Protective effect of peroxisome proliferator-activated receptor α activation against cardiac ischemia-reperfusion injury is related to up-regulation of uncoupling protein-3

Jong Wook Song

Department of Medicine

The Graduate School, Yonsei University

Protective effect of peroxisome proliferator-activated receptor α activation against cardiac ischemia-reperfusion injury is related to up-regulation of uncoupling protein-3

Directed by Professor Young Lan Kwak

Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

Jong Wook Song

June 2014

This certifies that the Doctoral Dissertation of Jong Wook Song is approved.

Thesis Supervisor : Young Lan Kwak

Thesis Committee Member#1 : Jae-Woo Kim

Thesis Committee Member#2 : Kyung Jong Yoo

Thesis Committee Member#3: Sahng Wook Park

Thesis Committee Member#4: Yon Hee Shim

The Graduate School
Yonsei University

June 2014

ACKNOWLEDGEMENTS

Over the past years I have received great support and encouragement from my committee members, colleagues and friends in my department, and my family and wife. I would never have been able to finish my dissertation without them.

Firstly, I would like to express my deep gratitude to Professor Young Lan Kwak. She has been a teacher, mentor and provided me with excellent atmosphere for a research. Also, I am sincerely grateful to Professor Jae-Woo Kim. This thesis would not have been possible without his insights and guidance. I would like to thank my dissertation committee of Professor Kyung Jong Yoo, Yon Hee Shim and Sahng Wook Park for their careful advices and sincere comments. In addition, thanks to Hyo Jung Kim for valuable and practical assistances. Finally, I would like to express my love and appreciation to my wife, my daughters and all my family members

Jong Wook Song

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Protective effect of peroxisome proliferator-activated receptor α activation against cardiac ischemia-reperfusion injury is related to up-regulation of uncoupling protein-3

Jong Wook Song

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Young Lan Kwak)

Peroxisome proliferator-activated receptor α (PPARα) is a ligand-activated nuclear receptor involved in transcriptional regulation of lipoprotein and fatty acid metabolism. PPARα activation was reported to have cardioprotective effect, while its mechanism remains elusive. This study investigated the effect of PPARα activation on expression of uncoupling protein (UCP) and protection against cardiac ischemia-reperfusion injury *in vitro* and *in vivo*.

H9c2 (neonatal rat cardiac myoblast) cells were incubated for 24 h with medium containing WY-14643, a PPARα ligand, or vehicle. UCP expression, intracellular reactive oxygen species (ROS) production and cell survival against hypoxia-reoxygenation were determined. WY-14643 increased the transcription of UCP3 mRNA and UCP3 protein level in H9c2 cells. The expression of UCP2 was not altered by WY-14643 treatment. WY-14643 decreased H_2O_2- or hypoxia-stimulated intracellular ROS production. Cell survival assessed by MTT assay or cell counting showed that WY-14643 treated H9c2 cells were more

resistant against hypoxia-reoxygenation injury than the untreated cells. Knocking-down UCP3 by siRNA prevented WY-14643 from decreasing the production of ROS. UCP3 siRNA abolished the effect of WY14643 on cell viability against hypoxia-reoxygention injury.

Degree of myocardial infarction following left anterior descending coronary artery occlusion and UCP expression was measured in rats treated with WY-14643 (20 mg/kg) or vehicle. Hemodynamic variables and 3-lead electrocardiogram were also recorded during ischemia-reperfusion. *In vivo*, UCP3 expression was increased in rats treated with WY-14643. Myocardial infarct size was smaller in rats treated with WY-14643 compared with those treated with vehicle (76 ± 8% vs. 42 ± 12%, control vs. WY-14643, respectively, $p < 0.05$). During reperfusion, the incidence of arrhythmia was higher in control group compared with WY-14643 group (9/10 vs. 3/10, $p < 0.05$).

In conclusion, PPARα activation by WY-14643 conferred the cell protective effect against hypoxia-rexoygenation. Administration of PPARα agonist WY-14643 also decreased the size of myocardial infarction and the incidence of reperfusion-induced arrhythmia. It is suggested that WY-14643 increases UCP3 expression resulting in concomitant attenuation of ROS production.

Key words : peroxisome proliferator-activated receptor α, uncoupling protein, ischemia-reperfusion, reactive oxygen species

Protective effect of peroxisome proliferator-activated receptor α activation against cardiac ischemia-reperfusion injury is related to up-regulation of uncoupling protein-3

Jong Wook Song

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Young Lan Kwak)

I. INTRODUCTION

Peroxisome proliferator-activated receptor α (PPARα) is one of the members of the nuclear hormone receptor superfamily. Upon activation by their cognate ligands, heterodimer of PPARα with 9-cis retinoic acid-activated receptors (RXRs) bind to DNA response elements in promoter regions and increases gene transcription.

PPARα is enriched in the heart tissue which has high capacity for fatty acid oxidation and regulates the expression of genes involved in cellular lipid metabolism.[1] Studies on murine models of knockout or overexpression of PPARα indicated its pivotal role in cardiac metabolic homeostasis. Transgenic mice with overexpression of PPARα showed an increased fatty acid uptake and oxidation with a concomitant decrease in glucose uptake and oxidation,[2, 3] and these metabolic changes were associated with left ventricular hypertrophy and systolic dysfunction.[4] In contrast, pharmacologic activation of PPARα conferred myocardial protection against acute ischemia-reperfusion injury model.[5-7] However, the mechanism of cardioprotection by PPARα activation remains unclear.

Uncoupling proteins (UCP) are inner mitochondrial carrier proteins that induce proton leak and dissipate the mitochondrial electrochemical gradient.[8] UCP1 was firstly discovered as a regulator of thermogenesis in brown adipose tissue. UCP2 and UCP3 were found to be expressed in various tissues including the heart while their role in the heart is still elusive. However, previous studies suggested that UCPs may have protective role during oxidative stress. Mitochondrial reactive oxygen species (ROS) generation is known to be proportional to electrochemical gradient across the inner membrane.[9] Mild uncoupling and decreased proton gradient across the mitochondrial inner membrane reduced ROS production,[10] which served as a critical mediator of ischemia-reperfusion injury. Treatment of dinitrophenol, a pharmacologic uncoupling agent, also showed cardioprotective effect.[11, 12] Overexpression of UCP2 in cardiomyocyte attenuated ROS production and increased tolerance to oxidative stress.[13] Moreover, UCP2 and UCP3 were up-regulated after ischemic preconditioning, which is one of the most potent endogenous cardioprotective mechanisms against ischemia-reperfusion injury.[14]

UCP3 is up-regulated by circulating free fatty acid and PPARα is shown to be a mediator of transcriptional activation of UCP3.[15, 16] Based on regulatory role of PPARα on the expression of UCP, we hypothesized that the mechanism of cardioprotective effect of PPARα against ischemia-reperfusion injury could involve increased expression of UCP, particularly UCP3, and attenuated ROS generation. This study aimed to investigate whether WY-14643, a PPARα ligand, conferred protection against acute myocardial ischemia-reperfusion injury and the cardioprotection involved up-regulation of UCP3 and decreased ROS production.

II. MATERIALS AND METHODS

1. Cell culture

The H9c2 cell line was obtained from ATCC (Manassas, VA, USA). The cells were cultured in Smooth Muscle Cell Medium containing smooth muscle cell growth supplement at 37°C in an atmosphere of 95% air and 5% CO_2, based on the manufacturer's recommendations. The cells were treated with WY-14643 or vehicle for 24 h before RT-PCR, western blot, ROS measurement or cell survival experiment. For cellular hypoxia-reoxygenation, cells were incubated for 20 h in an anoxic chamber, and reoxygenated for 2 h.

