondrial membrane potential and decreases myocyte apoptosis during H/R-induced model of injury. In isolated heart model, UB treatment decreased infarct size, improved functional recovery and decreased caspase-9 activation following global I/R in the heart.

2. Methods and Materials

Vertebrate Animals:

All animal procedures were reviewed and approved by the East Tennessee State University Institutional Committee on Animal Care and use. The study conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male Sprague-Dawley rats (average wt: 150-200 g) were purchased from Envigo (Indianapolis, IN). Male C57BL/6 mice (8 to 10 week-old) were purchased from Jackson Laboratories (Bar Harbor, ME). Rats and mice were anesthetized using a mixture of isoflurane (2.5%) and oxygen (0.5 l/min), and the heart was excised following a bilateral cut in the diaphragm. Animals were euthanized by exsanguination.

Cardiac myocyte isolation, treatment and hypoxia/reoxygenation:

Calcium-tolerant ARVMs were isolated from the hearts of adult male Sprague-Dawley rats (150-200 g) as described (Dalal et al. 2014; Dalal et al. 2016). ARVMs were cultured overnight in Dulbecco's modified Eagle's medium (DMEM; Mediatech) supplemented with 25 mM HEPES, 0.2% albumin, 5 mM creatine, 2 mM L-carnitine, 5 mM taurine, and 0.1% penicillin-streptomycin at a density of 30–50 cells/mm^2 on 100-mm tissue culture dishes or coverslips (Fisher Scientific) precoated with laminin (1 μg/cm^2). After changing the medium, cells were treated with AMD3100 (AMD; 10 μM) or mDivi1 (1 μM) for 30 min followed by treatment with UB (10 μg/mL; Sigma) or mutated UB proteins (mV70A and mF4A; 10 μg/mL; Boston Biochem) for 30 min. To simulate I/R, ARVMs were incubated at 37°C with 5% CO_2 and 0.1% O_2 in a hypoxic chamber (Pro-Ox Model C21, BioSpherix Ltd, Redfield, NY) for 2.5 h. After changing the media containing the corresponding treatments, ARVMs were then incubated at 37°C with 5% CO2 (reoxygenation) for indicated time points.

CXCR4 siRNA knockdown:

To knockdown CXCR4, ARVMs were washed with siRNA transfection medium (Santa Cruz Biotech) and transfected with CXCR4 siRNA (a mixture of 4 target-specific siRNAs; 80 nM; Santa Cruz Biotech) or negative control sequence siRNA (neg-siRNA; 80 nM; Santa Cruz Biotech) using INTERFERin transfection reagent (Polyplus) at 37°C. Following 5 h of incubation, 1 mL of serum free DMEM supplemented with antibiotics was added to the dishes. The cells were then incubated for 24 or 48 h. The cell lysates were analyzed by Western blot using anti-CXCR4 antibodies. For TUNEL-staining assay, transfected cells (24 h) were incubated with UB for 30 min followed by hypoxia (2.5 h) and reoxygenation (18 h).

Mitochondrial biogenesis:

To investigate if UB induces mitochondrial biogenesis, ARVMs were pretreated with UB (10 μg/mL) for 30 min followed by hypoxia (2.5 h) and reoxygenation (18 h). Total cellular DNA (nuclear and mitochondrial) was extracted using GeneJet DNA Kit (ThermoFisher Scientific). Total DNA (10 ng) was amplified for mitochondrial genes cytochrome b (CytB) and NADH oxidase 1 (ND1), and nuclear gene peptidyl-prolyl isomerase A (PPIA) using assay primers and Taqman Universal Master mix II with UNG (ThermoFisher Scientific). Primers sets for ND1 (Rn03296764_s1) and CytB (Rn03296746_s1) and PPIA (RN00690933_m1; a housekeeping gene) were obtained from ThermoFisher Scientific. Real-time quantitative PCR amplification was performed using a CFX96 Real Time System (Bio-Rad). Quantification of mitochondrial DNA content was calculated as the ratio of relative Cq values of CytB and ND1 with PPIA.

Apoptosis:

To detect apoptosis, ARVMs were incubated in a hypoxic chamber for 2.5 h, followed by 18 h of reoxygenation. TUNEL-staining assay was performed using *in situ* death detection kit according to the manufacturer's instructions (Roche Molecular Biochemicals). Hoechst 33258 (10 μM; Sigma, St Louis, MO) staining was used to localize TUNEL-positive nuclei. The percentage of TUNEL-positive cells (relative to total ARVMs) was measured by counting ~200 cells in 10 randomly chosen fields per coverslip for each experiment.

Measurement of mitochondrial membrane polarization:

ARVMs were plated on 50-mm MatTek dishes (MatTek, Ashland, MA) and incubated for 30 min in 3 mL of culture medium containing 200 nM TMRM (tetramethylrhodamine methyl ester) at 37°C and 5% CO_2 (Wu et al. 2013). The culture media was then substituted with PBS containing 5 mM glucose and 10 mM succinate with or without UB. After placing a cover slip over the cells, 15 μl of sterile oxyrase was added. Oxyrase selectively removes oxygen creating depletion of oxygen in the immediate surroundings of ARVMs. Images of cells were captured every 15 min using the Zeiss AxioCam MRm monochrome digital camera and analyzed with the Zeiss AxioVision 4.8.2 software. The number of polarized cells (exhibiting TMRM fluorescence) was counted at each time point and the data is expressed as a percentage of TMRM-positive cells prior to anoxia (basal).

ROS/superoxide detection:

The number of ROS-positive cells was determined using the Total ROS/superoxide detection kit (Enzo Life Sciences). The kit is designed to directly monitor real time ROS/superoxide production in live cells using fluorescent microscopy. For this, UB or mutated UB treated cells were exposed to 2.5 h of hypoxia followed by reoxygenation for 1 h. Cells were loaded with ROS/superoxide-responsive fluorescence probes for 1 h during reoxygenation. After washing with PBS, cells were visualized and counted using a fluorescent microscope. ROS-positive cells exhibit green fluorescence, while superoxide-positive cells exhibit red fluorescence. The percentage of ROS- and superoxide-positive cells (relative to total ARVMs) was determined by counting cells in 10 randomly chosen fields per coverslip for each experiment.

