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Curcumin attenuates IR-induced myocardial injury by activating SIRT3

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Abstract. – OBJECTIVE: Ischemia-reperfusion (IR) injury remains an unresolved and complicated situation in clinical practice. In this study, H9c2 cardiomyocytes were subjected to curcumin (Cur) treatment in the absence or presence of the silent information regulator 3 (SIRT3) inhibitor 3-TYP and were then subjected to IR.

MATERIALS AND METHODS: H9c2 cells and male Sprague-Dawley (SD) rats were cultured. MTT assay was performed to assess H9c2 cell viability. Cellular apoptosis was analyzed by TUNEL assay. The expressions of Bcl-2, Bax, SIRT3, and AcSOD2 were measured by Western-blotting. The activities of SOD, GSH-Px, and MDA were determined using commercially available kits. The myocardial infarct size was evaluated using TTC staining.

RESULTS: Cur significantly increased H9c2 cell viability, decreased the cell apoptotic index, and altered several biochemical parameters, including upregulation of the anti-apoptotic protein Bcl-2, downregulation of the proapoptotic protein Bax and AcSOD2, activation of SIRT3, increase in SOD and GSH-Px activity, and decrease in MDA content. In isolated rat hearts, Cur significantly improved cardiac function, decreased infarct size, and lowered lactate dehydrogenase levels. These protective effects induced by Cur were reversed by treatment with the SIRT3 inhibitor 3-TYP.

CONCLUSIONS: These results demonstrate that Cur protects cardiomyocytes and that rat hearts were exposed to IRI by activating SIRT3.

Key Words:
Curcumin, Ischemia-reperfusion, Anti-apoptosis, SIRT3.

Introduction

Despite major therapeutic advances, ischemic heart disease remains a leading cause of death and a major cause of global mortality and morbidity. However, ischemia-reperfusion (IR) injury (IRI) remains an unresolved and complicated situation in ischemic heart disease[1-3]. Many factors contribute to IRI, such as enhanced oxidative stress, which includes superoxide anion, hydrogen peroxide, and hydroxyl radical generation, during the acute reperfusion phase[4]. Curcumin (Cur;1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6heptadiene-3,5-dione, diferuloylmethane) is a phenolic natural product isolated from the rhizome of *Curcuma longa* (turmeric), which has been widely used as a herbal remedy in China and Southeast Asia for centuries[5-7]. In recent years, several studies[8,9] have demonstrated that curcumin is an antioxidant and anti-inflammatory agent. Recent reports[8-11] have also indicated that Cur attenuates IRI in the brain, kidney, retina, liver, and intestines under various experimental conditions. Some researchers[10,12] have also observed that Cur post-treatment attenuates IRI through the activation of pro-survival signaling pathways. Silent information regulator 3 (SIRT3) is a NAD-dependent histone deacetylase and is activated by an increase in NAD. Other studies[13-16] have shown that SIRT3 plays a key role in the longevity effects elicited by calorie restriction, that SIRT3 shows evident

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cardioprotective effects, and that intermittent pneumatic compression (IPC) and resveratrol treatment reduce myocardial IRI via SIRT3 activation. Therefore, this investigation was designed to evaluate the cardioprotective effect of Cur pretreatment on myocardial IRI and to investigate whether SIRT3 plays an important role in a clinically relevant model of IRI-associated severe cardiac stress. The involvement of SIRT3 signaling in mediating the protective mechanisms of Cur was also evaluated.

Our results suggest that the development of therapeutic approaches targeting SIRT3 activity may be a promising pathway to protect cardiomyocytes against IRI.

Materials and Methods

Materials

Cur, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), 2′,7′-dichlorofluorescein diacetate (2′7′-DCFH-DA), and triphenyltetrazolium chloride (TTC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kits were purchased from Roche (Mannheim, Baden-Württemberg, Germany). The kits used to measure lactate dehydrogenase (LDH), methane dicarboxylic aldehyde (MDA), superoxide dismutase (SOD), and glutathione (GSH) were purchased from the Institute of Jiancheng Bioengineering (Nanjing, Jiangsu, China). The SIRT3 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The SIRT3 inhibitor 3-TYP antibody was synthesized and characterized by the Innovative Drug Research Center (IDRC) at Chongqing University. The Bcl2, Bax, and β-actin antibodies were purchased from Cell Signaling (Boston, MA, USA). The rabbit anti-goat, goat anti-rabbit, and goat anti-mouse secondary antibodies were purchased from Zhongshan Company (Beijing, China). 10% fetal bovine serum (FBS), L-glutamine penicillin, and streptomycin were obtained from Gibco (Grand Island, NY, USA). H9c2 embryonic rat myocardium-derived cells, a well-characterized cell line that is widely used to study myocardial cell ischemia, were purchased from Tiancheng Technology (Shanghai, China).

Animals

Male Sprague-Dawley rats weighing 250-300 g were used. All experimental procedures were approved by the Research Commission on Ethics of the Fourth Military Medical University and were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH publication no. 80-23, revised in 1996).

Cell Culture and Treatments

H9c2 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 500 μg/mL penicillin, and 500 μg/mL streptomycin at 37°C in 5% CO_2 and 95% air. The Cur stock solution was prepared in dimethyl sulfoxide (DMSO) and immediately diluted with culture medium before the experiment. DMSO (0.01%) was used for the control group. After the treatments, the cells were harvested for further analysis.

Cardiomyocyte Preparation and Simulated Ischemia-Reperfusion (SIR) Treatment

The primary cultures of H9c2 cells were prepared using a previously reported method[11]. Briefly, the cardiomyocytes were exposed to an ischemic buffer containing the following reagents (in mM): NaCl (137), KCl (12), $MgCl_2$ (0.49), $CaCl_2{\cdot}2H_2O$ (0.9), and HEPES (4). This buffer was also supplemented with the following compounds (in mM): deoxyglucose (10), sodium dithionate (0.75), and lactate (20). The buffer pH was 6.5, and the cells were incubated for 1.5 h in a humidified cell culture incubator (21% O_2, 5% CO_2, 37°C). Reperfusion was initiated by returning the cells to the normal culture medium for 4 h in a humidified cell culture incubator (21% O_2, 5% CO_2, 37°C).

Preparation of Perfused Isolated Rat Hearts

As previously described[17], the male Sprague-Dawley rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal injection). The hearts were rapidly excised, placed in the ice-cold Krebs-Henseleit buffer (KHB), and mounted for retrograde perfusion using a modified Langendorff technique. To establish the IRI model, isolated perfused rat hearts were subjected to 45 min of ischemia followed by 120 min of reperfusion. The left ventricular developed pressure (LVDP) of the hearts was monitored using a transducer (Model 100 BP; BIOPAC Systems, Inc., Goleta, CA, USA).

