Resveratrol pretreatment protects rat hearts from ischemia/reperfusion injury partly via a NALP3 inflammasome pathway

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Abstract: Inflammatory responses are key players in myocardial ischemia/reperfusion (I/R) injury. Our previous studies showed that resveratrol alleviated I/R injury in myocardial I/R animal models, but whether the NALP3 inflammasome pathway contributes to the mechanisms remains to be elucidated. In this study, we explored the modulation effect of resveratrol on myocardial I/R-induced inflammatory responses in rats. Myocardial I/R animal models were induced by occlusion of the left anterior descending coronary arteries (LADs) for 30 min, followed by 2 h of reperfusion. Resveratrol was administered in different doses (2.5, 5, and 10 mg/kg) at the same time as the onset of reperfusion. The serum concentrations of the trinitrotoluene (TnT) and MB isoenzyme creatine kinase (CK-MB) were detected using an automatic biochemical analyzer. Myocardial ultrastructure and morphology were observed with an electron microscope and a light microscope. Myocardial ischemia and infarct sizes were evaluated using Evans blue and tetrazolium chloride (TTC) staining. The NALP3, Caspase1, interleukin 1β (IL-1β) and interleukin 18 (IL-18) mRNA levels were evaluated using RT-PCR. The NALP3 and Caspase1 protein expression levels were detected by western blotting. The IL-1β and IL-18 content in peripheral blood was measured by enzyme-linked immunosorbent assay (ELISA). The myocardial structure in myocardial ischemia reperfusion injury (MI/RI) rats was extensively damaged. After preconditioning with different concentrations of resveratrol (2.5, 5 and 10 mg/kg), the pathology and morphology were significantly improved in a dose-dependent manner. Our results showed that resveratrol treatment significantly reduced the infarct volume and myocardial fibrosis, resulting in myocardial cells that lined up in a more orderly fashion and dose-dependent decreases in TnT and CK-MB levels in the serum of the I/R rats. Resveratrol also significantly modulated mRNA and protein levels by down-regulating NALP3 and Caspase1 expression and IL-1β and IL-18 activation. These results suggest that the NALP3 inflammasome is activated during the myocardial I/R injury process and that the secretion of the inflammatory cytokines IL-1β and IL-18 mediates the cascade inflammatory response. Resveratrol may play an important role in protecting the myocardium against I/R injury in rats by inhibiting the expression and activation of the NALP3 inflammatory body. Therefore, the attenuation of the inflammatory response may be involved in the cardioprotective mechanisms of resveratrol in response to myocardial I/R injury.

Keywords: Resveratrol, I/R injury, NALP3, inflammasome body, Caspase1

Introduction

Myocardial infarction (MI) is a common and serious clinical occurrence and a future threat to public health worldwide. The underlying pathogenesis of MI occurs when atherosclerotic plaques become unstable and rupture, facilitating thrombus formation and resulting in occlusion of the coronary artery. Successful reperfusion of the myocardium after MI, such as percutaneous coronary intervention (PCI), thrombolytic or fibrinolytic therapy and coronary artery bypass graft (CABG), may effectively reduce the infarct size and improve the clinical outcome. However, the resulting ischemia/reperfusion (I/R) injuries may enhance the harmful effects on the myocardium [1]. Recent studies have demonstrated that inflammation plays an important role in myocardial I/R injury [2-4]. Experimental data have also shown that inhibition of the inflammatory responses could substantially reduce myocardial I/R injury [5-7].
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However, the mechanism by which the I/R stimuli trigger inflammatory responses in the heart remains unclear.

Recently, a new innate immune pathway involving the inflammasome was shown to be a key player in various types of sterile inflammatory diseases, such as pseudogout, Alzheimer’s disease, asbestosis, atherosclerosis, silicosis, type 2 diabetes mellitus (T2DM) and gout [8-14]. Currently, four inflammasome prototypes have been reported: the NLRC4 (IPAF) inflammasome, the AIM2 (absence in melanoma 2) inflammasome, the NLRP3 (NALP3) inflammasome and the NALP1 inflammasome [15, 16]. The NALP3 inflammasome, which contains pro-Caspase1 and the adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC), is the most studied and can recognize diverse endogenous metabolic danger signals, leading to sterile inflammation. Activation of the NLRP3 inflammasome leads to the production of the proinflammatory cytokine interleukin 1 (IL-1), resulting in an inflammatory response [17]. A previous investigation demonstrated that IL-1 inhibition reduced myocardial I/R injury [18]. Additionally, ASCs were detected in the myocardial tissues of MI patients [19]. NLRP3 also plays an important role in the production of inflammatory cytokines and in subsequent I/R injury in the liver [20]. However, whether the NALP3 inflammasome pathway is involved in myocardial I/R injury is unknown.