Western Analysis:

Cell lysates were prepared using lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.5% Nonidet P-40, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride]. Left ventricular (LV) lysates were prepared in RIPA buffer [10 mM Tris-HCl (pH 7.2), 158 mM NaCl, 1 mM EGTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride] using a tissue homogenizer. Equal amounts of total proteins (20 or 60 μg) were resolved on 10% SDS-polyacrylamide gels. The proteins were transferred onto PVDF membrane. The blots were then probed with primary antibodies directed against p-Akt (1:1000 dilution; Cell Signaling Tech.), p-GSK-3β (1:1000 dilution; Cell Signaling Tech.), CXCR4 (1:1000 dilution; Abcam), caspase-9 (1:1000 dilution; Millipore), and appropriate secondary antibodies. Membranes were then stripped and probed with Akt, GSK-3β (cell Signaling Tech.) or GAPDH (Santa Cruz Biotech.) antibodies to normalize protein loading. Band intensities were quantified using Kodak photo documentation system (Eastman Kodak Co.).

Ex vivo myocardial I/R (Langendorff) injury:

Global I/R experiments were performed in isolated mouse hearts as described (Wu et al. 2013). Briefly, the aorta was quickly cannulated and the heart was perfused ratrogradely using Krebs buffer containing: 118.5 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 24.8 mM $NaCHO_3$, 2.5 mM $CaCl_2$, and 10.6 mM glucose. The buffer was equilibrated with 95% O_2 and 5% CO_2, and maintained at 37°C. A fluid-filled silicon balloon was inserted in the left ventricle through the mitral valve for the measurement of left ventricular developed pressure (LVDP) using a pressure transducer (AD Instruments, Dunedin, New Zealand). Hearts were allowed to stabilize during a 20 min baseline period followed by 25 min of ischemia and 40 min of reperfusion. UB (10 μg/mL in Krebs buffer) treatment was started within 5 min prior to the starting of the ischemia. I/R hearts received Krebs buffer throughout the procedure. Immediately at the end of reperfusion, hearts were used for TTC staining to measure infarct size or snap frozen to prepare tissue lysates.

Infarct size:

Infarct size was measured using 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) staining. Each heart was sliced transversely to yield five slices. The slices were incubated in 1% TTC for 15 min at 37°C. Infarct size was measured using Bioquant image analysis software and calculated as the percentage of total white area divided by the total tissue area.

Statistical analysis:

Data are expressed as the mean ± SEM. Data were analyzed using Student's *t* test or a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Probability (P) values of <0.05 were considered to be significant.

Results

UB treatment inhibits H/R-induced apoptosis in ARVMs:

Measurement of apoptosis using TUNEL assay showed that H/R significantly increases the number of apoptotic ARVMs vs cells incubated under normoxic conditions (Control; CTL; Fig 1). The number of apoptotic ARVMs was significantly lower in UB+H/R samples vs H/R alone. Pretreatment with AMD3100 (AMD; an inhibitor of CXCR4) significantly negated the protective effects of UB (CTL, 3.3±0.7; H/R, 35.5±6.3*; UB+H/R, 9.2±2.2$; AMD+UB+H/R, 34.1±4.1*#; AMD+H/R, 24.3±5.0*; *p<0.05 vs CTL; $p<0.05 vs H/R; #p<0.05 vs UB+H/R; n=3-5; Fig 1A). Mutated UBs (mV70A or mF4A; unable to interact with CXCR4) had no effect on H/R-mediated increase in apoptosis (Fig 1B).

CXCR4 expression and siRNA-mediated knockdown:

In human leukemia cells, CXCR4 is identified as a receptor for extracellular UB (Saini et al. 2010). To investigate if ARVMs express CXCR4, cells were treated with UB for 24 h. Western blot analyses of total cell lysates, and cytosolic and membrane fractions showed expression of CXCR4 in ARVMs (Fig 2A) with majority of protein localized in the membrane fraction. UB treatment did not affect the expression or localization of CXCR4.

To investigate the role of UB/CXCR4 axis, ARVMs were transfected with CXCR4 siRNA. Western blot analysis of cell lysates using anti-CXCR4 antibodies showed ~50 and ~70% decrease in CXCR4 protein levels 24 and 48 h after CXCR4 siRNA transfection, respectively (Fig 2B). Non-targeting siRNA (neg siRNA) served as a negative control. Analysis of apoptosis using TUNEL-assay showed that knockdown of CXCR4 using siRNA significantly negates the protective effects of UB with respect to apoptosis (Fig 2C). Raw data from 4 different biological replicates is shown in supplementary data Figure S1.

Activation of Akt and GSK-3β:

PI3-kinase activates Akt, and activation of Akt plays an anti-apoptotic role (Kennedy et al. 1997). Western blot analysis of cell lysates using phospho-specific anti-Akt antibodies showed a significant increase in Akt phosphorylation (activation) in UB+H/R group vs control and H/R groups. AMD pretreatment significantly decreased UB-mediated increase in Akt phosphorylation (fold change vs CTL; H/R, 1.03±0.2; UB+H/R, 2.45±0.4$; AMD+UB +H/R, 1.06±0.2#; $P<0.05 vs CTL and H/R; #P<0.05 vs UB+H/R; n=3-7; Fig 3A). Treatment with mutated UB (mV70A) had no effect on Akt phosphorylation (Fig 3B).

Activation of GSK-3β plays a pro-apoptotic role in β-AR-stimulated apoptosis via the involvement of mitochondrial death pathway (Menon et al. 2007). Phosphorylation of an NH_2-terminal serine residue (Ser^9) inactivates GSK-3β. Akt is one of the upstream kinases involved in phosphorylation (Ser^9) and inactivation of GSK-3β (Hardt and Sadoshima, 2002). Western blot analysis of cell lysates using phospho-specific (Ser^9) anti-GSK-3β antibodies showed increased phosphorylation (inactivation) of GSK-3β in UB+H/R group when compared to CTL and H/R groups. Pretreatment with AMD significantly decreased UB-mediated increase in GSK-3β phosphorylation (Fig 4A), while treatment with mutated UB (mV70A) had no effect on GSK-3β phosphorylation (Fig 4B). Western blot images used

for Akt and GSK-3β signal intensities are presented in supplementary data Figures S2 and S3, respectively.

UB decreases the number of ROS- and superoxide-positive myocytes:

ROS are implicated in ischemic or hypoxic-toxicity with and without reperfusion (Levrand et al. 2006; Abramov et al. 2007). To determine the effect of UB on oxidative stress, we measured the number of ROS- and superoxide-positive ARVMs following H/R. H/R increased the number of ROS-positive ARVMs when compared to CTL. The UB+H/R group showed a significant decrease in total number of ROS-positive ARVMs vs H/R alone. Treatment with mutated UBs (mV70A and mF4A) had no effect on the H/R-mediated increase in the number of ROS-positive cells (number of ROS-positive cells; CTL, 7.2±2.3; H/R, 54.2±7.3*; UB+H/R, 11.6±2.6$; mF4A+H/R, 52.0±5.1*; mV70A+H/R, 57.9±3.0*; *p<0.05 vs CTL; $P<0.05 vs H/R; n=3-5; Fig 5A).

