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Inhibition of CTRP9, a novel and cardiac-abundantly expressed cell survival molecule, by TNF α -initiated oxidative signaling contributes to exacerbated cardiac injury in diabetic mice

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Abstract

Recently identified as adiponectin (APN) paralogs, C1q/TNF-related proteins (CTRPs) share similar metabolic regulatory functions as APN. The current study determined cardiac expression of CTRPs, their potential cardioprotective function, and investigated whether and how diabetes may regulate cardiac CTRP expression. Several CTRPs are expressed in the heart at levels significantly greater than APN. Most notably, cardiac expression of CTRP9, the closest paralog of APN, exceeds APN by >100-fold. Cardiac CTRP9 expression was significantly reduced in high-fat diet-induced diabetic mice. In H9c2 cells, tumor necrosis factor- α (TNF- α) strongly inhibited CTRP9 expression (>60 %), and significantly reduced peroxisome proliferator activated receptor- γ (PPAR γ), a known transcription factor promoting adiponectin expression. The inhibitory effect of TNF- α on PPAR γ and CTRP9 was reversed by Tiron or rosiglitazone. CTRP9 knockdown significantly enhanced, whereas CTRP9 overexpression significantly attenuated simulated ischemia/reperfusion injury in H9c2 cells. In vivo CTRP9 administration to diabetic mice significantly attenuated NADPH oxidase expression and superoxide generation, reduced infarct size, and improved cardiac function. To the best of our knowledge, this is the first study providing evidence that down-regulation of CTRP9, an abundantly expressed and novel cell survival molecule in the heart, by TNF- α -initiated oxidative PPAR γ suppression contributes to exacerbated diabetic cardiac injury. Preservation of CTRP9 expression or augmentation of CTRP9-initiated signaling mechanisms may be the potential avenues for ameliorating ischemic diabetic cardiac injury.

Keywords

Oxidative stress; Diabetes; Cytokines; Myocardial ischemia

Intensely investigated in the past decade, the cardioprotective protein adiponectin (APN) regulates energy homeostasis, increases insulin sensitivity, and has anti-inflammatory/vasculoprotective and anti-ischemic/cardio-protective properties [16, 18]. Although APN knockout mice develop insulin resistance when metabolically challenged (i.e., when subject to high-fat diet), in the absence of dietary stress, such animals manifest a relatively modest metabolic phenotype [8, 10, 11] suggesting the plausible existence of compensatory cytokines/proteins capable of providing cardioprotection in the absence of serum APN.

C1q/TNF-related proteins (CTRPs) are a protein family consisting of fifteen (CTRP1-CTRP15) newly identified APN paralogs [15]. In addition to sharing structural similarity with APN, CTRPs also regulate similar metabolic processes. CTRP1 and CTRP2 decrease blood glucose via activation of Akt, AMPK, ACC, and p42/44-MAPK in muscle [23]. CTRP3 diminishes glucose levels in both normal and insulin-resistant ob/ob mice, without modifying insulin or APN levels [14], and reduces hepatocyte glucose output via hepatic Akt activation and suppression of gluconeogenic glucose-6-phosphatase (G6Pase) gene expression [14]. CTRP9 and CTRP13 stimulate basal and insulin-mediated glucose uptake

[21]. Of the CTRP family members identified thus far, CTRP9 is the closest paralog of APN, and its globular C1q domain shares the highest degree of amino acid identity (51 %) with APN. More importantly, CTRP9 is the only molecule thus far identified capable of forming hetero-oligomers with APN, and APN/CTRP9 complexes can be found in the serum of APN and CTRP9 transgenic mice [22]. However, whether CTRP9 is similarly regulated as APN under diabetic conditions; thus contributing to exacerbated cardiac injury in diabetes, has not been previously investigated.