Experimental Protocol

Step 1: Evaluate the viability of H9c2 cells after Cur pretreatment. The H9c2 cells were randomly divided into control and Cur (2.5, 5, 10 μM) groups. The effects of 0.1 and 100 μM Cur on H9c2 cells were also explored. The MTT results demonstrated that 0.1 μM Cur did not improve cell viability (compared to the IR group) and that 100 μM Cur was not better than 10 μM Cur in improving cell viability. However, no statistical differences were observed when comparing the effects of 5 μM and 10 μM Cur on cell viability. Therefore, 5 μM Cur was selected for this study.

Step 2: Investigate the role of SIRT3 in the myocardial protection induced by pretreatment with Cur or the SIRT3 inhibitor 3-TYP in H9c2 cells. First, we assessed the apoptotic index of H9c2 cells. The H9c2 cells were randomly divided into control, IR, Cur (5 μM) + IR, Cur (5 μM) + 3-TYP (5 μM) + IR, and 3-TYP (5 μM) + IR groups. Then, we detected the effects of Cur pretreatment on SIRT3, acetylated SOD2, Bcl-2, and Bax expression in IR-injured H9c2 cells. The H9c2 cells were randomly divided into IR, Cur (5 μM) + IR, Cur (5 μM) + 3-TYP (5 μM) + IR, and 3-TYP (5 μM) + IR groups.

Step 3: Assess the role of SIRT3 in the protection of H9c2 cells induced by Cur or 3-TYP pretreatment against oxidative stress. We used commercially available kits to measure the intracellular SOD, GSH-Px, and MDA contents. The cardiomyocytes were randomly divided into IR, Cur (5 μM) + IR, Cur (5 μM) + 3-TYP (5 μM) + IR, and 3-TYP (5 μM) + IR groups.

Step 4: Investigate the role of SIRT3 in myocardial protection after pretreatment with Cur or SIRT3 inhibitor 3-TYP in isolated rat hearts. The hearts were randomly divided into IR, Cur (1 μM) + IR, Cur (1 μM) + 3-TYP (1 μM) + IR, and 3-TYP (1 μM) + IR groups.

Analysis of Cell Viability

An MTT assay was performed to assess H9c2 cell viability. After the H9c2 cells were treated and washed with PBS, 100 μL of 0.5 μM MTT solution in phenol red-free DMEM was added to the cells, and the samples were incubated for 4 h at 37°C. The results were determined by measuring absorbance at 490 nm, using a spectrophotometer (SpectraMax 190, Molecular Device, San Jose, CA, USA), and H9c2 cell viability was expressed as an optical density (OD) value. In addition, cell morphology was observed under an inverted/phase contrast microscope, and pictures were captured using an Olympus BX61 microscope (Tokyo, Japan).

Cellular Apoptosis Assay

Cellular apoptosis was analyzed by performing a TUNEL assay using an *in situ* cell death detection kit. A double-staining technique was used, according to the manufacturer's instructions. After the H9c2 cells were fixed in paraformaldehyde (4%) for 24 h, the TUNEL assay was performed to stain the nuclei of apoptotic cells (green), and 4′6-diamino-2-phenylindole (DAPI) was used to stain the nuclei of all the cells (blue). Pictures were taken at an original magnification of ×200. The apoptotic index was expressed as the number of positively stained apoptotic H9c2 cells/the total number of H9c2 cells counted ×100%.

Western-blot Analyses

The H9c2 samples were homogenized in lysis buffer containing 50 mmol/L Tris-HCL (pH 7.3), 150 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L dithiothreitol, 1% Triton X-100, and 1% protease inhibitor cocktail. The lysates were centrifuged for 15 min at 12,000 rpm. After the protein concentration was quantified, 30 μg of total protein was separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and, then, transferred to nitrocellulose membranes. The membranes were blocked for 1 h in Tris-buffered saline and Tween 20 (TBST, pH 7.6) containing 5% non-fat dry milk, and were then incubated overnight at 4°C with antibodies for SIRT3 (1:500 dilution), Bcl-2, Bax, AcSOD2, and β-actin (1:1000 dilution). After three TBST washing, the membranes were probed with appropriate secondary antibodies (1:5000 dilution) at room temperature for 90 min and then washed as described above. The protein bands were detected using chemiluminescence and quantified using the Quantity One software package (Bio-Rad, Hercules, CA, USA). The results of the control group were defined as 100%.

Measurement of the Intracellular Contents of SOD, GSH-Px, and MDA

As described previously[18], the activities of SOD, GSH-Px, and MDA were determined using commercially available kits. All procedures were performed in complete compliance with the man-

ufacturer's instructions. The enzyme activities were expressed as units per milligram of protein. The assay for measuring SOD activity was based on the ability of SOD to inhibit the oxidation of hydroxylamine by O_2^- produced from the xanthine-xanthine oxidase system. One unit of SOD activity was defined as the amount that reduced the absorbance at 550 nm by 50%. The assay used to measure GSH-Px activity was performed by quantifying the rate of oxidation of reduced glutathione to oxidized glutathione by H_2O_2, which is catalyzed by GSH-Px. One unit of GSH-Px was defined as the amount that reduced the GSH level by 1 μM at 412 nm in 1 min/mg protein. MDA was measured at 532 nm by its reaction with thiobarbituric acid to form a stable chromophoric product. The MDA level was expressed as nanomoles per milligram protein.

Myocardial Infarct Size

The myocardial infarct size *in vitro* was evaluated using TTC staining. Each treated heart was rapidly excised and serially sectioned along the long axis into six slices. This step was followed by incubation in 1% TTC for 10 min at 37°C to demarcate the viable and nonviable myocardium. The six-myocardial sections were individually weighed. The isolated hearts were subjected to global ischemia; therefore, the entire ventricle was considered as the infarcted area and the area at risk (AAR). The normalized infarct size was expressed as the ratio of the infarct size to the total AAR. An observer (blind protocol) was assigned to assess the percentage of the infarcted area using a computer-assisted planimetry technique (OPTIMAS v. 5.2; BioScan Inc., Edmonds, WA, USA) on randomly chosen fields under high-power magnification. The apoptotic index was expressed as the ratio of positively stained apoptotic myocytes to the total number of myocytes counted ×100%.

Measurement of LDH Release

Lactate dehydrogenase (LDH) release is usually measured to evaluate the presence of necrotic cell death. The levels of LDH in the coronary effluent were determined using an ELISA kit, according to the manufacturer's instructions. The amount of LDH released during the 60 min of reperfusion was measured by calculating the total amount of LDH from the coronary effluent of individual 5-min collections. The amount of LDH was normalized against the wet weight of the heart and expressed as IU/g.