Reperfusion is shown to increase superoxide radical production in isolated ischemic hearts (Yasmin et al. 1997; Levrand et al. 2006). As expected, H/R increased the number of superoxide-positive cells. Pretreatment with UB showed a significant decrease in superoxide-positive ARVMs vs H/R alone. Treatment with mutated UBs (mV70A and mF4A) had no effect on the H/R-mediated increase in the number of superoxide-positive cells (CTL, 5.6±1.3; H/R, 20.7±3.4*; UB+H/R, 7.6±2.8$; mF4A+H/R, 24.6±2.8*; mV70A +H/R, 22.3±5.2*; *P<0.05 vs CTL; $P<0.05 vs H/R; n=3-5; Fig 5B).

UB preserves mitochondrial membrane potential:

H/R induces mitochondrial damage, including loss of membrane potential (Dhar-Mascareño et al. 2005). To investigate if UB treatment protects mitochondrial polarization, ARVMs were subjected to anoxia. Mitochondrial polarization was monitored with TMRM potentiometric dye for 105 min under these conditions. We found a trend towards maintained mitochondrial polarization 60 min after UB treatment. However, the number of ARVMs with polarized mitochondria was significantly greater in UB-treated samples 105 min after UB treatment (Fig 6).

UB affects mitochondrial homeostasis:

Mitochondrial homeostasis is mainly regulated by mitochondrial fission/fusion and biogenesis (Friedman and Nunnari, 2014). Mitochondrial fission is regulated by dynamin-related protein-1 (DRP-1; a GTPase). To investigate the role of mitochondrial fission in H/R-induced apoptosis, ARVMs were pretreated with mdivi1 (1 μM; DRP-1 inhibitor) for 30 min followed by H/R. TUNEL-staining assay showed that pretreatment with mdivi1 significantly inhibits H/R-induced myocyte apoptosis (Fig 7A).

Analysis of mitochondrial biogenesis showed that H/R does not affect mitochondrial DNA content as measured by the ratio of relative Cq values of ND1 and CytB with PPIA. However, UB alone and pretreatment with UB followed by H/R significantly enhanced the DNA content of ND1 and CytB (Fig 7B&C).

UB decreases infarct size and improves functional recovery of the heart post-I/R:

To investigate if UB treatment modulates global I/R injury, isolated hearts were subjected to 25 min of global ischemia followed by 40 min of reperfusion in the presence or absence of UB. TTC staining showed a significant decrease in % infarct size in UB+I/R hearts versus I/R alone (% Infarct size; I/R, 39.5±6.9; UB I/R, 13.7±2.8*; *p<0.05 vs I/R; n=4-5; Fig 8A). LVDP is a measure of cardiac performance in langendorff perfused hearts. Percent LVDP recovery following ischemia was higher in UB+I/R group vs I/R (% LVDP recovery; I/R, 50.7±4.4; UB I/R, 67.3±4.3*; *p<0.05 vs I/R; n=6; Fig 8B).

Activation of caspase-9 in I/R hearts:

Pro-apoptotic caspase-9 is activated during I/R injury (Liu, 2018). Whole Western blot image is presented in supplementary data Figure S4. Presence of ~17 kDa protein on Western blot represents cleaved (active) caspase-9. Western blot analysis of heart homogenates showed decreased levels of active caspase-9 in UB+I/R hearts vs I/R (I/R, 1.63±0.27; UB+I/R, 0.98±0.08*; *p<0.05 vs I/R; n=4; Fig 9).

Discussion

A major finding of this study is that exogenous UB plays a protective role against H/R-induced myocytes apoptosis and global myocardial I/R-induced injury. The data presented here demonstrate that - 1) treatment of ARVMs with UB inhibits H/R-induced apoptosis; 2) ARVMs express CXCR4, and CXCR4 antagonism using AMD3100 and knockdown using siRNA negate the anti-apoptotic effects of exogenous UB, while two mutated UB protein, unable to bind to CXCR4, had no effect on H/R-mediated increase in apoptosis; 3) UB activates Akt (anti-apoptotic kinase), and inhibits H/R-mediated activation of GSK-3β (pro-apoptotic kinase); 4) UB inhibits H/R-mediated increase in the ROS and superoxide production, while maintaining mitochondrial membrane potential; 5) UB treatment induces mitochondrial biogenesis; and 6) exogenous UB decreases infarct size, improves heart function and decreases caspase-9 activation in an *ex vivo* model of global myocardial I/R injury.

Cardiac myocyte apoptosis is identified as an important process in the progression to heart failure (van Empel et al. 2005). Myocyte death due to apoptosis and necrosis contributes significantly to myocardial I/R injury (Scarabelli et al. 2002). Normal serum contains low levels of UB. Levels of UB increase in serum during a variety of pathological conditions (Majetschak, 2011; Scofield et al. 2015). UB treatment is shown to prolong skin graft survival in a mouse model (Earle et al. 2006). It also reduced lung edema formation and improved lung function during a lung I/R injury (Garcia-Covarrubias et al. 2008). In the heart, UB treatment preserved I/R-mediated decrease in heart function (Scofield et al. 2019). Previously, we have shown that stimulation of β-AR increases extracellular levels of UB in ARVMs, and treatment with UB inhibits β-AR-stimulated myocyte apoptosis *in vitro* as well as *in vivo* (Singh et al. 2010; Daniels et al. 2012). The data presented here provide evidence for the first time that UB decreases H/R-induced myocyte apoptosis. UB treatment also inhibited activation of pro-apoptotic protein, while decreasing infarct size and improving the functional recovery of heart in an *ex vivo* model of cardiac I/R injury. It is interesting to note

that UB alone, without superimposed I/R stress, does not affect myocyte apoptosis. This is consistent with our previous observation where anti-apoptotic effects of UB were only observed in the presence of β-AR stimulation (Singh et al. 2010). These data suggest that UB may serve as a survival factor during stress in the heart. However, the effects of UB on cell survival and apoptosis may be cell-type and/or tissue specific. Hematopoietic cells secrete UB and exogenous UB inhibits colony formation of normal myeloid and erythroid hematopoietic progenitor cells (Taniguchi et al. 1989; Daino et al. 1996). UB treatment alone inhibited the growth and survival of a number of hematopoietic cell lines through the induction of apoptosis (Daino et al. 2000).