2. Gene silencing with siRNA

Cells were seeded into 100-mm culture dishes 18–24 h prior to transfection. Cells were transfected with 80 nM control siRNA (Ambion, Austin, TX, USA), UCP3 siRNA (Genolution, Seoul, Korea), in serum-free medium using RNAiMAX (Invitrogen, Carlsbad, CA, USA). Following incubation for 6 h, the transfection medium was replaced with fresh medium, and the cells were incubated for an additional 48 h, at which point they were treated with the indicated reagents for the indicated time.

3. RT-PCR

Total RNA was isolated from cultured cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For quantitative RT-PCR, cDNA was synthesized from 5 mg of total RNA using random hexamer primers and SuperScript reverse transcriptase II (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. An aliquot (1/40) of the reaction was used for quantitative PCR using the SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies Korea, Seoul, Korea) and gene-specific primers. RT-PCR products were quantified using the ABI

PRISM 7300 RT-PCR System (Applied Biosystems, Life Technologies Korea, Seoul, Korea). RT-PCR was performed using the following primers: UCP2, 5'-CTCCC AATGT TTGCC CGAAA-3', 5'-ACTGG CCCAA GGCAG AGTTC-3'; UCP3, 5'-TATGG TGCGC ACAGA GGGTC-3', 5'-CACCA CATCC GTGGG TTGAG-3'; GAPDH, 5'-ACCAC AGTCC ATGCC ATCAC-3',5'-TCCAC CACCC TGTTG CTGTA-3'. GAPDH mRNA was also measured as an invariant control.

4. Western blot analysis

Cells treated with the indicated reagents were washed in ice-cold PBS and lysed in PRO-PREP Protein Extraction Solution (iNtRON Biotechnology, Seoul, Korea). An aliquot of the cell lysate was subjected to SDS-polyacrylamide gel electrophoresis and transferred onto a Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences, Corston, UK). Membranes blocked with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 overnight at 4°C were reacted with the indicated specific antibodies in TBS containing 1% BSA and 0.05% Tween-20 overnight at 4°C and then incubated with peroxidase-conjugated goat antibody diluted to 1:3000 for 2 h at room temperature. After extensive washing in TBS containing 0.1% BSA and 0.1% Tween-20, immunoreactive bands were detected using West-ZOL Plus (iNtRON Biotechnology, Seoul, Korea).

5. MTT assay

MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide, final concentration of 0.1 mg/ml) was added to the culture medium, and cells were incubated for an additional 4 h. The medium was removed and formazan crystals formed by the reduction of MTT by mitochondrial dehydrogenases in living cells were solubilized in acidified isopropanol, and measured spectrophotometrically at 570 nm.

6. Cell count assay

Cells were seeded at a density of 1×104 cells/well into 24-well plates, incubated for 24 h and were synchronized to quiescence by serum starvation for 12 h. Each day, the cells were trypsinized and cell numbers were determined using an automated cell counter, ADAM (NanoEnTek, Pleasanton, CA, USA), according to the manufacturer's instructions.

7. Measurement of reactive oxygen species

The cells were incubated with 5 lMH2DCFDA (DCF-DA, Calbiochem, San Diego, CA, USA) for a final 30 min, then harvested and washed twice with ice-cold PBS. The cells were immediately analyzed for fluorescence intensity using a FACSCalibur flow cytometer (Becton–Dickinson Immunocytometry Systems, San Jose, CA, USA) and CellQuestPro software.

Intracellular superoxide production was measured by lucigenin-amplified chemiluminescence or a fluorescent indicator, dihydroethidium (DHE), as described previously. To determine the level of superoxide anion by lucigenin, cells treated with each reagent and then, trypsinized and collected by centrifugation at 1,000 rpm for 5 min. After washing twice with ice-cold PBS, the cells were resuspended in HBSS buffer (5.4mM KCl, 0.3mM Na2HPO4, 0.4 mM KH2PO4, 4.2 mM NaHCO3, 1.3 mM CaCl2, 0.5 mM MgCl2, 0.6 mM MgSO4, 137 mM NaCl, 5.6 mM glucose, pH 7.4) containing 5 mM of lucigenin. The lucigenin-derived chemiluminescence was determined every 50 s for a total of 5 min, by a LB96V luminometer (EG&G Berthold, Bad Wildbad, Germany). For analysis of intracellular superoxide production, cells treated as described above were washed with ice-cold PBS and incubated for 30 min at 37 °C with 10 mM of DHE in PBS. Following incubation in a humidified chamber protected from light, the red fluorescence was detected through a 580 nm longpass filter using a fluorescence microscope (Olympus, Tokyo, Japan).

8. Animal preparation and experimental protocol

This study was approved by the institutional ethics committee for laboratory animal experiments and all experiments were conducted according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85-23, revised 1996).

Male Sprague-Dawley rats weighing 250 to 350 g were anesthetized with sodium pentobarbital 50 mg/kg ip bolus. Additional intermittent bolus of sodium pentobarbital 10 mg/kg every 1 h followed for the maintenance of anesthesia. The left jugular vein was cannulated for delivery of fluid and Patent Blue dye. The left carotid artery was cannulated for continuous monitoring of mean arterial pressure (MAP) and heart rate (HR). A 3-lead electrocardiogram was placed for detection of ischemic change and arrhythmia. The animals were ventilated via tracheostomy with 60% oxygen/air mixture at a tidal volume of 8 ml/kg. Respiratory rate was initially set to 50 breaths/min and adjusted to maintain arterial PCO_2 between 35 to 40 mmHg. After the exposure of heart via left thoracotomy, a snare was placed around the left anterior descending coronary artery (LAD). After the surgical preparation, all rats were stabilized for 30 min before LAD occlusion. Ischemia was induced by tightening the snare. Ischemia was confirmed by visual inspection of pale color on anterior wall of heart and ST segment elevation on electrocardiogram. After 30 min of ischemia, myocardium was reperfused by loosening the snare for 2 h.

Animals were randomly allocated into the two groups. The WY group received PPARα agonist WY-14643 (4-chloro-6-(2,3-xylidino)-2- pyrimidinylthioacetic acid, Sigma-Aldrich Korea, Seoul, Korea) 20 mg/kg ip 4 h before LAD occlusion for the measurement of infarct size, or 4 h before the excision of heart for RT-PCR and western blot analysis. The control group received the same volume of 5% dimethyl sulfoxide (DMSO).

9. Measurement of infarct size

The LAD was re-occluded after 2 h of reperfusion and 2 ml/kg of 10% Patent Blue was administered via left jugular vein. The heart was rapidly extracted and the right ventricle was carefully removed. The left ventricle was incubated at -20℃ for 20 min. Thereafter, the left ventricle was sectioned into 1 – 1.5 mm slices perpendicular to the apex-base axis. Then the slices were incubated in 2% triphenyltetrazolium chloride for 30 min at 37℃ to distinguish the necrotic tissue from the viable. Both planes of the slices were photographed and infarct size was calculated as area of necrosis / area at risk X 100 (%).

10. Statistical analysis

Values are expressed as mean ± SD. Data were analyzed by independent *t* test except the hemodynamic variables, which were analyzed by repeated measures ANOVA. SAS (version 9.1.3, SAS Institute, Inc., Cary, NC, USA) was used for statistical analysis. A p value less than 0.05 was considered statistically significant.

