C1q/TNF-related protein 9 decreases cardiomyocyte hypoxia/reoxygenation-induced inflammation by inhibiting the TLR4/MyD88/NF-κB signaling pathway

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Abstract. C1q/TNF-related protein 9 (CTRP9) acts as an adipokine and has been reported to exert numerous biological functions, such as anti-inflammatory and anti-oxidative stress effects, in ischemic heart disease. In the present study, the role of CTRP9 in neonatal rat cardiomyocytes (NRCMs) following hypoxia/reoxygenation (H/R) and the underlying mechanism was investigated. Adenoviral vectors containing CTRP9 or green fluorescent protein (GFP) were transfected into NRCMs. A H/R model was constructed 2 days after transfection by 2 h incubation under hypoxia followed by 4 h of reoxygenation. Lactate dehydrogenase (LDH), creatine kinase (CK) and CK-MB levels were detected by a biochemical analyzer using biochemical kits. In addition, cell viability was detected using trypan blue staining to determine the extent of cell injury. Inflammatory cytokines TNF-α, IL-6 and IL-10 were measured by ELISA. Western blotting and reverse transcription-quantitative PCR were used to evaluate the expression levels of CTRP9, toll-like receptor 4 (TLR4), myeloid differentiation primary response (MyD88) and NF-κB. The DNA binding activity of NF-κB was also detected using an electrophoretic mobility shift assay. The results indicated that transfection with adenoviral vectors containing CTRP9 could markedly enhance CTRP9 expression. CTRP9 overexpression increased cell viability and decreased the release of LDH, CK and CK-MB. In addition, CTRP9 overexpression reduced TNF-α and IL-6 levels whilst increasing IL-10 levels, but decreased the expression of TLR4, MyD88 and NF-κB. Furthermore, the DNA binding activity of NF-κB under H/R was also decreased by CTRP9 overexpression. In conclusion, the results of the present study suggested that CTRP9 could protect cardiomyocytes from H/R injury, which was at least partially due to the inhibition of the TLR4/MyD88/NF-κB signaling pathway to reduce the release of inflammatory cytokines.

Introduction

Ischemic heart disease (IHD) is a major cause of disability and mortality worldwide (1). According to the World Health Organization, the number of acute myocardial infarction cases is ~32.4 million per year worldwide as of 2020 (2). At present, timely revascularization is the most effective approach for reducing cardiomyocyte cell death (3). Nevertheless, reperfusion treatment can cause myocardial ischemia/reperfusion (I/R) injury (MIRI), which is a complex pathophysiological process that can cause additional myocardial damage (4). In recent years, evidence has shown that inflammation serves a crucial role in the pathogenesis of MIRI (5). Therefore, identification of effective approaches for reducing inflammation to attenuate MIRI remain in demand.

As a fat-derived plasma protein, C1q/TNF-related protein 9 (CTRP9) has reported beneficial effects on glucose metabolism and vascular function (6,7). Previous studies have indicated that CTRP9 can regulate the inflammatory response in the setting of various cardiovascular diseases, including atrial fibrillation (8), myocardial infarction (9), atherosclerosis (10) and heart failure (11). It was also demonstrated that CTRP9 is a potential protective factor against MIRI (12,13). Kambara et al (12) found that CTRP9 can protect the myocardium from I/R injury through an AMP-activated protein kinase (AMPK)-dependent mechanism. In another study, Zhao et al (13) revealed that cardiac-derived CTRP9 can protect against MIRI through...
calreticulin-dependent inhibition of apoptosis. However, to the best of our knowledge, the function of CTRP9 in MIRI remains unclear due to the complex molecular mechanism involved.

Previous studies have documented that I/R injury can induce the release of proinflammatory cytokines in cardiomyocytes by triggering toll-like receptor 4 (TLR4)/myeloid differentiation primary response 88 (MyD88)/NF-kB signaling (14,15). Inhibition of TLR4/MyD88/NF-kB-related signaling has been shown to decrease I/R injury-induced proinflammatory cytokine release and ameliorate cardiac dysfunction (16,17). Of note, TLR4/MyD88/NF-kB-related signaling transduction has also been found to be suppressed by CTRP9 during IHD (8,9). However, the effects of CTRP9 on inflammation and TLR4/MyD88/NF-kB activation during MIRI remain unclear.

In this experiment, adenoviral vectors containing CTRP9 were used to increase the expression of CTRP9 in neonatal rat cardiomyocytes (NRCMs), and then a hypoxia/reoxygenation (H/R) injury model was established to explore the role and mechanism of CTRP9 during MIRI.