In leukemia cell line, CXCR4 is identified as a receptor for UB (Saini et al. 2010). The hydrophobic surfaces surrounding Phe-4 and Val-70 play an important role UB/CXCR4 interaction (Saini et al. 2011). Evidence has been provided for the interaction of UB with CXCR4 in cardiac fibroblasts and microvascular endothelial cells (Steagall et al. 2014; Scofield et al. 2018). The data presented here suggest that ARVMs express CXCR4 with majority of the protein localized in the membrane fraction. Using CXCR4 antagonist, siRNA knockdown and two different UB mutants, we provide evidence for the involvement of CXCR4 in UB signaling and apoptosis. Use of two different UB mutants (F4A and V70A) further supports the significance of Phe-4 and Val-70 in UB/CXCR4 interaction. It should be noted that CXCR4 can act as a homodimer or heterodimer with CXCR7 (Levoye et al. 2009), β2-AR (LaRocca et al. 2010) or α1A-AR (Bach et al. 2014; Evans et al. 2016). Future investigations are needed to explore the possible involvement of these receptors in UB signaling.

Low to moderate levels of superoxide are produced during ischemia, followed by a burst of ROS production at the onset of reperfusion (Li and Jackson, 2002; Becker, 2004). Cardiac myocyte sarcolemma contains numerous uniport and antiport ion channels along with active transport mechanisms that are required to maintain cellular pH and membrane potential (Piper et al. 2004; Solaini and Harris, 2005). Mitochondria serve as the final arbitrators of life or death during an I/R insult. These organelles are not only required to produce ATP, but they also play a major role in cell survival and death (Green and Kroemer, 2004). Free radicals are produced within minutes of reperfusion and continue to be generated for hours after the restoration of blood flow to ischemic tissue (Bolli et al. 1989). Oxidant-mediated cellular injury can lead to damage in the cell membrane, impairment of the cell's ability to regulate ionic homeostasis, and contribute to mitochondrial injury, leading to a decrement in oxidative phosphorylation and opening of the mPTP (Li and Jackson, 2002). Mitochondrial dysfunction is a major contributor of myocyte loss during myocardial I/R injury. Mitochondrial damage and leakage of cytochrome c activates caspase-9 (Gogvadze et al. 2006). Caspase-9 is shown to be activated during ischemia and remains activated throughout reperfusion in the intact rat heart (Scarabelli et al. 2002). In cardiac myocytes, CXCR4 overexpression attenuates H/R-induced mitochondrial dysfunction (Cai et al. 2015). Inhibition of GSK-3β attenuates I/R injury by inhibiting mitochondrial permeability transition pore (mPTP) opening (Zhai et al. 2011). Previously, we have shown that GSK-3β plays a pro-apoptotic role in β-AR-stimulated apoptosis via the involvement of mitochondrial death pathway (Menon et al. 2007). UB treatment inhibited β-AR-stimulated activation of GSK-3β and apoptosis in vitro and in vivo (Singh et al. 2010; Daniels et al.

2012). Akt, a central regulator of myocyte survival, phosphorylates and inactivates GSK-3β (Hardt and Sadoshima, 2002). Here we show that UB treatment decreases caspase-9 activation in the heart following I/R. *In vitro*, UB treatment decreases the number of ROS- and superoxide-positive ARVMs during H/R. It also enhanced the number of ARVMs with polarized mitochondria during anoxic conditions. UB activated Akt, while inhibiting H/R-mediated increase in GSK-3β. CXCR4 antagonist, AMD3100, inhibited the protective effects of UB, while UB mutants (unable to bind CXCR4) had no effect. These data support the notion that UB/CXCR4 axis may help maintain normal mitochondrial function during myocardial I/R injury via the involvement of AKT/GSK-3β pathway/s.

Mitochondria change their morphology by undergoing either fusion or fission (Dimmer and Scorrano, 2006; Hausenloy and Scorrano, 2007). They can also undergo mitophagy and biogenesis. The fine balance between mitochondrial fusion/fission and biogenesis may be disrupted by stress factors, thereby predisposing the cell to mitochondrial death pathway and apoptosis (Frank et al. 2001; Hausenloy and Yellon, 2003). In fact, inhibition of mitochondrial fission is shown to protect the heart against I/R injury (Ong et al. 2010). Mitochondrial fission is regulated by DRP-1 (a GTPase). In cells of non-cardiac origin, recruitment of DRP-1 to mitochondria and subsequent fission is suggested to be regulated by the release of Ca^{++} from the endoplasmic reticulum (ER) and its uptake by mitochondria (Germain et al. 2005). Here, we show that inhibition of DRP-1 using mDivi1 inhibits H/R-induced apoptosis in ARVMs suggesting that H/R induces mitochondrial fission. UB treatment induced mitochondrial biogenesis as evidenced by increased CytB and ND1 DNA levels. These data suggest that exogenous UB may inhibit the crucial link between ER stress and mitochondrial fission, and/or enhance mitochondrial biogenesis, thereby playing a protective role in I/R-induced myocyte apoptosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Perspective

UB is found in normal plasma and is elevated in many disease states. Previously, we have shown that exogenous UB plays an anti-apoptotic role in β-AR-stimulated cardiac myocyte apoptosis *in vitro* and *in vivo* (Singh et al. 2010; Daniels et al. 2012). The data presented here suggest a protective role for exogenous UB in I/R-induced myocyte apoptosis and cardiac injury, and an important role for UB/CXCR4 axis in modulation of oxidative stress, and mitochondrial membrane potential and homeostasis. Further identification of signaling mechanisms leading to UB-mediated regulation of cardiac myocyte apoptosis and I/R injury may uncover novel strategies to improve cardiac remodeling and function.