Statistical Analysis

Data were analyzed using GraphPad Prism 5.0 statistical software (La Jolla, CA, USA). All the values are presented as the mean ± standard error of the mean (SEM). Group comparisons were performed using one-way ANOVA (SPSS 13.0), followed by Tukey's test. A difference of $p < 0.05$ was considered statistically significant.

Results

Effect of Pretreatment with Cur at Different Concentrations on Cell Viability

Compared with that in the IR group, pretreatment with Cur (2.5, 5, 10 μM) significantly increased cell viability; Cur treatment had no effect on cell viability compared to the control group. In addition, when the groups treated with different concentrations of Cur were compared with each other, cell viability was significantly increased in the Cur (5, 10 μM) groups compared with the Cur (2.5 μM) group (Figure 1).

Effect of Pretreatment with Cur and SIRT3 Inhibitor 3-TYP on the Apoptotic Index and Protein Expression in 3-TYP-injured Cardiomyocytes

Cur (5 μM) pretreatment significantly decreased the apoptotic index compared to that in the control and IR groups (Figure 2A). However,

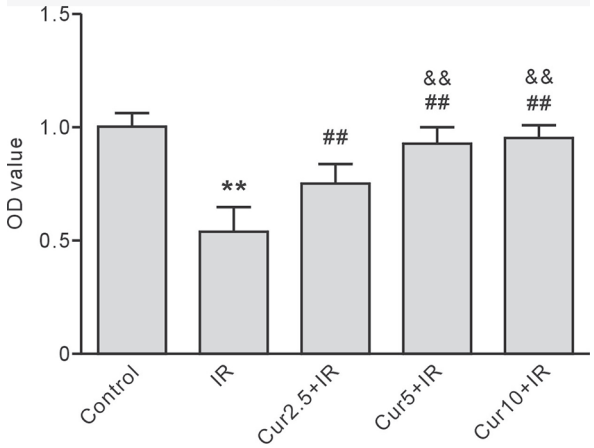

Figure 1. Effect of Cur on H9C2 cell viability following ischemia and reperfusion Each bar represents the mean ± SEM. Cur 2.5, 5, and 10 represent the concentrations of curcumin at 1, 5, and 20 mM, respectively. n = 6, $^{**}p < 0.01$ *vs.* control, $^{\#\#}p < 0.01$ *vs.* IR, $^{\&\&}p < 0.01$ *vs.* Cur 2.5 + IR. Cur, curcumin; Cur 2.5 + IR, ischemia/reperfusion with 2.5 mM Cur.

in the Cur pretreatment group, the protective effect of Cur pretreatment was abolished by SIRT3 inhibitor 3-TYP (Figure 2A and B). In addition, Cur pretreatment significantly increased Bcl-2 levels and decreased Bax levels in cardiomyocytes compared with those in the IR group. However, pretreatment with SIRT3 inhibitor 3-TYP (5 μM) abolished most of the protective effect and increased the passive effect mediated by Cur pretreatment (Figure 2C).

To further evaluate the role of SIRT3, the SIRT3 expression and its downstream protein AcSOD expression were measured. We found that Cur (5 μM) pretreatment significantly increased SIRT3 and decreased AcSOD levels compared to those in the IR group (Figure 3). More importantly, the Cur-induced protection effect on SIRT3 and AcSOD expression was abolished by SIRT3 inhibitor 3-TYP (5 μM) (Figure 3).

Effects of Cur Pretreatment and 3-TYP on Oxidative Damage Indicators in IR-injured Cardiomyocytes

Compared with the IR group, Cur (5 μM) markedly increased SOD and GSH-Px activities, and decreased MDA content. However, these alterations were blocked by SIRT3 inhibitor 3-TYP (5 μM). SIRT3 inhibitor 3-TYP had no effect on H9c2 cells subjected to IR (Figure 4).

Effects of Cur Pretreatment on Cardiac Function, Infarct Size, and LDH Release in Isolated Rat Hearts

Cur (1 μM) pretreatment significantly increased the functional recovery of post-ischemic hearts, as demonstrated by significantly higher LVDP throughout the reperfusion period in the Cur pretreatment groups compared with that in the IR group (Figure 5). LDH release (Figure 6C) and the myocardial infarct size were significantly increased in the hearts subjected to IR. Cur could significantly reduce infarct size and LDH release compared with the IR group (Figure 6A and B). Moreover, this effect was reversed by SIRT3 inhibitor 3-TYP (1 μM) (Figure 6A and B). However, 3-TYP had no effect on the hearts that suffered from IR (Figures 5 and 6).

Discussion

IRI in cardiomyocytes plays an important role in ischemic heart disease. It is harmful to the cardiovascular system and is responsible for cardiac failure, morbidity, and mortality after cardiac operations, and myocardial infarction[19,20]. Some reports[21] have shown that mitochondrial SIRT3 plays an essential role in mediating cell survival and that resveratrol protects cardiomyocytes from oxidative stress-induced apoptosis by activating SIRT3. Some studies[22] have also indicated that curcumin (Cur) pretreatment protects cardiomyocytes from IRI-induced apoptosis by activating SIRT1. However, the exact mechanism underlying the prevention of IRI in cardiomyocytes by Cur pretreatment, i.e., whether this occurs through SIRT3 activation, has not been elucidated.

Curcumin, the active component of the traditional Chinese drug *Curcuma longa*, has a wide spectrum of biological functions, including anti-inflammatory, antioxidant, cardioprotective, and anticancer properties, and lacks toxic and mutagenic activities[23-27]. Curcumin has been tested as a potential therapeutic agent in several pathological conditions, including cardiovascular disease and other vascular dysfunctions[3].

SIRT3 is a class III histone deacetylase (HDAC). AceCS2, the major mitochondrial NAD-dependent lysine deacetylase, regulates a variety of functions, and its inhibition may disrupt mitochondrial function, thereby impacting recovery from IRI[28]. The transcription factors FoxO3a and Ku70 are also substrates of SIRT3[29-31]. Overexpression of SIRT3 in brown adipocytes activates CREB phosphorylation and results in the stimulation of PGC-1α gene expression, and consequently decreases cellular reactive oxygen species (ROS) levels[32]. SIRT3 is highly expressed in cardiac cells and has attracted attention because of its direct link to the length of life. The hearts of SIRT3$^{-/-}$ mice are highly susceptible to hypertrophy and fibrosis, whereas SIRT3 overexpression protects cardiomyocytes against stress-mediated cell death[33]. Thus, SIRT3 can regulate cellular metabolism and gene expression. According to another study[34], SIRT3 deacetylates and activates SOD2.