III. RESULTS

1. Effect of WY-14643 on UCP expression *in vitro*

Figure 1 illustrates the expression of UCP2 and UCP3 in H9c2 cell line after 24 h incubation in the control medium or experimental media containing 0.1 to 100 μM of WY-14643. Cells treated with 50 μM of WY-14643 showed increased expression of PDK4 mRNA, a PPARα target gene. Expression of a PPARγ target gene aP2 was not altered after the treatment with WY-14643, indicating WY-14643 activated PPARα specifically (Figure 1A). Up-regulation of UCP3 mRNA was observed in cells treated with 10 μM or greater concentration of WY-14643 compared to the control ($p < 0.05$, Figure 1B and 1C). UCP3 protein was also increased after the treatment of WY-14643 10 μM or greater (Figure 1D). The expression of UCP2 was not altered by WY-14643 treatment. We next examined whether WY-14643 activated other cardioprotective signaling pathways. Treatment of WY-14643 at 50 μM also increased phosphorylation of Akt and STAT3, and protein level of heme oxygenase 1 (HO-1) as well as UCP3 (Figure 1E). Transfection of UCP3 siRNA silenced UCP3 mRNA (Figure 1F). Figure 1G shows that UCP3 siRNA also down-regulated UCP3 protein level.

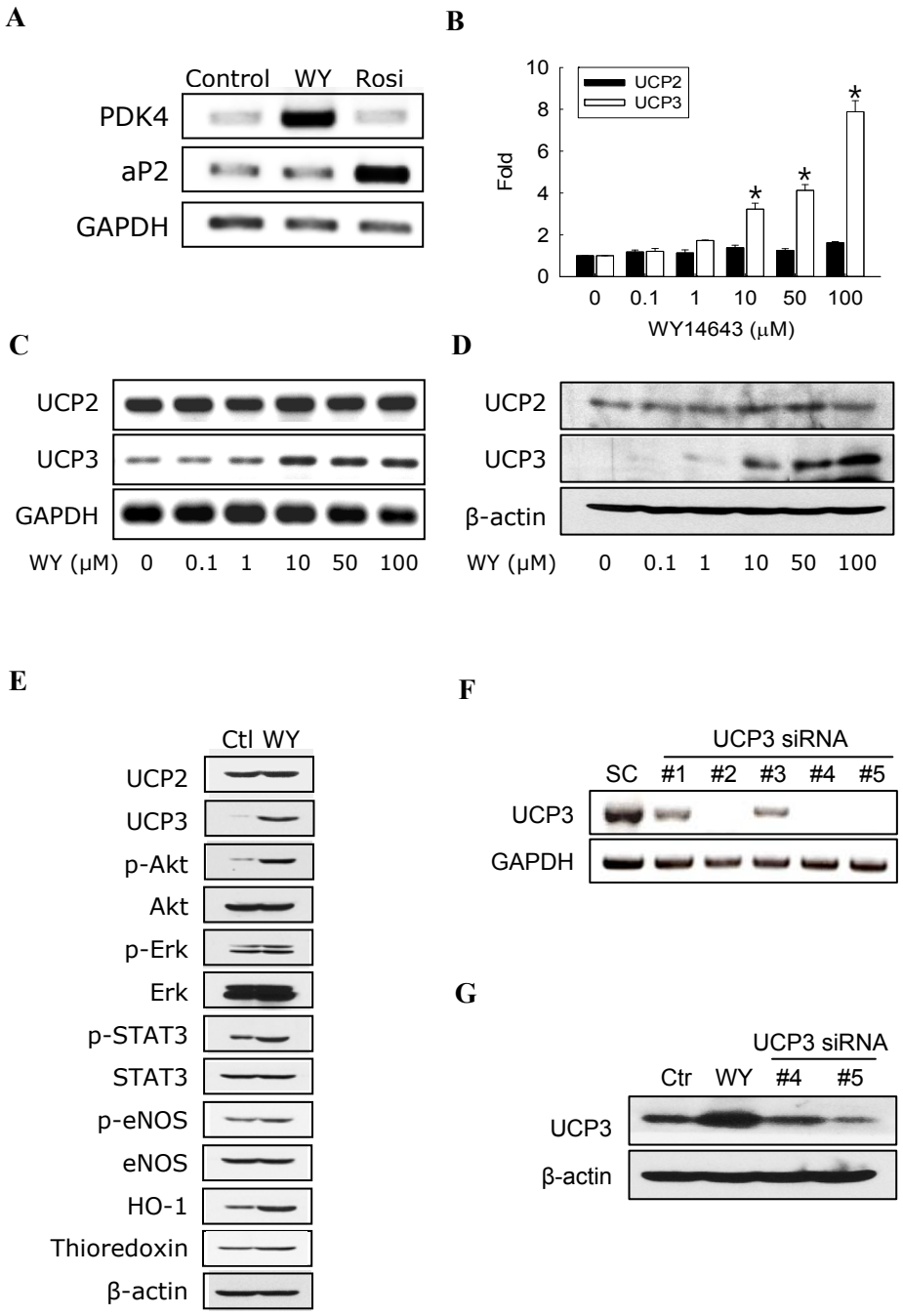

Figure 1. WY-14643 increased UCP3 expression in H9c2 cells. A, Treatment of WY-14643 at 50 μM increased expression of a PPARα target gene PDK4

mRNA, and expression of a PPARγ target gene aP2 was not altered after the treatment of WY-14643. B, Quantitative PCR shows that WY-14643 treatment at 10, 50 and 100 μM for 24 h increased mRNA levels of UCP3 compared with vehicle treatment. $*p < 0.05$ vs. WY-14643 0 μM (n = 3). C, RT-PCR shows increased UCP3 mRNA expression following WY-14643 treatment. D, Western blot of UCP3 shows increased protein level after WY-14643 treatment. E, Treatment of WY-14643 at 50 μM also increased phosphorylation of Akt and STAT3, and protein level of HO-1 as well as UCP3. F, Transfection of UCP3 siRNA silenced UCP3 mRNA (#2, #4, #5). G, Western blot of UCP3 shows that transfection of UCP3 siRNA downregulated UCP3 protein level. #5 was used for further experiment. WY, WY-14643; Rosi, rosiglitazone; HO-1, heme oxygenase-1.

2. WY-14643 decreased H_2O_2 or hypoxia-stimulated ROS production

After the treatment of WY-14643 or vehicle, ROS production was stimulated by 250 μM of H_2O_2 for 30 min. H_2O_2 increased DCF florescence ($p < 0.05$), and cells treated with 10 or 50 μM of WY-14643 showed reduced ROS level compared with the cells treated with vehicle ($p < 0.05$, Figure 2A). To assess whether WY-14643 could reduce oxidative stress and UCP3 up-regulation was involved in this process, cells treated with 50 μM of WY-14643 or vehicle were subjected to 20 h of hypoxia and ROS level was measured by DCF florescence. Hypoxia increased ROS generation ($p < 0.05$ vs. control) and WY-14643 attenuated hypoxia-induced increase in ROS ($p < 0.05$ vs. hypoxia). In cells transfected with UCP3 siRNA, WY-14643 treatment did not reduce hypoxia-induced ROS generation ($p < 0.05$ vs. control, Figure 2B). Lucigenin-amplified chemiluminescence also showed that WY-14643 reduced hypoxia-induced increase in intracellular ROS generation and depletion of UCP3 by siRNA abrogated the reduction of ROS after WY-14643 treatment (Figure 2C).