We and others have previously demonstrated that cardiomyocytes have the ability to produce biologically active APN [20]. However, compared to adipocytes, cardiomyocyte APN expression is extremely low (<1/120,000 of adipocytes). Physiologic/pathologic significance of locally produced APN by cardiomyocytes remains unclear. Initial tissue distribution study by Wong et al. [22] demonstrated that among 21 organs/tissues screened, the heart ranked third-most in CTRP9 expression. However, any biological function of this locally produced CTRP9 remains unknown.

Therefore, the aims of the present study were: (1) to determine the expression profile of CTRPs in the adult heart; (2) to determine whether cardiac CTRP9 expression is altered in diabetes, and if so, identify the involved mechanisms; and (3) to define the biological significance of locally produced CTRP9, and investigate effect of exogenous CTRP9 supplementation upon myocardial ischemia/reper-fusion (MI/R) injury in diabetic animals.

Materials and methods

Materials

Antibodies against CTRP9 were kindly provided by Dr. Wong (Department of Physiology, Johns Hopkins University School of Medicine, Baltimore, Maryland). Antibodies against APN and gp91^{phox} were purchased from Cell-Signaling (Danvers, MA). PPAR γ antibody was purchased from Santa Cruz (Santa Cruz, CA). siRNA against CTRP9 was obtained from Integrated DNA Technologies (IDT, Coralville, Iowa). Recombinant TNF- α was purchased from ProSpec (Rehovot 76704 Israel).

High-fat diet induced diabetes

High-fat diet induced type 2 diabetes model was established as previously reported [25]. In brief, adult (6-week old) male C57BL/6 J mice were randomized to receive high-fat diet (HFD, 60 % kcal, Research Diets Inc. D12492i) or normal diet (ND, 10 % kcal control, D12450Bi) containing the same protein content as HFD. All experiments in this study were performed in adherence with the National Institutes of Health Guidelines on the Use of Laboratory Animals, and were approved by the Thomas Jefferson University Committee on Animal Care.

Cell culture

H9c2 cardiac muscle cell line was purchased from American Type Culture Collection (Manassas, VA, USA), and cultured in DMEM complete culture medium supplemented with 10 % fetal calf serum (FCS). Cells were seeded in triplicate in six-well plates at 4×10^3 per

well in 2 ml DMEM. Cells were cultured for 2 days to ~80–90 % confluence, starved for 24 h in DMEM starvation medium (DMEM supplemented with 1 % BSA) for all experiments. After starvation, cells were utilized for experiments employing TNF- α (10 ng/ml), high glucose (33 mM, HG), or high free fatty acid (300 μ M palmitate) at passages 9–15.

Preparation and culturing of adult mouse cardiomyocytes

Mice were anesthetized with 2 % isoflurane. Hearts were removed and perfused at 37 °C for ~3 min with a Ca²⁺-free bicarbonate-based buffer. Enzymatic digestion was initiated by adding collagenase type B/D to the perfusion solution. After ~3 min of digestion, at which point the cardiac tissue became firm and swollen 50 μ M Ca²⁺ was added to the enzyme solution; ~7 min later, the left ventricle was removed, cut into several sections, and further digested in a shaker for 10 min at 37 °C in the same enzyme solution. The supernatant containing the dispersed myocytes was filtered into a sterilized tube, and centrifuged at 800 $\times g$ for 1 min. The cell pellet was then resuspended in bicarbonate-based buffer containing 125 μ M Ca²⁺. After the myocytes were pelleted by gravity for ~10 min, the supernatant was aspirated, and the myocytes were resuspended in bicarbonate-based buffer containing 250 μ M Ca²⁺. Myocytes were plated at $0.5\text{--}1 \times 10^4$ cells cm⁻² in culture dishes pre-coated with mouse laminin.