Fiorillo et al[12] confirmed that the myocardial protective effects of Cur are attributed not only to its antioxidant properties but also to other mechanisms. Heme oxygenase-1, JNK, eNOS, NF-kappa B, activating protein-1, peroxisome proliferator-activated receptor gamma, sirtuin 1, and the process of autophagy have been reported to play a role in the protective effects of Cur in endothe-

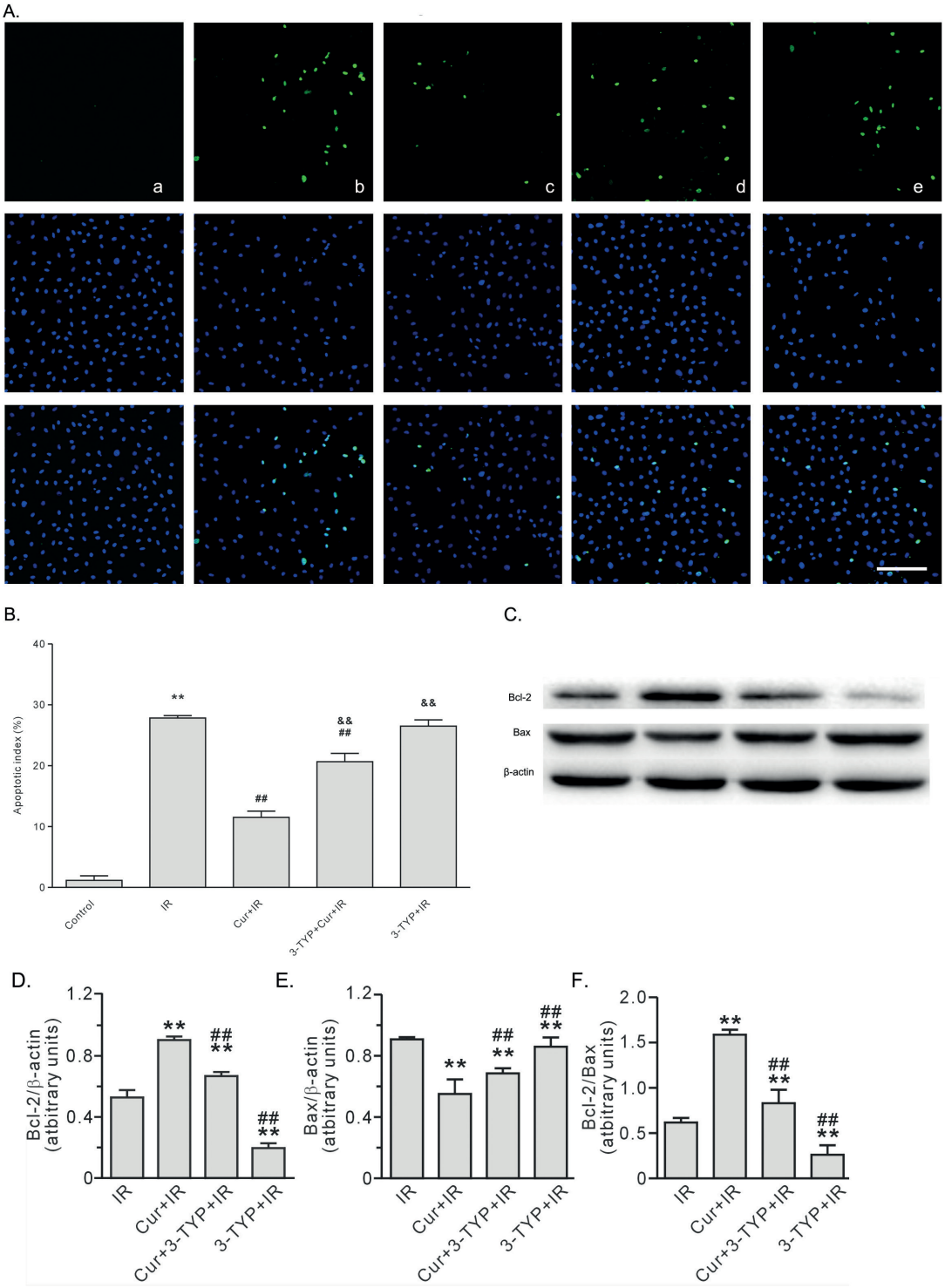

Figure 2. Effect of Cur and 3-TYP pretreatment on the apoptotic index and protein expression in H9c2 cells subjected to ischemia and reperfusion. ***(A)*** Representative images of the apoptotic cardiomyocytes are shown. The apoptotic cells were detected using immunofluorescent staining with TUNEL (green), and DAPI (blue) staining was used to label the nuclei. a. Control, b. IR, c. Cur + IR, d. Cur+3-TYP + IR, e. 3-TYP + IR. Scale bar, 20 mm ***(B)*** Group results regarding the apoptotic index. ***(C)*** Representative blots for Bcl-2, Bax protein, and β-actin. ***(D*** and ***E)*** Group results regarding densitometric analyses for Bcl-2 and Bax. ***(F)*** Group results regarding the ratio of Bcl-2 and Bax. Each bar represents the mean ± SEM. n = 6, $^{**}p < 0.01$ *vs.* IR, $^{\#\#}p < 0.01$ *vs.* Cur + IR. Cur + IR, ischemia/reperfusion with 5 mM Cur. 3-TYP, 3-(1H-1,2,3-triazol-4-yl)pyridine; CuR + 3-TYP + IR, ischemia/reperfusion with 5 mM Cur and 5 mM 3-TYP; 3-TYP + IR, ischemia/reperfusion with 5 mM 3-TYP.