Materials and methods

Chemicals and reagents. Adenoviruses encoding CTRP9 (Ad-CTRP9) or green fluorescent protein (GFP; Ad-GFP) were constructed and amplified by Gene Company, Ltd.. Lactate dehydrogenase (LDH; cat. no. A020-1-2), creatine kinase (CK; cat. no. A032-1-1) and CK-myocardial band (CK-MB; cat. no. E006-1-1) biochemical kits were obtained from Nanjing Jiancheng Bioengineering Institute. The following primary antibodies were purchased from Abcam: TLR4 (cat. no. ab13867; 1:1,000 dilution), MyD88 (cat. no. ab219413; 1:800 dilution), NF-kB (cat. no. ab220803; 1:1,000 dilution) and GAPDH (cat. no. ab8245; 1:1,000 dilution). HRP-conjugated anti-rabbit (cat. no. bs-0295G; 1:1,000 dilution) and HRP-conjugated anti-mouse secondary antibodies for another 2 h (1:2,000 dilution) were obtained from Bioss. A LightShift Chemiluminescent electrophoretic mobility shift assay (EMSA) kit (cat. no. SIDET201) was purchased from Viagene Biotech, Inc..

NRCM culture. The NRCMs were isolated from ~2-day old Sprague Dawley rats (n=5; weight, 8±2 g; sex, unknown), which were provided by the Experimental Animal Center of Southern Medical University (Shenzhen, China). The Sprague Dawley rats (housed at 23±2˚C with 50% relative humidity, 12-h light/dark cycles and free access to water) were anesthetized with 3% sodium pentobarbital (30 mg/kg) and their large blood vessels carefully excised. The obtained heart tissues were rinsed in ice-cold PBS to remove any residual blood. Next, 0.08% collagenase and 0.125% trypsin were used to digest the tissues at 37˚C for 7 min. Finally, the NRCMs (fusiform or polygonal, type II and 0.125% trypsin were used to digest the tissues at 37˚C for 12 h. Next, NRCMs were moved into a normal incubator (37˚C) for an additional 4 h to induce reoxygenation. The primary cardiomyocytes were randomly separated into the following four groups: Control, H/R, Ad-CTRP9 and Ad-GFP groups (both of which also underwent H/R). Each experiment was repeated ≥5 times.

Cell viability assay. Cell viability assay was performed to assess the cytotoxicity of adenovirus on NRCMs. Briefly, after the NRCMs (5x10^5/ml) were transfected with adenoviruses at various multiplicities of infection (MOI=5, 20, 50, 100 and 200) at 37˚C for 48 h, they were stained with 0.4% trypan blue 37˚C for 3 min and observed under a light microscope (magnification, x200). The ratio of unstained cells to total cell number was calculated to estimate cell viability.

Determination of markers of myocardial injury. In the present study, the supernatant of cultured NRCMs was collected and an automatic biochemical analyzer (Jinan Tianheng Technology Co., Ltd.) was used with the aforementioned biochemical kits, according to the manufacturers’ protocols, to determine the LDH, CK and CK-MB levels.

Measurement of TNF-α, IL-6 and IL-10 levels. TNF-α, IL-6 and IL-10 were measured using ELISA kits (R&D Systems, Inc.) according to the manufacturer's protocol to determine the TNF-α, IL-6 and IL-10 levels in supernatants. Samples was centrifuged 500 x g at 4˚C for 10 min to obtain supernatants.

Western blotting. Western blotting was performed to analyze protein expression (19). Briefly, NRCMs were first homogenized and lysed in RIPA buffer (cat. no. R0278; MilliporeSigma). Next, the protein was extracted and the concentration was determined using a BCA assay (Beyotime Institute of Biotechnology). Subsequently, 10% SDS-PAGE was used to separate the extracted proteins (40 µg), which were then electrophoretically transferred onto PVDF membranes. The membranes were then blocked with 5% non-fat dry milk in PBS with 0.05% Tween-20 for 2 h at room temperature. Next, the membranes were incubated with antibodies against TLR4, MyD88 and NF-kB overnight at 4˚C. The next day, the membranes were incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies for another 2 h at room temperature. Finally, a Pierce™ ECL Western Blotting Substrate kit (cat. no. 32109; Pierce; Thermo Fisher Scientific, Inc.) was used to detect protein expression. An Odyssey Infrared Imaging system (model 9120; LI-COR Biosciences) was used to capture images of the membranes and Quantity One 1-D
software (version 4.6.9; Bio-Rad Laboratories, Inc.) was used to quantify the protein bands.