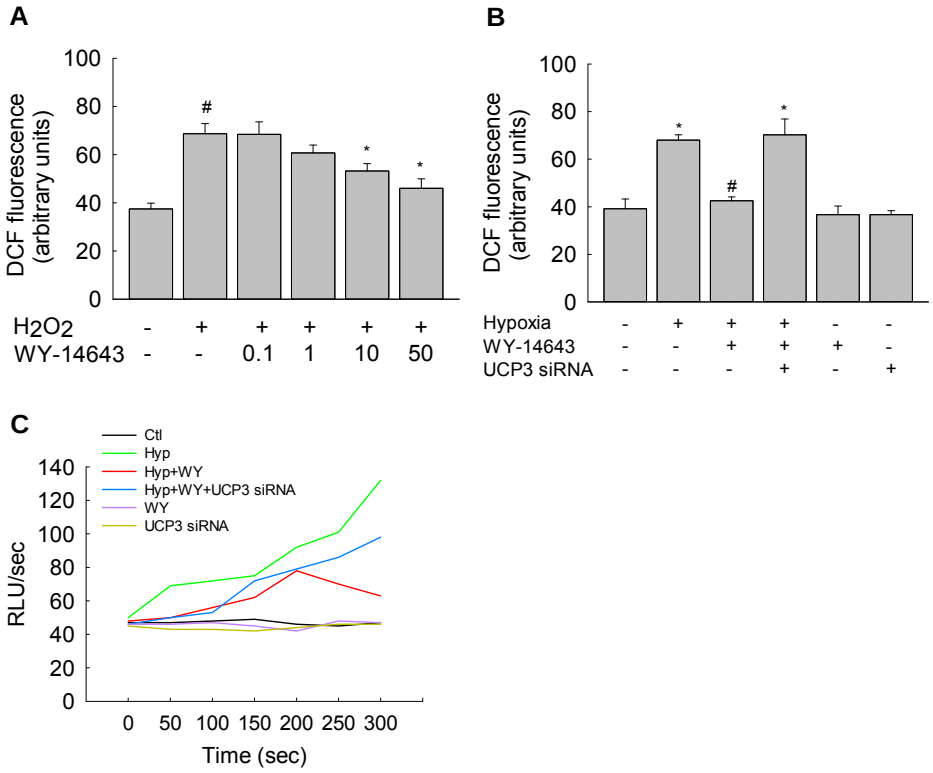

Figure 2. WY-14643 decreased H_2O_2 or hypoxia-stimulated intracellular ROS production. A, H_2O_2 increased ROS level and WY-14643 treatment at 10 μM and 50 μM for 24 h attenuated the increase in ROS by H_2O_2. *$p < 0.05$ vs. H_20_2 (+) WY (-). $^{\#}p < 0.05$ vs. H_20_2 (-) WY (-) (n = 3). B, cells treated with 50 μM of WY-14643 or vehicle were subjected to 20 h of hypoxia and ROS level was measured by DCF fluorescence. Hypoxia increased ROS generation and WY-14643 attenuated hypoxia-induced increase in ROS. UCP3 siRNA abrogated the effect of WY-14643 on ROS reduction. *$p < 0.05$ vs control. $^{\#}p < 0.05$ vs. Hyp (n = 3). C, cells treated with 50 μM of WY-14643 or vehicle were subjected to 20 h of hypxia and ROS level was measured by Lucigenin-amplified chemiluminescence. Hypoxia increased ROS generation and WY-14643 attenuated hypoxia-induced increase in ROS. UCP3 siRNA abrogated the effect of WY-14643 on ROS reduction. Hyp; Hypoxia, WY; WY-14643 treatment.

3. Cell survival against hypoxia-reoxygenation injury

Cell survival experiments were conducted to evaluate the protective effect of WY-14643 against hypoxia-reoxygenation injury. H9c2 cells incubated in control medium or experimental media treated with 0.1 to 100 μM of WY-14643 for 24 h were subjected to 20 h of hypoxia and 2 h of reoxygenation prior to MTT assay or cell counting. WY-14643 treatment at 10, 50 and 100 μM decreased cell death after hypoxia-reoxygenation ($p < 0.05$ vs. control, Figure 3). To examine the role of UCP3 in WY-14643 induced protection, cell survival experiment was also conducted in cells transfected with UCP3 siRNA. Cell protective effect of 50 μM of WY-14643 against hypoxia-reoxygenation was attenuated in cells transfected with UCP3 siRNA ($p < 0.05$ vs. control, Figure 4A and 4B). Treatment of PI3K inhibitor wortmannin 100 nM, MEK inhibitor PD98059 50 μM, or HO-1 inhibitor zinc protoporphyrin-IX 100 nM also decreased protective effect of WY-14643 ($p < 0.05$ vs. control). The degrees of decrease in cell survival following treatment of these inhibitors were less compared with the cells transfected with UCP3 siRNA ($p < 0.05$ vs. hypoxia (+) WY-14643 (+) UCP3 siRNA (+), Figure 4C).

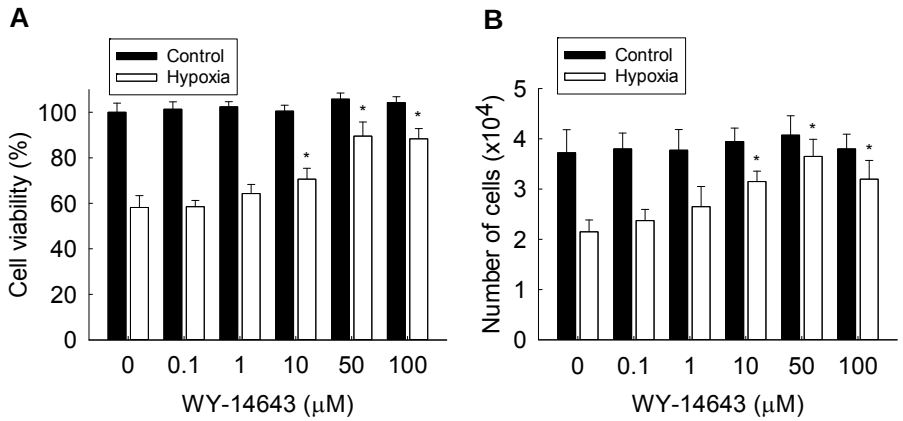

Figure 3. H9c2 cells treated with WY-14643 were more resistant against hypoxia-reoxygenation injury. Cells were incubated with medium containing 0 to 100 μM of WY-14643 for 24 h before 20 h of hypoxia and 2 h of reoxygenation. A, MTT assay shows that cell viability was increased with treatment of WY-14643 10 μM or greater. *$p < 0.05$ vs. WY-14643 0 μM (n = 3). B, Cell counting also shows increased number of viable cells following WY-14643 treatment at 10, 50 and 100 μM. *$p < 0.05$ vs. WY-14643 0 μM (n = 3).