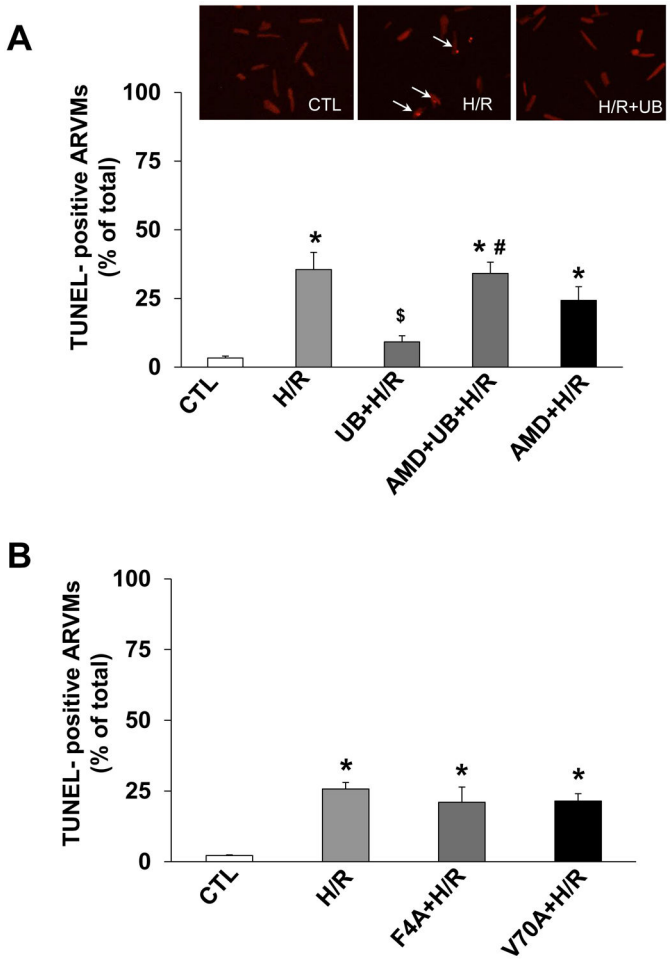

Figure 1. UB inhibits H/R-induced apoptosis in ARVMs:

A. ARVMs were pretreated with AMD3100 (AMD; 10 μM) for 30 min followed by treatment with UB (10 μg/mL) for 30 min. The cells then underwent hypoxia (2.5 h) and reoxygenation (18 h). Apoptosis was measured using TUNEL-staining assay. Top panels show TUNEL-stained images of ARVMs. *$p<0.05$ vs CTL; $^{\$}p<0.05$ vs H/R; $^{\#}p<0.05$ vs UB; n = 3-5. **B.** ARVMs were treated with mutated UBs (mV70A and mF4A; 10μg/mL) for 30 min followed by hypoxia (2.5 h) and reoxygenation (18 h). *$P<0.05$ versus CTL; n=3.

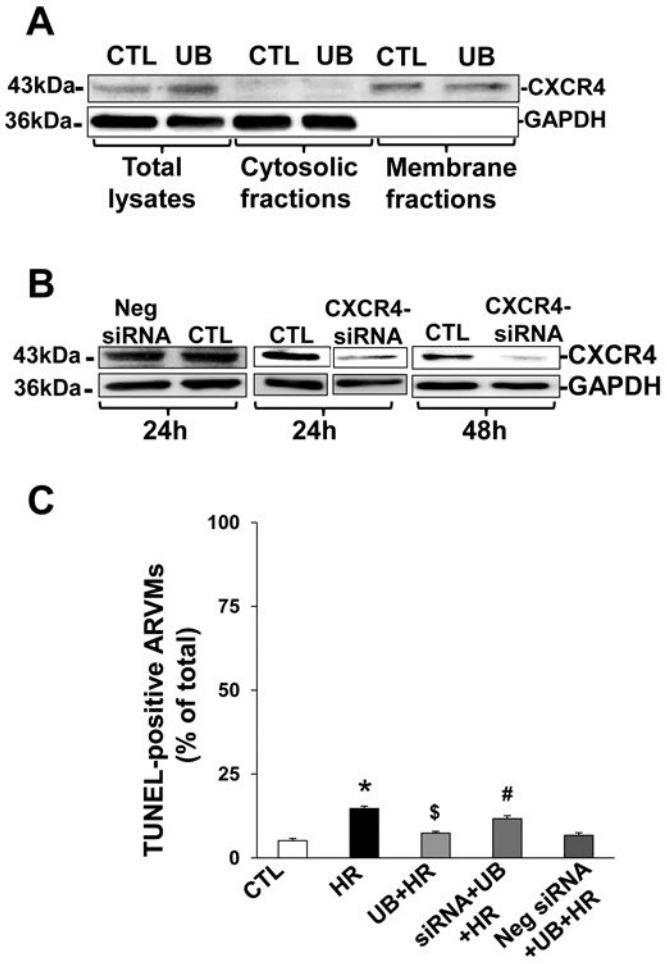

Figure 2. CXCR4 expression, siRNA-mediated knockdown of CXCR4 and role in apoptosis:
A. Total cell lysates, and membrane and cytosolic fractions were analyzed by Western blot using anti-CXCR4 antibodies. **B.** CXCR4 siRNA transfection for 24 and 48 h decreases protein levels of CXCR4. **C.** CXCR4 knockdown using siRNA decreases H/R-mediated increase in the number of apoptotic ARVMs. *p<0.05 vs CTL; $p<0.05 vs H/R; #P<0.05 vs UB+HR, n=4.

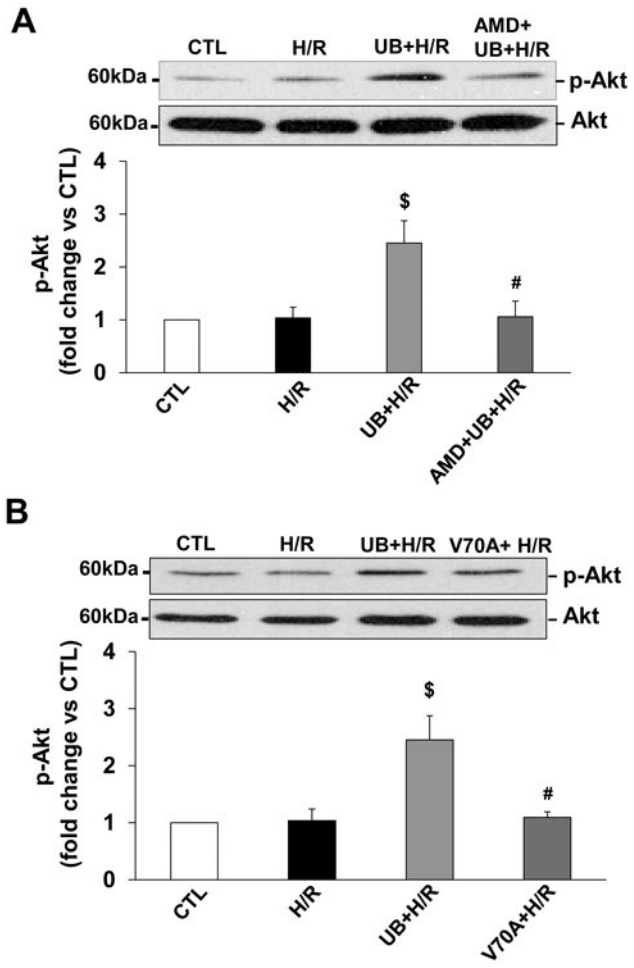

Figure 3. Activation of Akt:

A. ARVMs were pretreated with AMD3100 (AMD; 10 μM) for 30 min followed by treatment with UB (10 μg/mL) for 30 min. The cells then underwent hypoxia (2.5 h) and reoxygenation (15 min). **B.** ARVMs were treated with mutated UBs (mV70A; 10μg/mL) for 30 min followed by hypoxia (2.5 h) and reoxygenation (15 min). Cell lysates (20 μg) were analyzed by Western blot using phospho-specific anti-Akt antibodies. The lower panels exhibit mean data normalized to total Akt. $^{\$}P<0.05$ vs H/R; $^{\#}P<0.05$ versus UB+H/R; n=7 for CTL, H/R, and UB+H/R; n=4 for AMD+UB+H/R; n=3 for V70A+H/R.

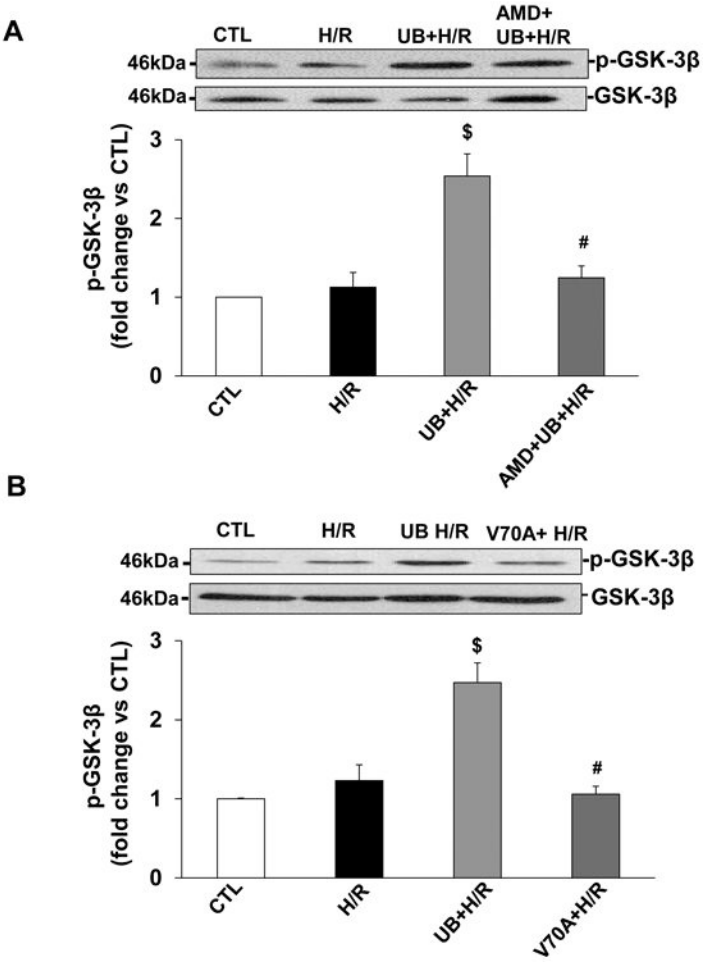

Figure 4. Activation of GSK-3β:

A. ARVMs were pretreated with AMD3100 (AMD; 10 μM) for 30 min followed by treatment with UB (10 μg/mL) for 30 min. The cells then underwent hypoxia (2.5 h) and reoxygenation (15 min). **B.** ARVMs were treated with mutated UB (mV70A; 10μg/mL) for 30 min followed by hypoxia (2.5 h) and reoxygenation (15 min). Cell lysates (20 μg) were analyzed by Western blot using anti-phospho-GSK-3β antibodies. The lower panels exhibit mean data normalized with total GSK-3β. $^{\$}P<0.05$ versus H/R; $^{\#}P<0.05$ versus UB+H/R; n=7 for CTL, H/R, and UB+H/R; n=4 for AMD+UB+H/R; n=3 for V70A+H/R.

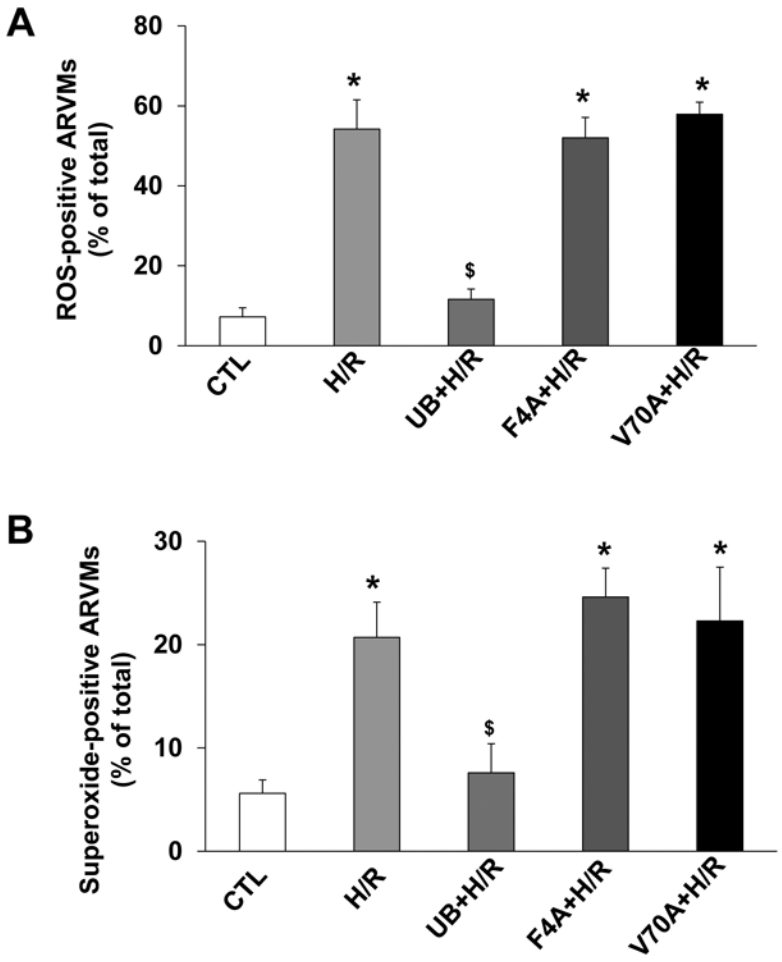

Figure 5. UB decreases the number of H/R-induced ROS- and superoxide-positive ARVMs: ARVMs were pretreated with UB or mutated UBs (mUB V70 and mUB F4; 10μg/mL) for 30 min. The cells then underwent hypoxia (2.5 h) and reoxygenation (1 h). At time of reoxygenation, cells were loaded with total ROS/superoxide detection stain for 1 h. The number of green (ROS-positive; **A**) and red (superoxide-positive; **B**) fluorescent cells vs total cells were counted. *P<0.05 versus CTL; $P<0.05 versus UB+H/R; n=3-5.