Resveratrol (trans-3,5,4'-trihydroxystilbene) is one of the most biologically active polyphenols present in red wine and has been pharmacologically evaluated for a variety of illnesses [21], including cancer, cardiovascular disease, neurological problems and diabetes. In its synthetic or purified form, resveratrol can impair the activity and production of nitric oxide (NO) [22], decrease endothelin-1 (ET-1) expression [23, 24], reduce the synthesis of lipids in the rat liver [25], decrease low-density lipoprotein-cholesterol oxidation [26], inhibit platelet activation/aggregation [27-30], inhibit the activity of some protein kinases [31], and strongly inhibit intracellular and extracellular reactive oxygen species (ROS) production [32, 33]. Previous studies have shown that resveratrol protected from myocardial I/R injury via NO-dependent and NO-independent mechanisms in rats [34, 35]; however, whether NALP3 inflammasome mechanisms are involved in its cardioprotective activities are not clear.

In the present study, we hypothesized that different concentrations of resveratrol conferred cardioprotection against I/R injury. Furthermore, we explored whether resveratrol exerted its anti-I/R injury effect via inhibition of the activation of the NALP3 inflammasome pathway.

Materials and methods

Materials

Resveratrol was purchased from Sigma Chemical (St. Louis, MO, USA) and was dissolved in DMSO on the day of administration. All antibodies were obtained from Abcam Technology Inc. (Cambridge, MA, USA). Trinitro-toluene (TnT), MB isoenzyme creatine kinase (CK-MB), IL-1β and IL-18 assay kits were purchased from Sigma Chemical Company (St. Louis, MO, USA). The real-time PCR kits were obtained from TOYOBO Biotechnology Company (Japan).

Animal preparation

The procedures and animal care were approved by the Animal Care and Use Committee of Wuhan University and conformed to the principles of laboratory animal care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). Fifty healthy male Sprague-Dawley (SD) rats (270-300 g body wt) were purchased from Tongji Medical School, Huazhong University of Science and Technology (HUST), China. The rats were randomly assigned to five groups containing 10 rats each: the Sham-operated group, the I/R group and three groups of rats pretreated with given dosages of resveratrol, including the 2.5 mg/kg dosage group (L-Res group), the 5 mg/kg dosage group (M-Res group), and the 10 mg/kg dosage group (H-Res group). The dosages were adjusted based on the data collected from pretests and relevant documents. The I/R model was established among the three drug groups and the I/R group by occluding the left anterior descending coronary artery (LAD) for 30 min, followed by 2 h of reperfusion as previously described [36].
Sham-operated animals underwent all surgical procedures without I/R. All five groups were intraperitoneally injected with resveratrol (2.5, 5, and 10 mg/kg) or vehicle (dimethyl sulfoxide, 0.1 ml/kg) 1 h prior to LAD occlusion.

Pathological examination by light microscopy

The formalin-fixed, paraffin-embedded sections of the rat left ventricular myocardium were stained with hematoxylin and eosin. Then, the histomorphology changes were observed under a light microscope (≤ 400× magnification).

Pathological examination by transmission electron microscopy

The specimens were pre-fixed in 2.5% glutaraldehyde and rinsed with phosphate-buffered saline (PBS). Next, the specimens were fixed in 1% osmium tetroxide, dehydrated in ethanol and subsequently embedded in epoxy resin (Epon 812); then, the specimens were sectioned into 70-nm ultra-thin slices and stained with uranyl acetate and lead citrate. The ultrastructural changes were observed with a transmission electron microscope (Hitachi, Ltd, Japan).

Myocardial infarct size

The ischemic area and infarct size were estimated using the triphenyl tetrazolium chloride (TTC)-Evans blue technique. After 2 h of reperfusion, the coronary artery was re-occluded, and 2.0 ml of methyl blue (3%) was injected intravenously to denote the area at risk. Then, the heart was excised, and the atria were removed. The ventricular tissue was sliced into 1-mm sections, which were incubated in tetrazolium dye (1% 2,3,5-triphenyltetrazolium chloride/0.9% NaCl, pH 7.4) at 37°C for 40 min. Sections were placed in 10% formaldehyde in saline for 2 days prior to the excision of the infarct (white) tissue. The infarct tissue was quantitated as the percentage of the occluded zone.