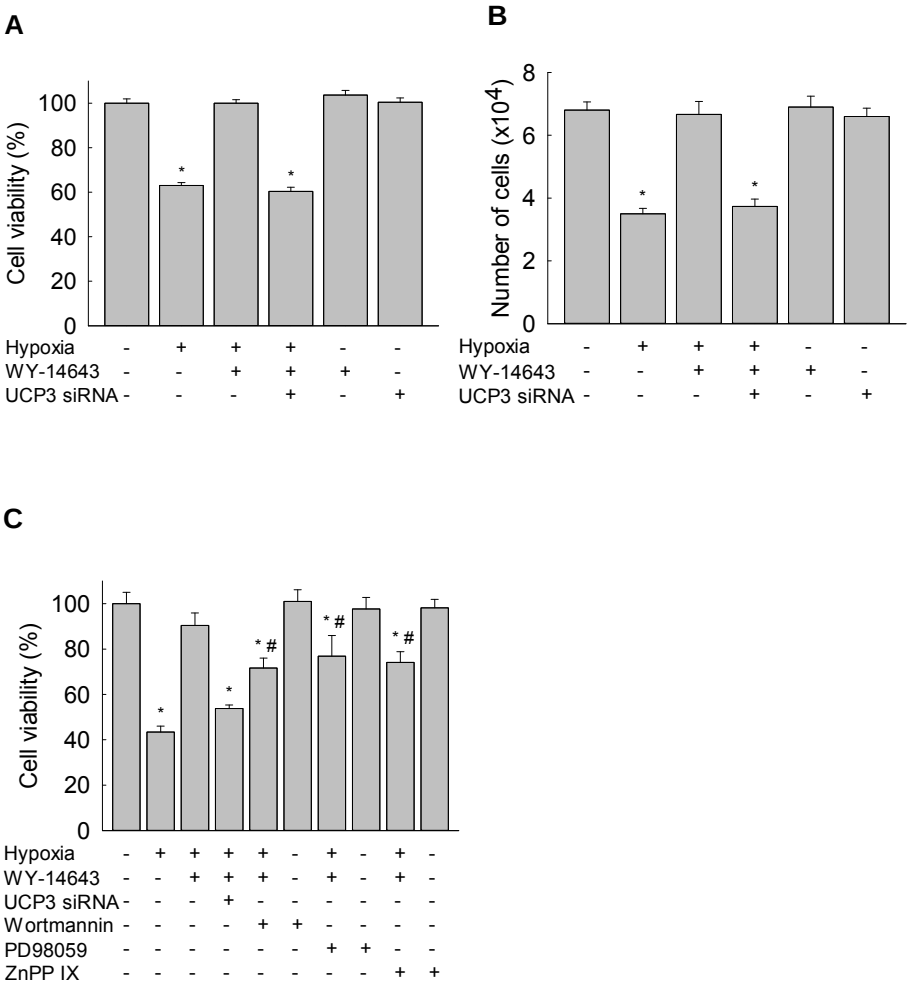

Figure 4. Depletion of UCP3 abrogated cell protective effect of WY-14643. Cells were incubated with medium containing 50 μM of WY-14643 for 24 h before 20 h of hypoxia and 2 h of reoxygenation. MTT assay (A) and cell counting (B) show that cell viability was increased with treatment of WY-14643 50 μM while the enhanced viability was attenuated in cells transfected with UCP3 siRNA. *$p < 0.05$ vs. control (n = 3). C, Wortmannin, PD98059, or zinc protoporphyrin-IX (ZnPP IX) decreased protective effect of WY-14643. Mitigated cell survival with treatment of Wortmannin, PD98059, or ZnPP IX were less significant compared with that of cells transfected with UCP3

siRNA.*$p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. hypoxia (+) WY-14643 (+) UCP3 siRNA (+) (n = 3).

4. Effect of WY-14643 on UCP expression *in vivo*

Animals received WY-14643 20 mg/kg ip 4 h before excision of heart. UCP3 mRNA expression was increased in rats received WY-14643 ($p < 0.05$ vs. control, Figure 5A and 5B). UCP3 protein level was increased with administration of WY-14643, and expression of HO-1, phosphorylation of Akt and Erk were also increased (Figure 5C). UCP2 expression was not increased after WY-14643 administration.

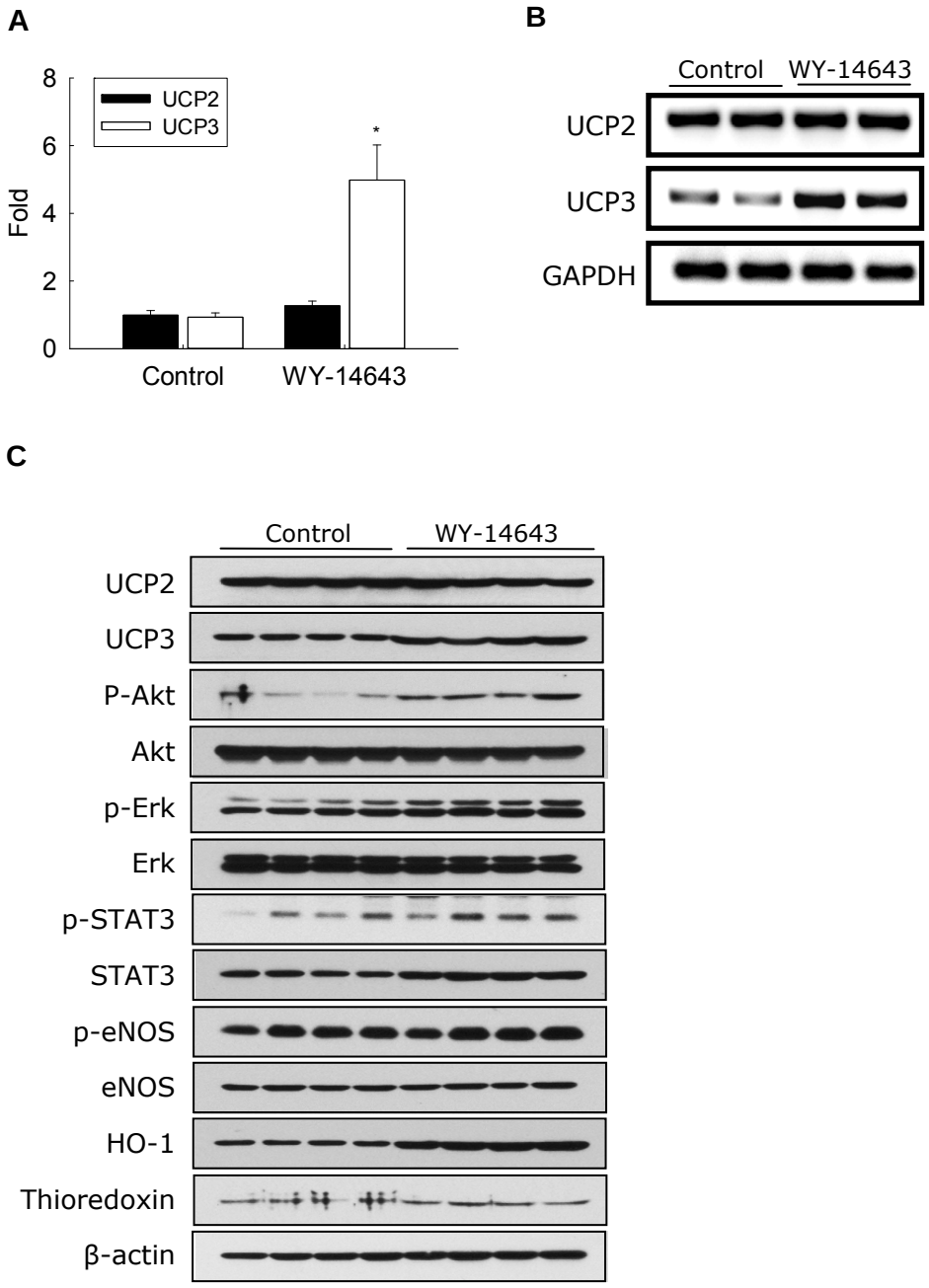

Figure 5. WY-14643 increased UCP3 expression in rats. WY-14643 20 mg/kg was administered 4 h before the extraction of heart. A, Quantitative PCR shows that WY-14643 increased mRNA levels compared with vehicle. *$p < 0.05$ (n =

3). B, RT-PCR shows increased UCP3 mRNA expression following WY-14643 administration. C, Western blot shows increased expression of UCP3 and HO-1, and phosphorylation of Akt and Erk after WY-14643 administration.