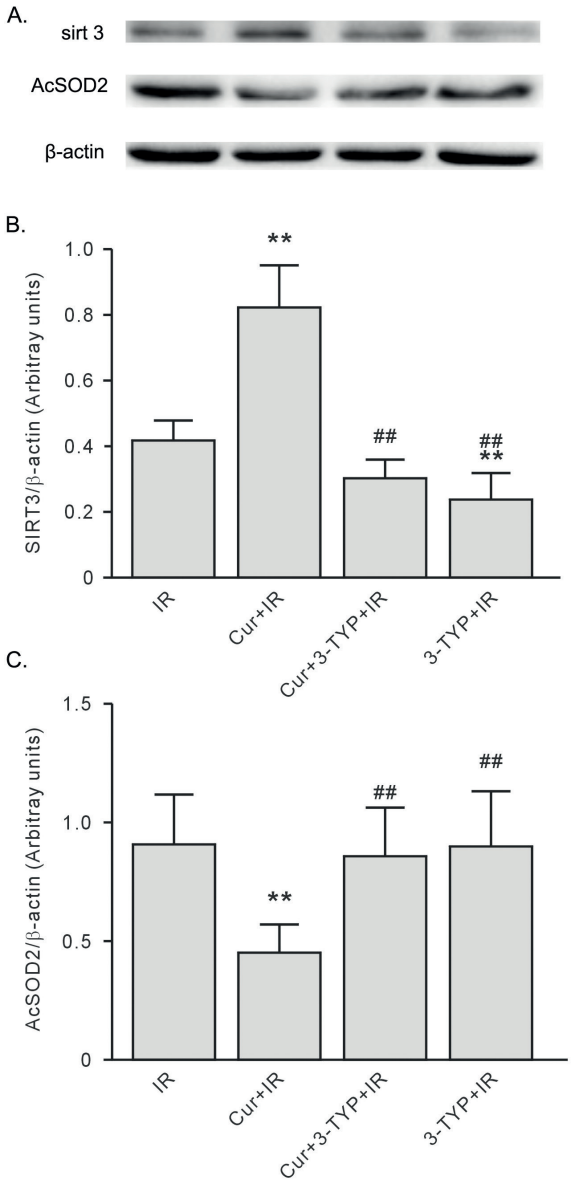

Figure 3. Effect of Cur and 3-TYP pretreatment on SIRT3 and acetylated SOD2 expression in H9c2 cells subjected to ischemia and reperfusion. (A) Representative blots for SIRT3, AcSOD2, and β-actin protein levels. (B) Group results regarding densitometric analyses for SIRT3. (C) Group results regarding densitometric analyses for AcSOD2. Each bar represents the mean ± SEM. n = 6, $^{**}p < 0.01$ *vs.* IR, $^{\#\#}p < 0.01$ *vs.* Cur + IR. The definitions of the abbreviations are as provided in the legends for Figures 1 and 2.

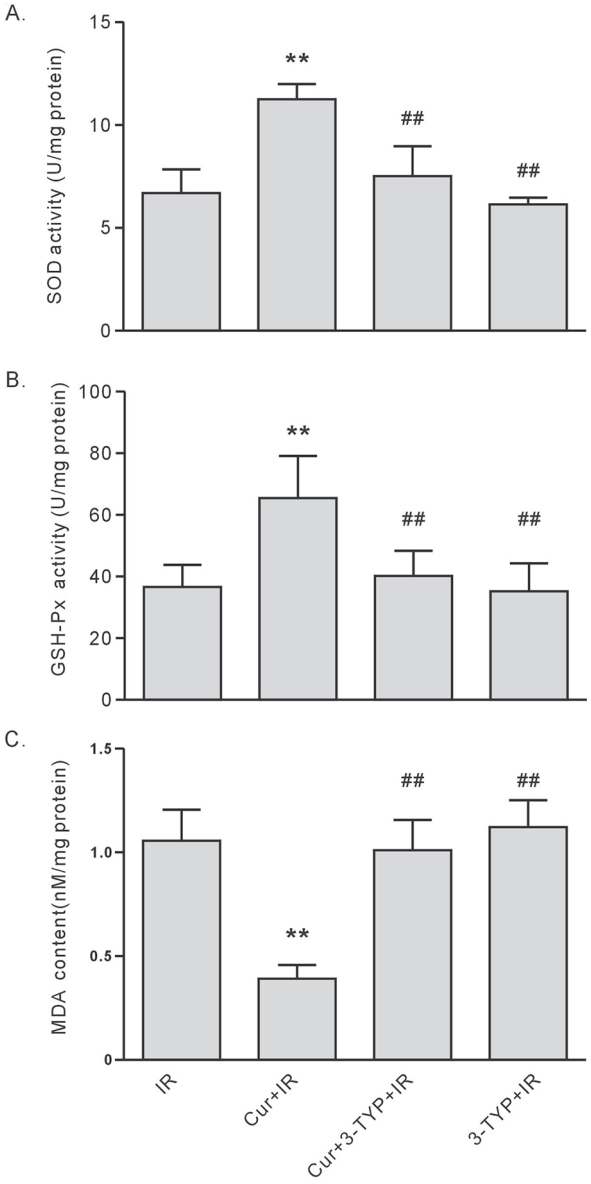

Figure 4. Effects of Cur and 3-TYP pretreatment on oxidative damage in H9c2 cells subjected to ischemia and reperfusion. ***(A)*** Total SOD activity in cardiomyocytes; ***(B)*** GSH-Px enzymatic activity in cardiomyocytes; ***(C)*** MDA production in cardiomyocytes. Each bar represents the mean ± SEM. n = 6, $^{**}p < 0.01$ *vs.* IR, $^{\#\#}p < 0.01$ *vs.* Cur + IR. The definitions of the abbreviations are as provided in Figures 1 and 2.