Plasma TnT and CK-MB analysis

TnT and CK-MB released from the cells into the whole blood were measured with a commercially available assay kit (Sigma Chemical Co.). An aliquot of serum was mixed with 1 ml TnT or CK-MB reagent, and the absorbance at 340 nm caused by nicotinamide adenine dinucleotide (NADH) production was followed for 5 min at 30°C. The change in the rate of absorbance was directly proportional to the TnT or CK-MB activity.

Determination of the IL-1β and IL-18 levels in serum samples

The enzyme-linked immunosorbent assay (ELISA) method was employed to determine the levels of IL-1β and IL-18 in serum samples. The cardiac muscle tissues were cut into small pieces, weighed, and homogenized with an UP-200H homogenizer (DR. HEESCHER, Germany). The resulting homogenates were centrifuged at 4,000 rpm for 15 min at 4°C. The supernatants were collected for the analyses of IL-1β and IL-18 using rat IL-1β and IL-18 ELISA kits (Xitang Company Shanghai, China).

Reverse transcription-polymerase chain reaction

Total RNA from rat myocardial tissues were extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A total of 2 μg of RNA was converted into cDNA using the cDNA synthesis kit (TaKaRa, Japan). The expression levels of target genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. The 20-μl reaction volume included 1 μl of the total RNA (1 μg/μl), and the reaction was performed incubating the samples at 42°C for 15 min and then at 95°C for 2 min. The cDNA was stored at -20°C prior to further use. SYBR Green real-time PCR was performed with the SYBR ExScript RT-PCR kit (TaKaRa, Japan) in 50-μl reaction volumes. This 50-μl reaction mixture contained 25 μl of 2x SYBR Green PCR Master Mix, 2 μl of the sense and antisense primers (1 μl/each primer), 1 μl of 50x ROX Reference Dye, 4 μl of the cDNA template, and 18 μl of sterilized distilled H₂O (dH₂O). The PCR reaction conditions included pre-denaturing at 95°C for 10 s, then 40 cycles of 95°C for 5 s, 60°C for 30 s and 72°C for 1 min. Subsequent reaction and quantification analyses were performed using an Opticon-2 Real-time PCR reactor (MJ Research, US). In this experiment, GAPDH was used as the

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The primers for NALP3, Caspase1, IL-1β, IL-18 and GAPDH were designed according to the reference sequences provided by the GenBank accession numbers. For NALP3 amplification, the 221-bp sequence was generated using the sense and antisense primers F5'-TTGTGTGACAGGGCTAAAG-3' and R5'-CCGCAGATCACACTTCAAC-3', respectively. For Caspase1 amplification, the 154-bp sequence was generated using the sense and antisense primers F5'-GTGGTCCTCAAGTTTTGC-3' and R5'-CCGACTCTCCGGAGAAAGATG-3', respectively. For IL-1β amplification, the 211-bp sequence was generated using the sense and antisense primers F5'-CTGTGACCTTGGGATGATG-3' and R5'-AGGGATTTTGTGGGTGCTTTG-3', respectively. For IL-18 amplification, the 218-bp sequence was generated using the sense and antisense primers F5'-ATATACGACGACAGCCACAGC-3' and R5'-TGGCACACGTTCTGAAGA-3', respectively. Likewise, the 251-bp GAPDH amplification was generated using the sense and antisense primers F5'-CCATGTTCGGAGGGTGTGGT-3' and R5'-GCCAGTAGGCGAGGATGATGTTCT-3', respectively. For quantification, the Ct value of NALP3 (or Caspase1, or IL-1β, or IL-18) minus the GAPDH value was regarded as ΔCt, and the ΔΔCt method was used for calculation and statistical analysis [37].

Western blotting

To determine the protein levels of NALP3, Caspase1, and GAPDH in cardiac muscle samples, the proteins were extracted, and Western blot analysis was performed using the standard protocol. Briefly, the rat myocardial tissues...
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were harvested in lysis buffer. After centrifugation, equal amounts of protein samples were subjected to SDS-PAGE and were transferred onto nitrocellulose membranes. Non-specific binding was blocked with 5% non-fat dry milk. The membrane was rinsed and immunoblotted with each primary antibody at the recommended dilution and then incubated with a horseradish peroxidase-conjugated secondary antibody. Bands were visualized using an enhanced chemiluminescence (ECL) detection kit (Pierce).