siRNA-mediated CTRP9 knockdown and simulated ischemia–reperfusion of H9c2 cells

H9c2 cells were grown in normal growth medium in a 6-well plate to approximate 80 % confluence. 50 nM of CTRP9 siRNA (AGGAGAGAGGUUCA AUGGCUUAUUU) or scramble siRNA were transfected into H9c2 cells using the Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY) per manufacturer's instructions. 48 h after transfection, the efficiency of siRNA-mediated CTRP9 knockdown was determined by Western blotting. H9c2 cells were subjected to simulated ischemia–reperfusion (SI/R) as described in our previously published study [3, 20]. In brief, culture medium was quickly replaced with hypoxia-hypoglycemic medium, and H9c2 cells were placed in a Napco 8000WJ hypoxia (1 % O₂–5 % CO₂–94 % N₂) incubator (Thermo Scientific, Waltham, MA). After 6 h of hypoxia-hypoglycemic culture, the hypoxia-hypoglycemic medium was replaced with normal culture medium. After 3 h reoxygenation, caspase-3 activity was determined via fluorometric assay.

Quantitative real-time PCR

Animals were anesthetized with 2 % isoflurane and epididymal fat pad, and heart were removed. Total tissue RNA was prepared via RNeasy Mini Kit (Qiagen, Valencia, CA). First strand cDNA was synthesized from total RNA using the Transcriptor First Strand cDNA Synthesis Kit. Real-time PCR was performed on the ABI7900 using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) per manufacturer's protocol. Forward/reverse primer sets for CTRPs, APN, and 18S were established according to previously published protocols by other investigators and our laboratory [5, 22, 24].

Western blot analysis

Proteins were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels, transferred to nitro-cellulose membranes, and incubated with primary antibodies (anti-CTRP9, Anti-APN, Anti-PPAR γ and anti-gp91^{phox}) followed by horseradish peroxidase-conjugated secondary antibody. The blot was developed with a SuperSignal Chemiluminescence detection kit (Pierce, Rockford, IL), and observed with a Kodak Image Station 400 (Rochester, NY).

Myocardial ischemia/reperfusion in diabetic mice

After 8 weeks of feeding with HD when typical type 2 diabetes was developed [25], mice were anesthetized with 2 % isoflurane and subjected to either sham MI or MI via left anterior descending coronary artery slip-knot ligation as described in our previous studies [18]. Twenty minutes after MI, both HFD mice and ND mice were randomized to receive either vehicle (MI + Vehicle, $n = 13$) or globular domain of CTRP9 (MI + gCTRP9, 3 $\mu\text{g/g}$, intraperitoneal injection; $n = 13$). Expression and purification of gCTRP9 has been reported in detail in our previous study [26]. In brief, the globular domain of mouse CTRP9 gene was generated by PCR and cloned into the prokaryotic protein expression vector pET45b (Novagen, Merck, USA). The construct were verified by DNA sequencing. Globular CTRP9 prokaryotic expression vector was transferred into the BL21(DE3) bacterium protein expression host, grown in Lysogeny broth medium, and shaken overnight at 37 °C. Protein expression inducer IPTG was added to medium (final concentration 1 mM). The solution was shaken for 4–5 h, and subject to 5,000 rpm centrifugation. Protein was purified in native condition by Ni–NTA resin per manufacturer's instructions (Novagen, #70666–3, Merck, USA). Endotoxin was removed by endotoxin-removing column (Sterogene ActiCleanEtoc resin, Carlsbad, CA), desalted, and concentrated by centrifugation (Millipore Centricon, Plus-20). Purified proteins were stored at –70 °C until use. The utilized CTRP9 dose was selected from our recently published study demonstrating that supplementation of CTRP9 at this dose restored plasma CTRP9 levels in diabetic mice to normal physiological concentrations [26]. After 30 min of MI, the slip-knot was released, and reperfusion commenced. After 3 h (for all assays except cardiac function and infarct size) or 24 h (for cardiac function and infarct size only) reperfusion, the ligature around the coronary artery was retied, and 1 ml of 2 % Evans blue dye was injected into the left ventricular cavity. The isolated Evans-blue-negative stained cardiac portion (ischemic/reperfused tissue, or area-at-risk) was utilized for immunohistological, biochemical, and Western blot studies as described below.