**Reverse transcription-quantitative PCR (RT-qPCR).** RT-qPCR was performed to detect mRNA levels. Briefly, TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from NRCMs. Obtained RNA (~4.0 µg) was then reverse transcribed into cDNA using SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, Inc.) at 37˚C for 60 min. Next, qPCR was performed using a SYBR green Master Mix kit (Thermo Fisher Scientific, Inc.) on a 7500 ABI PRISM system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The qPCR thermocycling conditions were as follows: 45˚C for 2 min; 95˚C for 10 min, immediately followed by 45 cycles of 95˚C for 30 sec and 60˚C for 30 sec. The mRNA expression of CTRP9, TLR4, MyD88 and NF-κB were normalized to that of GAPDH. The $2^{-\Delta\Delta Cq}$ method was used to calculate changes in mRNA expression (20). The following primers were used: CTRP9 forward, 5'-GGCTTCTACTGGTTATGGACGC-3' and reverse, 5'-GGAGCCCTGACCTCCTTGGAT-3'; TLR4 forward, 5'-TGCTCA GACATGCGAGTTTC-3' and reverse, 5'-CTGGAGTCAAGG CTTTTCACA-3'; MyD88 forward, 5'-GAGATCCGCGAGTTTGAGAC-3' and reverse, 5'-CTGTCTTCTCGTCTGGTGCG TA-3'; NF-κB forward, 5'-GGCAGACTCCTTATCAA CC-3' and reverse, 5'-GAGGTGTCTGCTCCACATGTAAG-3' and GAPDH forward, 5'-CGCTAACATCAAATGGGGTG-3' and reverse, 5'-TGTGCTGACAATCTTTAGGGAG-3'.

**EMSA.** The binding activity of NF-κB was detected using an electrophoretic mobility shift assay. Briefly, nuclear extracts were prepared from the NRCMs and stored at -80˚C for the EMSA assay. The protein concentration was measured using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc.). Equal amounts (5 µg) of nuclear protein were incubated with poly (2'-deoxyinosinic-2'-deoxycytidylic acid) and synthesized NF-κB binding consensus oligonucleotides (sense, 5'-AGT TGAGGGGACCGACGC-3'; antisense, 5'-GGCTTGG GAAAAGCTCCCTCAACT-3') for 20 min at room temperature using a LightShift EMSA Optimization and Control kit. Subsequently, protein-DNA complexes were separated via electrophoresis on a 6.5% non-denaturing polyacrylamide gel, transferred to a nylon membrane and cross-linked by UV light.

**Figure 1.** Transfection efficiency and cell viability in NRCMs after transfection with Ad-CTRP9 at different MOIs. (A) Cell viability following Ad-CTRP9 transfection at different MOIs (0-200). (B) NRCMs were transfected with Ad-CTRP9 (MOI=50) for 48 h and observed using fluorescence microscopy. Magnification, x100. Data are expressed as the mean ± SD (n=5). *P<0.05 vs. 0 MOI. Ad-CTRP9, adenovirus encoding CTRP9; MOI, multiplicity of infection; NRCM, neonatal rat cardiomyocytes.

The membrane was incubated with streptavidin-horseradish peroxidase and detected via enhanced chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.).

**Statistical analysis.** SPSS 22.0 software (IBM Corp.) was used for data analysis. Data are presented as the mean ± SD (n=5). Student's unpaired t-test and one-way ANOVA were used for comparisons between groups. If interactions were significant, a Tukey post hoc test was used for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**NRCM viability.** As shown in Fig. 1A, Ad-CTRP9 exerted no toxic effects on NRCM viability at MOI of <100. However, cell viability was 86.8% at an MOI of 200. After 48 h of transfection, GFP expression was assessed using a fluorescence microscope at an MOI of 50 (Fig. 1B). The transfection efficiency at MOI of 20 was only ~84.7%, whereas that at MOI of 50 and 100 were ~92.6 and ~93.8% respectively, with no clear differences between MOI of 50 and 100 (data not shown). Considering these aforementioned results, MOI of 50 was used due to its higher transfection efficiency but minimal effects on NRCM viability.
Ad-CTRP9 induces CTRP9 upregulation in cardiomyocytes after H/R. Following adenoviral infection and the establishment of the H/R injury model, CTRP9 expression was examined by RT-qPCR. As shown in Fig. 2, the mRNA expression levels of CTRP9 were significantly reduced in the H/R and Ad-GFP groups compared with those in the control group. However, the mRNA expression of CTRP9 was significantly increased in the Ad-CTRP9 group compared with that in the Ad-GFP or H/R groups.