Statistical analysis

Data were expressed as the means ± SD of three or more individual experiments. Statistical comparisons among groups were performed with one-way analysis of variance (ANOVA) and the Student-Newman-Keuls (SNK)-q test using SPSS 13.0 statistical analysis software. *P values < 0.05 were considered significant.

Results

Resveratrol protects against ischemic injury mediated by I/R

Light microscope evaluation: In the Sham group, the myocardia were arranged regularly, with cell membranes that remained integrated.
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No apparent apomorphosis, necrosis, or other pathological changes were observed. In contrast, the I/R group showed evidence of myocardial necrosis, disorganized and ruptured myocardial fibers, and interstitial edema, with large numbers of erythrocytes and tiny numbers of inflammatory cells in the interstitial tissues. After resveratrol administration, the amelioration of the acute injury was apparent by histology. Well-arranged myocardial fibers, an integrated structure, and myocardial interstitial blood were observed in the M-Res group, whereas decreasing amounts of myocardial necrosis and local swelling were evident in the H-Res group (Figure 1).

Transmission electron microscopic evaluation: The Sham group had a grossly normal myocardium ultrastructure and shape: the myocardial membrane was intact, the myofilaments were well-arrayed, and the mitochondria retained a clear and integrated structure with compact cristae. The I/R group had various forms of damage in the myocardial ultrastructure: the myocardial membrane was damaged, with myocardial edema, and the myofilaments were dissolved and partially ruptured. Additionally, the mitochondria were swollen and partially vacuolized, with ruptured and lysed cristae. Compared with the I/R group, resveratrol administration significantly mitigated the ultrastructural damage in a dose-dependent manner. The L-Res group displayed no apparent morphological amelioration compared with the I/R group, whereas the M-Res group had a staggered normal myocardial fiber structure, with damaged myocardia that were morphologically recuperating; the H-Res group displayed a roughly complete myocardial structure in which the myocardial membrane was grossly intact, myocardial fibers were arranged regularly with clear ino-comma zonation, and the mitochondria retained their normal shape with intensive cristae (Figure 2).

Myocardial infarct size: The ischemic area and infarct size were estimated after 30 min of ischemia and 2 h of reperfusion. There were no significant differences in the size of the area at risk among the experimental groups (Figure 3A), indicating that similar amounts of tissue were jeopardized by occlusion of the left coro-
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In the I/R group, the necrotic area constituted 43.2 ± 1.5% of the area at risk. Resveratrol administration reduced the necrotic area in a dose-dependent manner. Resveratrol pre-administration at doses of 5 and 10 mg/kg body wt reduced the necrotic area to 30.4 ± 1.9%* and 25.5 ± 1.2%** of the area at risk, respectively (Figure 3B).

Plasma TnT and CK-MB levels in I/R: The biochemical indicators of myocardial cellular damage (TnT and CK-MB release) were measured in the five groups. Low TnT and CK-MB activities were recorded in the Sham group (0.03 ± 0.01 µg/l and 780 ± 115.4 µg/l, respectively), whereas large increases in TnT and CK-MB were observed in the serum samples from the other four groups (especially in the I/R group). In contrast, resveratrol administration markedly inhibited TnT and CK-MB release in a dose-dependent manner during I/R (Figure 4).

IL-1β and IL-18 activity levels in peripheral blood sera by ELISA: The activity levels of IL-1β and IL-18 in peripheral blood were determined by ELISA. Low IL-1β and IL-18 activity levels were detected in the Sham group (260.63 ± 46.35 pg/ml and 292.45 ± 46.37 pg/ml, respectively), whereas large increases in IL-1β and IL-18 activity levels were observed in the sera from the other four groups, (especially in the I/R group). In contrast, resveratrol administration markedly inhibited IL-1β and IL-18 release in a dose-dependent manner during I/R (Figure 5).

Resveratrol suppresses I/R-mediated myocardial IL-1β and IL-18 expression: As shown in Figures 6 and 7, IL-1β and IL-18 mRNA expression levels in the resveratrol-treated groups decreased markedly compared with the I/R group.

Resveratrol decreases myocardial NALP3 expression mediated by I/R: Rats in the Sham group expressed low levels of NALP3 in vivo. After I/R, the NALP3 mRNA and protein levels were significantly up-regulated by 12.7- and 4.38-fold, respectively (P < 0.05). Furthermore, the enhanced expression of NALP3 mediated by I/R was reduced by resveratrol pretreatment in a dose-dependent manner, as shown in Figures 8 and 9. The NALP3 mRNA and protein...
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expression levels in the H-Res, M-Res and L-Res groups decreased significantly compared with the I/R group.