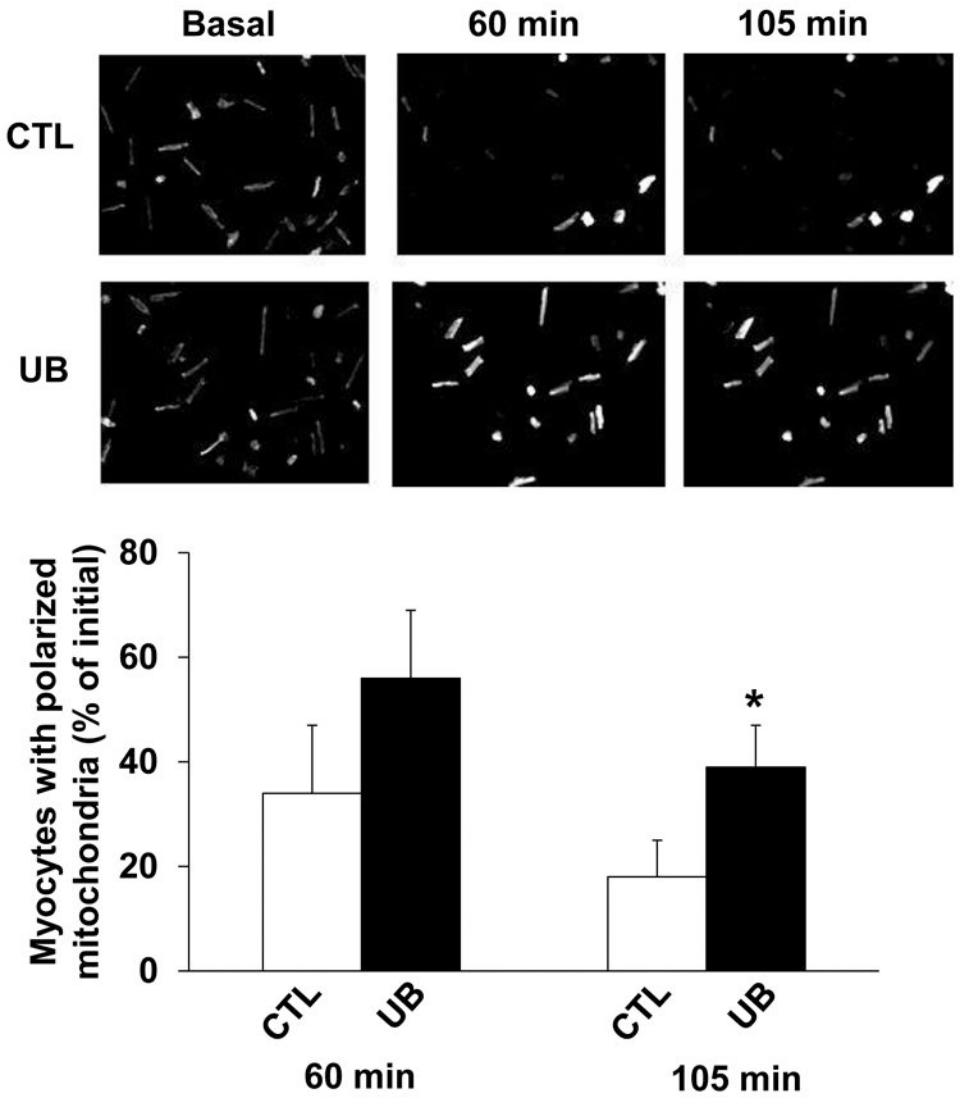

Figure 6. UB treatment helps maintain mitochondrial membrane polarization:

ARVMs were incubated with oxyrase to create anoxic conditions. Mitochondrial membrane polarization was measured using TMRM loading. Upper panel depicts images of ARVMs obtained using fluorescent microscope before anoxia (basal) and at indicated time points during anoxia. The number of polarized cells (i.e., those exhibiting TMRM fluorescence) was counted at each time point of oxyrase incubation, and the data is expressed as a percent of TMRM-positive cell prior to anoxia (basal). *P<0.05 vs CTL; n=4.

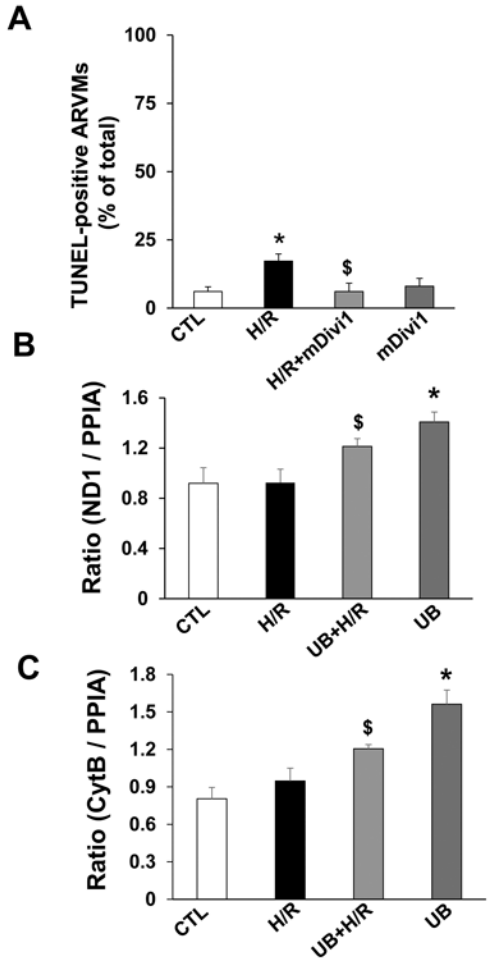

Figure 7.