CTRP9 attenuates H/R-induced NRCM injury. LDH, CK and CK-MB are enzymes that are released by cardiomyocytes following severe injury, and their levels are used to estimate the severity of myocardial damage (17). To assess the effects of CTRP9 on H/R-induced cellular damage, cell viability, as well as LDH, CK and CK-MB activity in the cell culture supernatant, were evaluated. Cell viability was significantly suppressed by H/R compared with that in cells that underwent normoxic treatment in all three of the transfection groups (Fig. 3A). However, after H/R, cell viability was significantly increased in the Ad-CTRP9 group compared with that in the control group (Fig. 3A). LDH (Fig. 3B), CK (Fig. 3C) and CK-MB (Fig. 3D) activities were also significantly increased in the H/R group compared with those in the control group. However, the H/R-induced enzyme release was significantly reversed by CTRP9 overexpression (Fig. 3B-D).

CTRP9 alleviates inflammation after H/R injury. MIRI is closely associated with an excessive inflammatory response (5). In addition, CTRP9 has been shown to be involved in the progression of inflammation in the heart (9). ELISA was therefore used to measure TNF-α, IL-6 and IL-10 expression in cell culture supernatant. The levels of TNF-α and IL-6 were found to be significantly increased following
H/R compared with those in the control group, but the levels of the anti-inflammatory cytokine IL-10 was significantly downregulated (Fig. 4). However, CTRP9 overexpression significantly decreased the levels of TNF-α and IL-6 whilst increasing the levels of IL-10 compared with those in the Ad-GFP or H/R groups (Fig. 4).

CTRP9 inhibits TLR4/MyD88/NF-κB signaling. To further understand the possible mechanism underlying the CTRP9-mediated mitigation of H/R damage, the expression of components of TLR4/MyD88/NF-κB signaling was determined by western blotting and RT-qPCR. As shown in Fig. 5, H/R significantly upregulated the protein (Fig. 5A) and mRNA (Fig. 5B) expression levels of TLR4, MyD88 and NF-κB compared with those in the control group. However, CTRP9 overexpression after the onset of H/R significantly reversed these aforementioned increases. Similarly, the binding activity of NF-κB to DNA was markedly increased after H/R, but decreased following Ad-CTRP9 transfection (Fig. 5B). Therefore, this suggests that H/R injury may be ameliorated by CTRP9 overexpression, possibly through suppression of TLR4/MyD88/NF-κB signaling.

Discussion

MIRI leads to a range of pathological changes, including activation of the inflammatory response, which can lead to cell injury (21). Effectively reducing inflammation can improve the outcomes of MIRI in animal and cell models (22). Previous studies have found that CTRP9 exerts protective effects against ischemic heart injury through a variety of signaling pathways, such as the protein kinase A and ERK1/2 signaling pathways (23-25). In particular, cardiac-derived CTRP9 has been found to protect against MIRI through the inhibition of apoptosis and endoplasmic reticulum stress (13,26). However, to the best of our knowledge, the role of CTRP9 and possible underlying mechanism in MIRI has not been completely elucidated. In the present study, a H/R model was established to simulate MIRI and CTRP9 was found to alleviate MIRI by significantly reducing myocardial inflammation, which was characterized by the upregulation of cell viability and reducing the release of CK, CK-MB and LDH. In addition, it was observed that CTRP9 overexpression markedly inhibited the TLR4/MyD88/NF-κB signaling pathway. These aforementioned results suggest that CTRP9 may possess the ability to ameliorate H/R-induced inflammation by regulating the TLR4/MyD88/NF-κB signaling pathway.

Previous studies have found that CTRP9 can regulate the inflammatory response in various pathological processes, such as myocardial infarction and endothelial dysfunction (27-29). In db/db mice, Li et al (30) found that overexpression of CTRP9 may reduce retinal inflammation and protect the blood-retinal barrier. Liu et al (9) revealed that overexpression of CTRP9 restored cardiac function following myocardial infarction by regulating TLR4/MD2/MyD88 and AMPK/NF-κB signaling in a rat model of myocardial infarction.
inflammation. In another study, Zhang et al (31) demonstrated that overexpression of CTRP9 attenuated a mouse model of atherosclerosis by inhibiting AMPK/NLR family pyrin domain containing 3 signaling. Qian et al (32) showed that overexpression of CTRP9 alleviated airway inflammation in a mouse model of asthma. Zhao et al (33) demonstrated that Ad-CTRP9 transfection weakened neuro-inflammation by activating adiponectin receptor 1 following intracerebral hemorrhage in mice. Therefore these previously reported biological activities of CTRP9 aforementioned attracted the interest of the research community. The present study demonstrated that CTRP9 overexpression may reverse the H/R-induced upregulated expression of TNF-α and IL-6 in addition to reverting the H/R-induced reduction in the levels of the anti-inflammatory cytokine IL-10.