Resveratrol inhibits I/R-mediated myocardial Caspase1 expression: Caspase1 expression levels in the I/R group and the three resveratrol-treated groups were significantly increased compared with the Caspase1 expression level in the Sham group. Additionally, the overexpression of the Caspase1 response to I/R at the mRNA and protein levels was markedly inhibited by resveratrol pretreatment compared to I/R, but only in the I/R group (Figures 10, 11).

Discussion

The successful establishment of an animal model of I/R injury represents an important foundation for investigations into the pathogenesis of myocardial I/R injury (MI/RI). Rats are the best experimental animal model for MI/RI for several reasons, including: more than 90% of rat genes are similar to those in humans, their evolution occurred in proximity to humans, their breeding is simple and inexpensive, the fixed position of the rat coronary artery is clear and contains little collateral circulation, myocardial necrosis appears earlier and the incidence of arrhythmia is higher after tying, and the experimental model exhibits repeatability and stability [38]. Moreover, because the myocardial ischemia pathological processes caused by ligation of the anterior descending coronary artery are similar to the processes observed in the clinic, this technique is now recognized as an optimal preparation method to model I/R [39]. In this study, we produced a cardiac I/R model through ligation of the anterior descending artery of SD rats and recorded the limb lead using an electrocardiogram. The results showed that the II lead of the S-T segment appeared to be significantly elevated instantly after ligation of the LAD. The QRS wave amplitude increased and broadened, demonstrating the success of the ischemia model; moreover, the success of the model was further demonstrated by the significant depression of the visible elevation of the S-T segment at the time of reperfusion after release of the thread (decline rate > 50%) [40].

Acute myocardial infarction (i.e., MI/RI) is characterized by a very complex cascade of processes involving interactions between many biochemical materials, including endothelial cells, mesenchymal cells, and circulating cells. Inflammation is a key mediator of MI/RI and can trigger a strong immune response, resulting in vascular endothelial cell activation, increasing permeability and expression of adhesion molecules [41-43]. The secretion and release of reactive oxygen species, cytokines, chemokines and adhesion molecules increase the inflammatory response. Inflammatory cytokines, such as IL-1β and IL-18α, play very important roles in mediating the inflammatory response and apoptosis in MI/RI pathogenesis [44]. The NALP3 inflammasome can function in a pro-Caspase1 environment, and its activation results in the production of active Caspase1. Activated Caspase1 can cleave pro-IL-1β and pro-IL18, resulting in the secretion of active IL-1β and IL-18 and mediating the inflammatory response [45]. However, whether the myocardial protection of resveratrol occurs through inhibition of NALP3 inflammation has not been previously reported.
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In this study, we found that resveratrol treatment significantly reduced the infarct volume and myocardial fibrosis; myocardial cells were lined up in a more orderly fashion, and decreased levels of TNF, CK-MB, IL-1β and IL-18 were detected in the serum of the I/R rats. Our results also showed that myocardial IL-1β and IL-18 mRNA expression levels significantly dropped in the M-Res and H-Res groups compared with I/R group and that the expression of NALP3 and Caspase1 in the rat myocardium decreased at the mRNA and protein levels after 2 h of ischemia-reperfusion following 30 min of ischemia. The inhibiting effect of resveratrol was most obvious in the H-Res and M-Res groups. A low dosage of resveratrol may lessen this effect. Based on the above results, we speculate that pretreatment with an appropriate dose of resveratrol could weaken myocardial I/R injury, which may, in part, be attributed to resveratrol’s role in diminishing NALP3 expression in the myocardium. In turn, this effect reduces Caspase1 and accordingly reduces the expression of the inflammatory factors IL-1β and IL-18.

In this preliminary study, we demonstrated that resveratrol induces a myocardial protective effect due to its relationship with NALP3 inflammation. We explored new potential intracellular targets of cardiovascular disease through resveratrol treatment. However, whether other factors are involved MI/RI in addition to the NALP3 inflammasome signaling pathway still needs to be further explored. We propose that the role of NALP3 in MI/RI can be explained in a future study using gene silencing or the application of NALP3-specific blockers.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 81170133 to J. Yang; Grant No. 81200088, 81470387 to J. Yang), and the National Science Foundation of Yichang city, China (Grant No. A12301-01) as well as Hubei Province’s Outstanding Medical Academic Leader program, China.

Disclosure of conflict of interest

None.

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Int J Clin Exp Pathol 2015;8(8):8731-8741
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