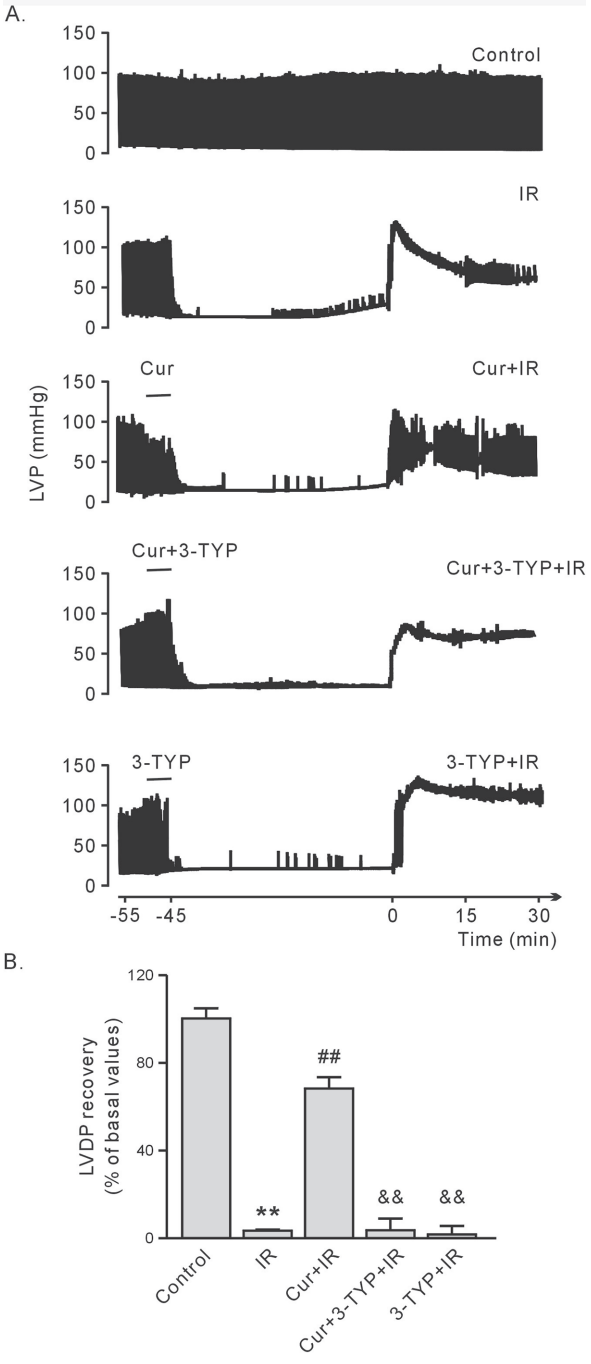

Figure 5. Effects of Cur and 3-TYP pretreatment on the function of isolated hearts subjected to ischemia and reperfusion. ***(A)*** Representative images of LVDP curves, as monitored using an MP150 pressure transducer system. ***(B)*** Group results on the recovery of LVDP at the end of perfusion. Each bar represents the mean ± SEM. n = 6, $^{**}p < 0.01$ *vs.* the control group, $^{\#\#}p < 0.01$ *vs.* IR, $^{\&\&}p < 0.01$ *vs.* Cur + IR. The definitions of the abbreviations are as provided in Figures 1 and 2.

lial cells with oxidative stress-induced injury[35-42]. However, the role of the SIRT3 signaling pathway in this process has not been previously identified.

Samant et al[43] investigated the role of SIRT3 in mediating the response to myocardial IR and showed that compared to control hearts, SIRT3(+/−) hearts were more vulnerable to IR, and showed less functional recovery and greater infarct size, suggesting that decreased SIRT3 levels increase the susceptibility of the hearts to IRI.

Oxidative stress in cardiomyocytes plays an important role in the pathogenesis of both heart failure and IRI[44]. Superoxide dismutases (SODs) are a class of enzymes that catalyze the detoxification of superoxide into oxygen and hydrogen peroxide. SODs are believed to be present in all oxygen-metabolizing organisms, and their physiological role is to balance the levels of intracellular ROS, which are products of aerobic metabolism that are normally produced in the mitochondria. Mitochondrial SOD2 is thought to play a crucial role in controlling the level of oxygen and producing a large flux of ROS. Mutations in SOD2 are associated with ageing and various human diseases, including idiopathic cardiomyopathy, sporadic motor neuron disease, and cancer[45].

Oxidative stress-induced SOD2 expression is believed to be an important cellular defense mechanism[45,46]. Chen et al[34] demonstrated that increased ROS levels stimulate SIRT3 transcription, leading to SOD2 deacetylation and activation. According to another report[47], SOD2 deacetylation by SIRT3 regulates SOD2 enzymatic activity. SIRT3 deacetylates SOD2 in response to ionizing radiation, indicating that SOD2 is a major downstream signal of SIRT3-mediated mitochondrial-derived O_2^- reduction. SOD2 activity is tightly regulated by acetylation at its lysine residues[48]. Our data corroborate these previous findings and suggest an inverse relationship between the activation of SIRT3 signaling and the inhibition of AcSOD2 expression. The SIRT3 inhibitor 3-TYP significantly increased AcSOD2 expression and reduced SIRT3 activity, thus abolishing the antiapoptotic effect of Cur.

Conclusions

Our findings suggest that Cur treatment exerts a profound cardioprotective effect against IRI-induced apoptosis. This protection appears to be largely due to modulation of the SIRT3 signaling pathway. Thus, Cur may be a promising candidate

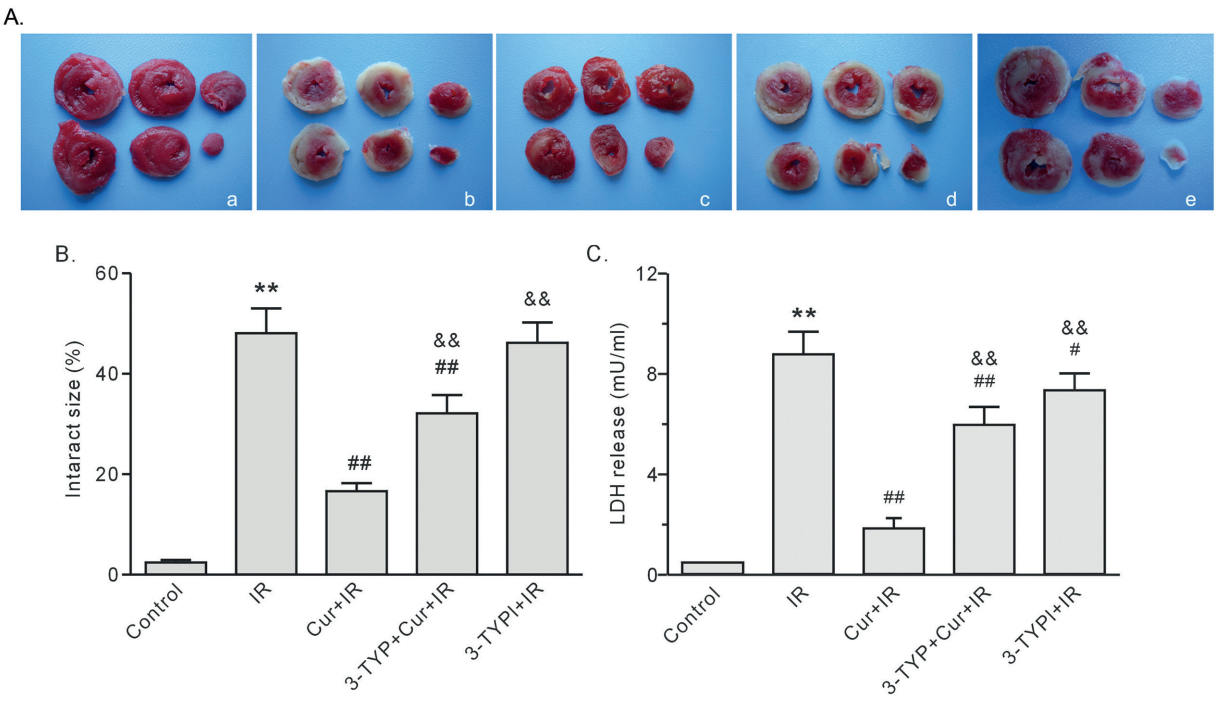

Figure 6. Effects of Cur and 3-TYP pretreatment on myocardial necrosis in isolated rat hearts subjected to ischemia and reperfusion. ***(A)*** Representative TTC staining of heart slices. a. Control, b. IR, c. Cur + IR, d. Cur + 3-TYP + IR, e. 3-TYP + IR; ***(B)*** Group results for myocardial infarct size; ***(C)*** Group results for LDH release. Each bar represents the mean ± SEM. n = 6, $^{**}p < 0.01$ *vs.* control, $^{\#\#}p < 0.01$ *vs.* IR, $^{\&\&}p < 0.01$ *vs.* Cur + IR. The definitions of the abbreviations are as provided in Figures 1 and 2.

for the treatment of IRI to decrease morbidity and mortality after cardiac operations and myocardial infarction.