H/R can directly decrease cardiomyocyte contractility and induce inflammation in cardiomyocytes by activating the TLR4/MyD88-dependent signaling pathway (16). The TLR4 signaling pathway has been extensively studied, where exerts its effects through the MyD88-mediated activation of NF-κB (34,35). As a transcription factor, NF-κB has been confirmed to be closely associated with inflammation activation (36). TLR4 signaling-related inflammatory activation is closely linked to myocardial injury during MIRI (37). A number of endogenous factors that can negatively regulate TLR4 signal transduction directly have been found (9,38). Among them, CTRP9 has particularly garnered interest (9). MyD88 and NF-κB are downstream molecules of TLR4, and the activation of MyD88 and NF-κB are partly dependent on TLR4; Liu et al (9) found that CTRP9 could directly bind to TLR4 to regulate the downstream molecules of MyD88 and NF-κB. In addition, ample evidence suggests that CTRP9 exerts pleiotropic effects, such as anti-inflammation, on a variety of pathological conditions by suppressing NF-κB, such as cardiac hypertrophy (39) and osteoarthritis (40). In terms of the possible involvement of CTRP9 in the suppression of TLR4/MyD88/NF-κB-related inflammatory signaling, the present study suggest that CTRP9 may exert protective effects on cardiomyocytes following H/R insult by conferring anti-inflammatory effects through suppressing TLR4/MyD88/NF-κB signaling downstream. However, it should be noted that the cause-effect relationship between TLR4 signaling and inflammation after overexpression of CTRP9 require further study. TLR4-knockout mice need to be established for further study, which will contribute to the understanding of the relationship between TLR4 signaling and inflammation after overexpression of CTRP9.

In conclusion, the present study indicated that the overexpression of CTRP9 could alleviate H/R by attenuating inflammation in a TLR4/MyD88/NF-κB-dependent manner. However, the pathophysiological process of MIRI is complex, such that the possibility of other signaling pathways being involved in the protective effects of CTRP9 in MIRI cannot be ruled out. Nevertheless, results from the present study suggest that CTRP9 can represent a novel therapeutic target for MIRI.

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Clemastine fumarate protects against myocardial inflammation by inhibiting the TLR4/NF-κB signaling pathway. Exp Ther Med 20: 343‑350, 2020.

By RNA interference attenuates ischemia/reperfusion‑induced myocardial inflammation and improves cardiac dysfunction after myocardial infarction. J Cell Physiol 234: 1873‑1874, 2019.

C1q/TNF‑related protein‑9 promotes macrophage polarization and improves cardiac dysfunction after myocardial infarction. J Cell Physiol 234: 1873‑1874, 2019.

C1q/TNF‑related protein‑9 promotes macrophage polarization and improves cardiac dysfunction after myocardial infarction. J Cell Physiol 234: 1873‑1874, 2019.

C1q/TNF‑related protein‑9 regulates the fate of implanted mesenchymal stem cells via protein kinase A signaling pathway. Cell Physiol Biochem 41: 22‑32, 2017.

C1q/TNF‑related protein‑9 attenuates retinal inflammation and protects blood‑retinal barrier in db/db mice. Eur J Pharmacol 853: 289‑298, 2019.

C1q/TNF‑related protein‑9 protects against myocardial injury following ischemia‑reperfusion through AMP‑activated protein kinase (AMPK)‑dependent mechanism. J Biol Chem 287: 18065‑18073, 2012.

C1q/TNF‑related protein‑9 attenuates atherosclerosis through AMPK‑NLPR3 inflammasome singling pathway. Int Immunopharmacol 77: 105934, 2019.

C1q/TNF‑related protein‑9 alleviates airway inflammation in asthma. Int Immunopharmacol 81: 106238, 2020.

C1q/TNF‑related protein‑9 alleviates myocardial ischemia/reperfusion injury via calreticulin‑dependent inhibition of apoptosis. Cell Death Dis 9: 723, 2018.

C1q/TNF‑related protein‑9 alleviates myocardial ischemia/reperfusion injury via calreticulin‑dependent inhibition of apoptosis. Cell Death Dis 9: 723, 2018.

C1q/TNF‑related protein‑9 protects against myocardial ischemia/reperfusion injury via calreticulin‑dependent inhibition of apoptosis. CellDeath Dis 9: 723, 2018.

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