5. Myocardial infarct size following LAD occlusion

Area at risk was similar between the groups (41 ± 6% vs. 39 ± 10%, control vs. WY-14643, respectively, $p > 0.05$, Figure 6). Myocardial infarct size was significantly smaller in rats treated with WY-14643 compared with those treated with vehicle (76 ± 8% vs. 42 ± 12%, control vs. WY-14643, respectively, $p < 0.05$, Figure 6).

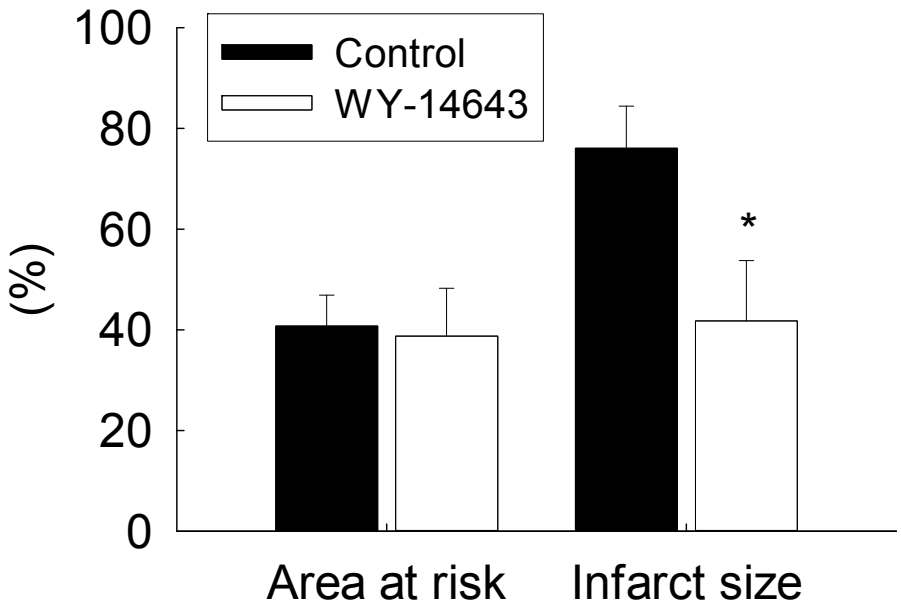

Figure 6. WY-14643 decreased myocardial infarct size. WY-14643 20 mg/kg was administered 4 h before the occlusion of left anterior descending coronary artery. Left panel, Area at risk was similar between the groups. Right panel, WY-14643 decreased the percentage of myocardial infarct size to area at risk. *$p < 0.05$ vs. control (n = 10).

6. Hemodynamic variables and incidence of arrhythmia

Hemodynamic variables including mean arterial pressure, heart rate and rate pressure product showed no significant differences between the groups (Table 1). During reperfusion, incidence of arrhythmia was significantly higher in the control group compared with the WY-14643 group (9/10 vs. 3/10, $p < 0.05$). In the control group, ventricular tachycardia was detected in 2 rats and premature ventricular complexes were detected in 7 rats. In rats received WY-14643, ventricular tachycardia was detected in one rat and premature ventricular complexes were detected in 2 rats.

Table 1. Hemodynamic data during ischemia-reperfusion

	MAP (mmHg)		Heart Rate (beats/min)		RPP (mmHg·beats/min)	
	Control	WY	Control	WY	Control	WY
Baseline	94 ± 15	107 ± 11	440 ± 40	450 ± 29	41438 ± 8847	48432 ± 6877
Before ischemia	97 ± 13	94 ± 24	435 ± 43	452 ± 42	42506 ± 7402	42428 ± 10754
Before reperfusion	89 ± 23	82 ± 24	436 ± 62	441 ± 76	39042 ± 12347	36719 ± 15041
15 min after reperfusion	77 ± 19	91 ± 27	401 ± 56	426 ± 57	31403 ± 11025	39381 ± 13750
30 min after reperfusion	77 ± 15	88 ± 15	419 ± 60	418 ± 62	32688 ± 9924	37592 ± 10024
60 min after reperfusion	75 ± 26	91 ± 15	419 ± 53	418 ± 60	32117 ± 13794	38494 ± 8261
2 h after reperfusion	87 ± 17	91 ± 22	425 ± 59	405 ± 43	37259 ± 9170	37268 ± 9514

MAP; mean arterial pressure, RPP; rate pressure product, WY; WY-14643 group.

IV. DISCUSSION

In the current study, administration of WY-14643, a PPARα agonist, reduced myocardial infarct size following ischemia-reperfusion injury in rats. Treatment of WY-14643 in H9c2 cell line also improved cell survival against hypoxia-reoxygenation. Activation of PPARα by WY-14643 was associated with increased expression of UCP3 *in vitro* and *in vivo*, which resulted in the attenuation of ROS production. Furthermore, depletion of UCP3 by siRNA abolished protective effect of WY-14643, implicating critical role of UCP3 in PPARα mediated cardioprotection.

PPARα is well known to be involved in myocardial fatty acid and glucose metabolism. It was demonstrated that cardiac specific PPARα-overexpression increased myocardial fatty acid uptake and oxidation, and concomitantly decreased glucose uptake and oxidation.[2] These metabolic alterations were associated with ventricular hypertrophy and contractile dysfunction, which was improved by low triglyceride diet.[2, 4] The PPARα-overexpressed mice also showed impaired recovery of myocardial function following ischemia-reperfusion injury.[17] These data suggests that chronic activation of PPARα might be detrimental to cardiac function. However, previous studies have shown that acute activation of PPARα by pharmacologic agonists can protect myocardium against ischemia-reperfusion injury.[5-7, 18] Mechanisms or molecular targets involved in PPARα-mediated cardioprotection have not been fully elucidated yet. It was proposed that cardioprotection by PPARα might be mediated through metabolic and anti-inflammatory effect. PPARα agonist inhibited pro-inflammatory cytokine production, matrix metalloproteinase expression and activation of NF-κB.[5] In accordance with the findings from PPARα-overexpressed mice, increased fatty acid oxidation with concomitantly decreased glucose oxidation was also observed in rodents treated with PPARα agonist compared with vehicle after acute ischemia-reperfusion injury.[5]

Although elevated serum free fatty acid concentration is known to aggravate myocardial infarction,[19] given that PPARα-overexpressed mice showed systolic dysfunction with cardiac metabolic fuel shift, direct causal relationship between altered cardiac metabolism and cardioprotection against acute ischemia-reperfusion injury is still unclear. Other mechanisms including activation of the phosphatidylinositol 3-kinase/Akt and nitric oxide signaling pathway were also suggested.[6, 7]