A. Inhibition of mitochondrial fission inhibits H/R-induced apoptosis: ARVMs were pretreated with mDivi1 (1 μM) for 30 min followed by hypoxia (2.5 h) and reoxygenation (18 h). Apoptosis was measured using TUNEL-staining assay. *p<0.05 vs CTL; $p<0.05 vs H/R; n=3-6. **B&C. UB treatment increases mitochondrial biogenesis.** ARVMs were pretreated with UB (10 μg/mL) for 30 min followed by hypoxia (2.5 h) and reoxygenation (18 h). Total cellular DNA (nuclear and mitochondrial) was amplified for mitochondrial genes cytochrome b (CytB) and NADH oxidase 1 (ND1), and nuclear gene peptidyl-prolyl isomerase A (PPIA). **B** and **C** exhibit the ratio of ND1/PPIA and CytB/PPIA, respectively. *P<0.05 vs CTL and H/R; n=3-4.

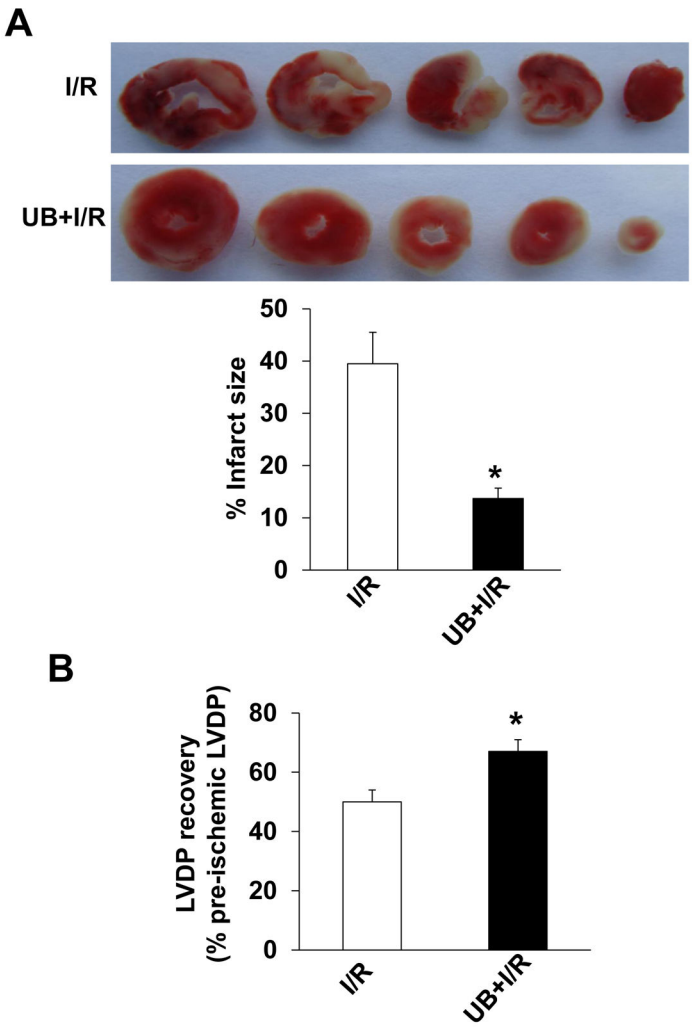

Figure 8. UB decreases infarct size and improves LVDP recovery:

Mice hearts underwent global ischemia for 25 min, followed by 40 min reperfusion. **A.** Upper panel depicts representative TTC-stained hearts exhibiting infarct regions (white area) in I/R and UB+I/R hearts. The lower panel exhibits % infarct size (n=4-5). **B.** Recovery of left ventricular developed pressure (LVDP) following ischemia. *P<0.05 vs I/R; n=6.

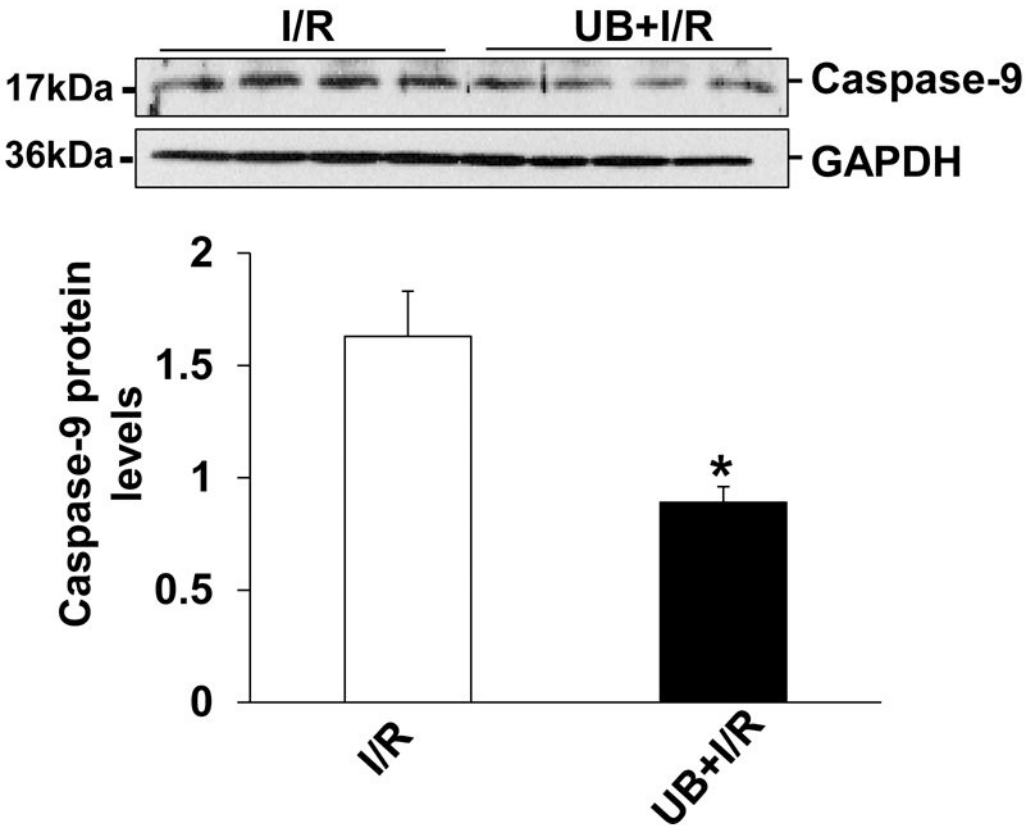

Figure 9. UB decreases activation of caspase-9:

Total heart lysates (60 μg) were analyzed by Western blot using anti-caspase-9 antibodies. The lower panel exhibits mean data normalized with GAPDH. *P<0.05 versus I/R; n=4.