Acknowledgments

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Conflict of Interest

The Authors declare that they have no conflict of interest.

References

1) Abete P, Testa G, Ferrara N, De Santis D, Capaccio P, Viati L, Calabrese C, Cacciatore F, Longobardi G, Condorelli M, Napoli C, Rengo F. Cardioprotective effect of ischemic preconditioning is preserved in food-restricted senescent rats. Am J Physiol Heart Circ Physiol 2002; 282: H1978-1987.
2) Bulvik BE, Berenshtein E, Meyron-Holtz EG, Konijn AM, Chevion M. Cardiac protection by preconditioning is generated via an iron-signal created by proteasomal degradation of iron proteins. PLoS One 2012; 7: e48947.
3) Chen KH, Chao D, Liu CF, Chen CF, Wang D. Curcumin attenuates airway hyperreactivity induced by ischemia-reperfusion of the pancreas in rats. Transplant Proc 2010; 42: 744-747.
4) Zweier JL, Talukder MA. The role of oxidants and free radicals in reperfusion injury. Cardiovasc Res 2006; 70: 181-190.
5) Duvoix A, Blasius R, Delhalle S, Schnekenburger M, Morceau F, Henry E, Dicato M, Diederich M. Chemopreventive and therapeutic effects of curcumin. Cancer Lett 2005; 223: 181-190.
6) Holt PR, Katz S, Kirshoff R. Curcumin therapy in inflammatory bowel disease: a pilot study. Dig Dis Sci 2005; 50: 2191-2193.
7) Salh B, Assi K, Templeman V, Parhar K, Owen D, Gomez-Munoz A, Jacobson K. Curcumin attenuates DNB-induced murine colitis. Am J Physiol Gastrointest Liver Physiol 2003; 285: G235-243.
8) Kunwar A, Barik A, Sandur SK, Indira Priyadarsini K. Differential antioxidant/pro-oxidant activity of dimethoxycurcumin, a synthetic analogue of curcumin. Free Radic Res 2011; 45: 959-965.
9) Lopresti AL, Hood SD, Drummond PD. Multiple antidepressant potential modes of action of curcumin: a review of its anti-inflammatory, monoaminergic,

antioxidant, immune-modulating and neuroprotective effects. J Psychopharmacol 2012; 26: 1512-1524.

10) Cosentino V, Fratter A, Cosentino M. Anti-inflammatory effects exerted by Killox®, an innovative formulation of food supplement with curcumin, in urology. Eur Rev Med Pharmacol Sci 2016; 20:1390-1398.

11) Srivastava G, Mehta JL. Currying the heart: curcumin and cardioprotection. J Cardiovasc Pharmacol Ther 2009; 14: 22-27.

12) Fiorillo C, Becatti M, Pensalfini A, Cecchi C, Lanzilao L, Donzelli G, Nassi N, Giannini L, Borchi E, Nassi P. Curcumin protects cardiac cells against ischemia-reperfusion injury: effects on oxidative stress, NF-kappaB, and JNK pathways. Free Radic Biol Med 2008; 45: 839-846.

13) Hsu CP, Zhai P, Yamamoto T, Maejima Y, Matsushima S, Hariharan N, Shao D, Takagi H, Oka S, Sadoshima J. Silent information regulator 1 protects the heart from ischemia/reperfusion. Circulation 2010; 122: 2170-2182.

14) Samuel SM, Thirunavukkarasu M, Penumathsa SV, Paul D, Maulik N. Akt/FOXO3a/SIRT1-mediated cardioprotection by n-tyrosol against ischemic stress in rat in vivo model of myocardial infarction: switching gears toward survival and longevity. J Agric Food Chem 2008; 56: 9692-9698.

15) Chen CJ, Yu W, Fu YC, Wang X, Li JL, Wang W. Resveratrol protects cardiomyocytes from hypoxia-induced apoptosis through the SIRT1-FoxO1 pathway. Biochem Biophys Res Commun 2009; 378: 389-393.

16) Nadtochiy SM, Redman E, Rahman I, Brookes PS. Lysine deacetylation in ischaemic preconditioning: the role of SIRT1. Cardiovasc Res 2011; 89: 643-649.

17) Zhang JY, Kong LH, Lai D, Jin ZX, Gu XM, Zhou JJ. Glutamate protects against Ca(2+) paradox-induced injury and inhibits calpain activity in isolated rat hearts. Clin Exp Pharmacol Physiol 2016; 43: 951-959.

18) Blanquicett C, Kang BY, Ritzenthaler JD, Jones DP, Hart CM. Oxidative stress modulates PPAR gamma in vascular endothelial cells. Free Radic Biol Med 2010; 48: 1618-1625.

19) Heusch G, Boengler K, Schulz R. Inhibition of mitochondrial permeability transition pore opening: the Holy Grail of cardioprotection. Basic Res Cardiol 2010; 105: 151-154.

20) Heusch G, Musiolik J, Gedik N, Skyschally A. Mitochondrial STAT3 activation and cardioprotection by ischemic postconditioning in pigs with regional myocardial ischemia/reperfusion. Circ Res 2011; 109: 1302-1308.

21) Yu W, Fu YC, Zhou XH, Chen CJ, Wang X, Lin RB, Wang W. Effects of resveratrol on H(2)O(2)-induced apoptosis and expression of SIRTs in H9c2 cells. J Cell Biochem 2009; 107: 741-747.

22) Yang Y, Duan W, Lin Y, Yi W, Liang Z, Yan J, Wang N, Deng C, Zhang S, Li Y, Chen W, Yu S, Yi D, Jin Z. SIRT1 activation by curcumin pretreatment attenuates mitochondrial oxidative damage induced by myocardial ischemia reperfusion injury. Free Radic Biol Med 2013; 65: 667-679.

23) DeWeerdt S. Diagnostics: a testing journey. Nature 2011; 474: S20-21.