ROS is a critical mediator of ischemia-reperfusion injury.[20] Mitochondrial electron transport system is one of the major sources of cellular ROS generation. Return of oxygen supply during reperfusion induces large burst of ROS. Lipid peroxidation resulted from ROS breakdown cell membranes. In addition, ROS trigger mitochondrial permeability transition pore opening, which causes loss of mitochondrial inner membrane potential, ceased ATP production and initiation of apoptosis.[21] Since mitochondria is a major target of damage from ROS as well as a source of production, reducing ROS burst during reperfusion may be important for maintaining mitochondrial function and cardioprotection. The production of ROS is proportional to mitochondrial membrane potential, and mild uncoupling of mitochondrial respiration is thought to reduce ROS production.[22] Indeed UCP2 overexpression in rat neonatal cardiomyocyte increased viability against oxidative stress via reduced ROS production and mitochondrial Ca^{2+} overload.[13] Pharmacologic uncoupling agents such as dinitrophenol also conferred cardioprotective effect.[11, 12, 23] Recently, a crucial role of UCP3 in protection against myocardial ischemia-reperfusion injury was demonstrated in a cardiac UCP3 knockout mouse model.[24]

In this study, treatment of WY-14643, a PPARα ligand, up-regulated the expression of UCP3, attenuated ROS production and improved cell survival against hypoxia-reoxygenation. While WY-14643 also increased phosphorylation of kinases known to be involved in cardioprotective signaling pathways, protective effect of WY-14643 were nearly completely attenuated in

cells transfected with UCP3 siRNA, which suggests that UCP3 may play a central role in cardioprotective effect of PPARα activation. Previous studies indicated that cardiac UCP3 level is regulated by PPARα.[15, 16] In contrast to UCP3, UCP2 mRNA and protein levels were not affected by administration of PPARα ligand in the current study. Normally, level of transcripts for UCP2 is more dominant than UCP3 in rat heart.[25] The exact physiologic roles of UCP2 or UCP3 in intact heart are not yet completely understood. However, considering ectopic overexpression of UCP1 in mouse heart could also improve the severity of ischemia-reperfusion injury,[26] it may be speculated that both UCP2 and UCP3, once activated during ischemia-reperfusion, can contribute to reduced ROS generation and increased UCP3 level by PPARα activation raises ability to resist oxidative stress.

Reperfusion injury can be manifested by myocardial stunning or arrhythmia as well as myocardial infarction. ROS play a significant role in the development of myocardial stunning, a reversible contractile dysfunction following reperfusion,[27] and arrhythmias.[28] It was also shown that stability of the inner mitochondrial membrane potential is associated with reperfusion-induced arrhythmias.[29] Treatment of WY-14643 was associated with reduced incidence of arrhythmia in the current study. In addition, although not statistically significant, there was a trend to higher mean arterial pressure and rate pressure product in rats received WY-14643 during early phase of reperfusion. Regarding myocardial contractility, activation of UCP might have detrimental influence since increase in uncoupled respiration can lower mitochondrial ATP production. Indeed, increased UCP expression was shown in the failing human heart.[30] However, it might be possible that inefficiency in energy production is offset by benefits of reduced oxidative stress in acute ischemia-reperfusion.

Clinically, fibrates are synthetic PPARα agonists widely used to treat dyslipidemia. Fibrates stimulate fatty acid oxidation, improve lipoprotein metabolism, and exert anti-atherogenic effect by decreasing vascular

inflammation via PPARα activation.[31] Fibrates has been shown to be effective in prevention of cardiovascular events, particularly coronary disease.[32] Results of our study is in accordance with previous studies showing beneficial effect of PPARα activation, and suggests a possible role of PPARα agonists as a therapeutic agent for acute ischemia-reperfusion injury as well as a preventive medicine.

V. CONCLUSION

PPARα activation by WY-14643 conferred H9c2 cytoprotective effect against hypoxia-rexoygenation. Administration of PPARα agonist WY-14643 also decreased the degree of myocardial infarction and incidence of reperfusion-induced arrhythmia. The mechanism of protective effect of WY14643 involved increased UCP3 expression and resultant attenuation of ROS production.

REFERENCES

1 Madrazo JA, Kelly DP. The PPAR trio: regulators of myocardial energy metabolism in health and disease. J Mol Cell Cardiol 2008; 44 (6): 968-75.

2 Finck BN, Lehman JJ, Leone TC, Welch MJ, Bennett MJ, Kovacs A, *et al.* The cardiac phenotype induced by PPARalpha overexpression mimics that caused by diabetes mellitus. J Clin Invest 2002; 109 (1): 121-30.

3 Park SY, Cho YR, Finck BN, Kim HJ, Higashimori T, Hong EG, *et al.* Cardiac-specific overexpression of peroxisome proliferator-activated receptor-alpha causes insulin resistance in heart and liver. Diabetes 2005; 54 (9): 2514-24.

4 Finck BN, Han X, Courtois M, Aimond F, Nerbonne JM, Kovacs A, *et al.* A critical role for PPARalpha-mediated lipotoxicity in the pathogenesis of diabetic cardiomyopathy: modulation by dietary fat content. Proc Natl Acad Sci U S A 2003; 100 (3): 1226-31.

5 Yue TL, Bao W, Jucker BM, Gu JL, Romanic AM, Brown PJ, *et al.* Activation of peroxisome proliferator-activated receptor-alpha protects the heart from ischemia/reperfusion injury. Circulation 2003; 108 (19): 2393-9.

6 Bulhak AA, Sjoquist PO, Xu CB, Edvinsson L, Pernow J. Protection against myocardial ischaemia/reperfusion injury by PPAR-alpha activation is related to production of nitric oxide and endothelin-1. Basic Res Cardiol 2006; 101 (3): 244-52.

7 Bulhak AA, Jung C, Ostenson CG, Lundberg JO, Sjoquist PO, Pernow J. PPAR-alpha activation protects the type 2 diabetic myocardium against ischemia-reperfusion injury: involvement of the PI3-Kinase/Akt and NO pathway. Am J Physiol Heart Circ Physiol 2009; 296 (3): H719-27.

8 Krauss S, Zhang CY, Lowell BB. The mitochondrial uncoupling-protein homologues. Nat Rev Mol Cell Biol 2005; 6 (3): 248-61.

9 Korshunov SS, Skulachev VP, Starkov AA. High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. FEBS Lett 1997; 416 (1): 15-8.

10 Negre-Salvayre A, Hirtz C, Carrera G, Cazenave R, Troly M, Salvayre R, *et al.* A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation. FASEB J 1997; 11 (10): 809-15.

11 Minners J, van den Bos EJ, Yellon DM, Schwalb H, Opie LH, Sack MN. Dinitrophenol, cyclosporin A, and trimetazidine modulate preconditioning in the isolated rat heart: support for a mitochondrial role in cardioprotection. Cardiovasc Res 2000; 47 (1): 68-73.

12 Rodrigo GC, Lawrence CL, Standen NB. Dinitrophenol pretreatment of rat ventricular myocytes protects against damage by metabolic inhibition and reperfusion. J Mol Cell Cardiol 2002; 34 (5): 555-69.

13 Teshima Y, Akao M, Jones SP, Marban E. Uncoupling protein-2 overexpression inhibits mitochondrial death pathway in cardiomyocytes. Circ Res 2003; 93 (3): 192-200.

14 McLeod CJ, Aziz A, Hoyt RF, Jr., McCoy JP, Jr., Sack MN. Uncoupling proteins 2 and 3 function in concert to augment tolerance to cardiac ischemia. J Biol Chem 2005; 280 (39): 33470-6.

15 Young ME, Patil S, Ying J, Depre C, Ahuja HS, Shipley GL, *et al.* Uncoupling protein 3 transcription is regulated by peroxisome proliferator-activated receptor (alpha) in the adult rodent heart. FASEB J 2001; 15 (3): 833-45.