24) Nemmar A, Subramaniyan D, Ali BH. Protective effect of curcumin on pulmonary and cardiovascular effects induced by repeated exposure to diesel exhaust particles in mice. PloS One 2012; 7: e39554.

25) Yang D, Li T, Liu Z, Arbez N, Yan J, Moran TH, Ross CA, Smith WW. LRRK2 kinase activity mediates toxic interactions between genetic mutation and oxidative stress in a Drosophila model: suppression by curcumin. Neurobiol Dis 2012; 47: 385-392.

26) Yallapu MM, Othman SF, Curtis ET, Bauer NA, Chauhan N, Kumar D, Jaggi M, Chauhan SC. Curcumin-loaded magnetic nanoparticles for breast cancer therapeutics and imaging applications. Int J Nanomedicine 2012; 7: 1761-1779.

27) Yang Y, Duan W, Zhou J, Yan J, Liu J, Zhang J, Jin Z, Yi D. Protective effects of adenosine on the diabetic myocardium against ischemia-reperfusion injury: role of calpain. Med Hypotheses 2012; 79: 462-464.

28) Lindahl T, Satoh MS, Poirier GG, Klungland A. Post-translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks. Trends Biochem Sci 1995; 20: 405-411.

29) Jacobs KM, Pennington JD, Bisht KS, Aykin-Burns N, Kim HS, Mishra M, Sun L, Nguyen P, Ahn BH, Leclerc J, Deng CX, Spitz DR, Gius D. SIRT3 interacts with the daf-16 homolog FOXO3a in the mitochondria, as well as increases FOXO3a dependent gene expression. Int J Biol Sci 2008; 4: 291-299.

30) Sundaresan NR, Gupta M, Kim G, Rajamohan SB, Isbatan A, Gupta MP. Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. J Clin Invest 2009; 119: 2758-2771.

31) Sundaresan NR, Samant SA, Pillai VB, Rajamohan SB, Gupta MP. SIRT3 is a stress-responsive deacetylase in cardiomyocytes that protects cells from stress-mediated cell death by deacetylation of Ku70. Mol Cell Biol 2008; 28: 6384-6401.

32) Kong X, Wang R, Xue Y, Liu X, Zhang H, Chen Y, Fang F, Chang Y. Sirtuin 3, a new target of PGC-1alpha, plays an important role in the suppression of ROS and mitochondrial biogenesis. PloS One 2010; 5: e11707.

33) Pillai VB, Samant S, Sundaresan NR, Raghuraman H, Kim G, Bonner MY, Arbiser JL, Walker DI, Jones DP, Gius D, Gupta MP. Honokiol blocks and reverses cardiac hypertrophy in mice by activating mitochondrial Sirt3. Nat Commun 2015; 6: 6656.

34) Chen Y, Zhang J, Lin Y, Lei Q, Guan KL, Zhao S, Xiong Y. Tumour suppressor SIRT3 deacetylates and activates manganese superoxide dismutase to scavenge ROS. EMBO Rep 2011; 12: 534-541.

35) Motterlini R, Foresti R, Bassi R, Green CJ. Curcumin, an antioxidant and anti-inflammatory agent, induces heme oxygenase-1 and protects endothelial cells against oxidative stress. Free Radic Biol Med 2000; 28: 1303-1312.

36) Scapagnini G, Foresti R, Calabrese V, Giuffrida Stella AM, Green CJ, Motterlini R. Caffeic acid phenethyl ester and curcumin: a novel class of heme oxygenase-1 inducers. Mol Pharmacol 2002; 61: 554-561.

37) Kumar A, Kingdon E, Norman J. The isoprostane 8-iso-PGF2alpha suppresses monocyte adhesion to human microvascular endothelial cells via two independent mechanisms. FASEB J 2005; 19: 443-445.

38) Chai H, Yan S, Lin P, Lumsden AB, Yao Q, Chen C. Curcumin blocks HIV protease inhibitor ritonavir-induced vascular dysfunction in porcine coronary arteries. J Am Coll Surg 2005; 200: 820-830.

39) Chiu J, Khan ZA, Farhangkhoee H, Chakrabarti S. Curcumin prevents diabetes-associated abnormalities in the kidneys by inhibiting p300 and nuclear factor-kappaB. Nutrition 2009; 25: 964-972.

40) Farhangkhoee H, Khan ZA, Chen S, Chakrabarti S. Differential effects of curcumin on vasoactive factors in the diabetic rat heart. Nutr Metab (Lond) 2006; 3: 27.

41) Chung S, Yao H, Caito S, Hwang JW, Arunachalam G, Rahman I. Regulation of SIRT1 in cellular functions: role of polyphenols. Arch Biochem Biophys 2010; 501: 79-90.

42) Han J, Pan XY, Xu Y, Xiao Y, An Y, Tie L, Pan Y, Li XJ. Curcumin induces autophagy to protect vascular endothelial cell survival from oxidative stress damage. Autophagy 2012; 8: 812-825.

43) Samant SA, Zhang HJ, Hong Z, Pillai VB, Sundaresan NR, Wolfgeher D, Archer SL, Chan DC, Gupta MP. SIRT3 deacetylates and activates OPA1 to regulate mitochondrial dynamics during stress. Mol Cell Biol 2014; 34: 807-819.

44) Murdoch CE, Zhang M, Cave AC, Shah AM. NADPH oxidase-dependent redox signalling in cardiac hypertrophy, remodelling and failure. Cardiovasc Res 2006; 71: 208-215.

45) Ghneim HK. The kinetics of the effect of manganese supplementation on SOD2 activity in senescent human fibroblasts. Eur Rev Med Pharmacol Sci 2016; 20: 1866-1880.

46) Qiu X, Brown K, Hirschey MD, Verdin E, Chen D. Calorie restriction reduces oxidative stress by SIRT3-mediated SOD2 activation. Cell Metab 2010; 12: 662-667.

47) Pi H, Xu S, Reiter RJ, Guo P, Zhang L, Li Y, Li M, Cao Z, Tian L, Xie J, Zhang R, He M, Lu Y, Liu C, Duan W, Yu Z, Zhou Z. SIRT3-SOD2-mROS-dependent autophagy in cadmium-induced hepatotoxicity and salvage by melatonin. Autophagy 2015; 11: 1037-1051.

48) Tao R, Coleman MC, Pennington JD, Ozden O, Park SH, Jiang H, Kim HS, Flynn CR, Hill S, Hayes McDonald W, Olivier AK, Spitz DR, Gius D. Sirt3-mediated deacetylation of evolutionarily conserved lysine 122 regulates MnSOD activity in response to stress. Mol Cell 2010; 40: 893-904.