16 Murray AJ, Panagia M, Hauton D, Gibbons GF, Clarke K. Plasma free fatty acids and peroxisome proliferator-activated receptor alpha in the control of myocardial uncoupling protein levels. Diabetes 2005; 54 (12): 3496-502.

17 Sambandam N, Morabito D, Wagg C, Finck BN, Kelly DP, Lopaschuk GD. Chronic activation of PPARalpha is detrimental to cardiac recovery after ischemia. Am J Physiol Heart Circ Physiol 2006; 290 (1): H87-95.

18 Wayman NS, Hattori Y, McDonald MC, Mota-Filipe H, Cuzzocrea S, Pisano B*, et al.* Ligands of the peroxisome proliferator-activated receptors (PPAR-gamma and PPAR-alpha) reduce myocardial infarct size. FASEB J 2002; 16 (9): 1027-40.

19 van der Vusse GJ, Glatz JF, Stam HC, Reneman RS. Fatty acid homeostasis in the normoxic and ischemic heart. Physiol Rev 1992; 72 (4): 881-940.

20 Murphy E, Steenbergen C. Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. Physiol Rev 2008; 88 (2): 581-609

21 Kim JS, Jin Y, Lemasters JJ. Reactive oxygen species, but not Ca2+ overloading, trigger pH- and mitochondrial permeability transition-dependent death of adult rat myocytes after ischemia-reperfusion. Am J Physiol Heart Circ Physiol 2006; 290 (5): H2024-34.

22 Brand MD, Affourtit C, Esteves TC, Green K, Lambert AJ, Miwa S*, et al.* Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. Free Radic Biol Med 2004; 37 (6): 755-67.

23 Brennan JP, Southworth R, Medina RA, Davidson SM, Duchen MR, Shattock MJ. Mitochondrial uncoupling, with low concentration FCCP, induces ROS-dependent cardioprotection independent of KATP channel activation. Cardiovasc Res 2006; 72 (2): 313-21.

24 Ozcan C, Palmeri M, Horvath TL, Russell KS, Russell RR, 3rd. Role of uncoupling protein 3 in ischemia-reperfusion injury, arrhythmias, and preconditioning. Am J Physiol Heart Circ Physiol 2013; 304 (9):

H1192-200.

25 Alan L, Smolkova K, Kronusova E, Santorova J, Jezek P. Absolute levels of transcripts for mitochondrial uncoupling proteins UCP2, UCP3, UCP4, and UCP5 show different patterns in rat and mice tissues. J Bioenerg Biomembr 2009; 41 (1): 71-8.

26 Hoerter J, Gonzalez-Barroso MD, Couplan E, Mateo P, Gelly C, Cassard-Doulcier AM, *et al.* Mitochondrial uncoupling protein 1 expressed in the heart of transgenic mice protects against ischemic-reperfusion damage. Circulation 2004; 110 (5): 528-33.

27 Bolli R. Causative role of oxyradicals in myocardial stunning: a proven hypothesis. A brief review of the evidence demonstrating a major role of reactive oxygen species in several forms of postischemic dysfunction. Basic Res Cardiol 1998; 93 (3): 156-62.

28 Manning AS, Hearse DJ. Reperfusion-induced arrhythmias: mechanisms and prevention. J Mol Cell Cardiol 1984; 16 (6): 497-518.

29 Akar FG, Aon MA, Tomaselli GF, O'Rourke B. The mitochondrial origin of postischemic arrhythmias. J Clin Invest 2005; 115 (12): 3527-35.

30 Murray AJ, Anderson RE, Watson GC, Radda GK, Clarke K. Uncoupling proteins in human heart. Lancet 2004; 364 (9447): 1786-8.

31 Lefebvre P, Chinetti G, Fruchart JC, Staels B. Sorting out the roles of PPAR alpha in energy metabolism and vascular homeostasis. J Clin Invest 2006; 116 (3): 571-80.

32 Jun M, Foote C, Lv J, Neal B, Patel A, Nicholls SJ, *et al.* Effects of fibrates on cardiovascular outcomes: a systematic review and meta-analysis. Lancet 2010; 375 (9729): 1875-84.

ABSTRACT (IN KOREAN)

Peroxisome proliferator-activated receptor α 활성화가 심장 허혈-재관류 손상에 미치는 영향; uncoupling protein의 역할

<지도교수 곽영란>

연세대학교 대학원 의학과

송 종 욱

Peroxisome proliferator-activated receptor α (PPARα) 는 핵 수용체군에 속하는 전사인자로 지단백 및 지방산 대사에 관련되어 있는 것으로 잘 알려져 있다. 급성 허혈-재관류 손상에 있어 PPARα 항진제의 투여는 심장 보호 효과가 있다는 보고들이 있었으나 아직 그 기전은 명확하게 밝혀지지 않았다. 이 연구에서는 PPARα 항진제가 uncoupling protein (UCP)의 발현을 증가시키는지 확인하고 이것이 허혈-재관류 손상에서의 보호 효과와 관계가 있는지 알아보고자 하였다.

H9c2 세포에 PPARα 항진제인 WY-14643을 24시간 처리 후 UCP의 발현, 반응성 산소종의 발생 정도 및 저산소-재산소화 손상에서 세포 생존을 측정하였다. 백서에서 WY-14643 20 mg/kg 또는 위약을 복강내 투여 후 4시간 후 심장을 적출해 UCP의 발현을 측정하였다. 또한 심근경색정도 측정을 위해 WY-14643 20 mg/kg 또는 위약의 복강내 투여 4시간 후 좌전하행동맥 폐쇄를 통한 허혈-재관류 손상을 유도하였다. 허혈-재관류 손상 동안 평균동맥압, 심박수 등 혈역학 변수들 및 심전도 상 부정맥의 발생 여부도 측정하였다.

H9c2 세포에서 WY-14643 처리는 UCP3의 발현을 증가시켰다. UCP3와 달리 UCP2의 발현은 변화하지 않았다. WY-14643은 과산화수소 혹은 저산소로 인해 유도된 반응성 산소종의 발생을 감소시켰다. 또한 WY-14643을 처리한 세포는 저산소-재산소화

후 세포 생존이 향상되었다. UCP3의 siRNA를 transfection시킨 세포에서는 WY-14643을 처리하더라도 반응성 산소종의 발생 감소나 세포 생존의 향상이 관찰되지 않았다. 백서에서도 WY-14643 투여는 UCP3의 발현을 증가시켰으며 UCP2의 발현은 변화하지 않았다. WY-14643을 투여 받은 백서는 유의하게 심근경색의 정도가 적었다 (76 ± 8% vs. 42 ± 12%, 대조군 vs. 실험군, $p < 0.05$). 혈역학 변수들은 두 군간 차이가 없었으며 WY-14643을 투여 받은 군에서 재관류 시 부정맥의 발생 빈도가 더 낮았다 (9/10 vs. 3/10, $p < 0.05$).

결론적으로 PPARα 항진제는 허혈-재관류 손상에서 심근경색의 크기를 줄이고 재관류 시 부정맥의 발생 빈도를 낮췄다. PPARα 항진제는 세포에서도 반응성 산소종의 발생량을 줄이고 심근경색의 크기도 감소시켰으며 이러한 PPARα 항진제의 심장보호효과에는 UCP3의 발현 증가가 관계가 있는 것으로 생각된다.

핵심되는 말 : peroxisome proliferator-activated receptor α, uncoupling protein, 허혈-재관류 손상, 반응성 산소종