Quantification of superoxide production

Myocardial superoxide content (in the area-at-risk) was determined by lucigenin-enhanced luminescence as described previously [18]. Approximately 30 mg protein of ischemic left ventricular region was separated, immediately minced, and incubated in 5 ml of oxygen-equilibrated Krebs-Henseleit solution containing 10 μM HEPES- NaOH (pH 7.4) for 20 min at room temperature. The samples were then placed into glass tubes containing 10 μM lucigenin in a final volume of 1 ml Krebs-Henseleit solution. Superoxide levels were

reported as relative light units (RLU) after background luminescence subtraction and were normalized to milligram wet tissue weight.

Determination of myocardial apoptosis

Myocardial apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining and caspase-3 activity as described previously [19]. TUNEL staining was performed via In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, German) per manufacturer's protocol. In brief, cardiomyocytes from at least four slides per block randomly selected were evaluated immunohistochemically to determine the number and percentage of cells exhibiting apoptotic-positive staining. The slides were covered with the mounting medium containing DAPI for detection of total nuclei. 10 fields were randomly chosen from each slide, and a total of 100 cells per field were counted within a defined rectangular field area ($\times 20$ objective). The index of apoptosis was determined (number of apoptotic myocytes/the total number of myocytes counted $\times 100\%$) from a total of 40 fields per heart, and the assays were performed in a blinded manner. The caspase-3 activity assay utilized the fluorogenic substrate DEVD-7-amino-4-trifluoromethyl-coumarin (AFC). In brief, cells or mouse heart tissue were lysed by $1\times$ caspase-3 lysis buffer (50 mM HEPES, pH 7.4, 0.1 % CHAPS 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate, 5 mM DTT, 0.1 mM EDTA, 0.1 % Triton X-100), and total protein concentration was determined by the Bradford method (Bio-Rad). To each well of a 96-well plate, supernatant containing 50 $\mu\text{g}/50\ \mu\text{l}$ of protein was loaded and incubated with 3.645 μg of Ac-DEVD-AFC (Biomol, P-409) in 50 μl $2\times$ Assay buffer (100 mM HEPES, 200 mM NaCl, 0.2 % CHAPS, 2 mM EDTA, 10 % glycerol, 10 mM DTT, pH 7.4) at 37 °C for 1.5 h. AFC was cleaved from Ac-DEVD-AFC by activated caspase-3, and the free AFC was quantified with Spectra Max M5 fluorescence microplate reader (excitation wavelength, 400 nm; emission wavelength, 508 nm, Molecular Devices, Sunnyvale, CA) by AFC standard curve (Biomol, KI-108). Caspase-3 activity was expressed as nanomoles of AFC formation per hour per milligram of protein.

Determination of infarct size and cardiac function

Myocardial infarct size was assessed via Evans blue/2,3,5-triphenyl tetrazolium chloride double-staining method and expressed as a percentage of infarct area over ischemic area (AAR, area-at-risk) [19]. Cardiac function was determined by echocardiography and left ventricular catheterization methods 24 h after reperfusion before thoracotomy, as described previously [18, 19]. In brief, at the end of the 24-hour reperfusion period, mice were re-anesthetized and cardiac function was determined by invasive hemodynamic evaluation methods. The left ventricular ejection fraction (LVEF), left ventricular end-diastolic pressure (LVEDP), and the first derivative of the left ventricular pressure (dP/dt_{max}) were obtained by use of computer algorithms and an interactive videographics program (Po-Ne-Mah Physiology Platform P3 Plus, Gould Instrument Systems, Valley View, Ohio).

Statistical analysis

All values in the text and figures are presented as mean $\pm$ SE of the mean of n independent experiments. Analysis of variance was performed across all investigated groups first. Data (except Western blot density) were subjected to t test (two groups) or one-way ANOVA

(three or more groups) followed by Bonferroni correction for post hoc *t* test. Western blot densities were analyzed with Kruskal–Wallis test followed by Dunn post hoc test. *p* values less than or equal to 0.05 were considered statistically significant.

Results

CTRP9 is highly expressed in adult heart

As expected, adipose APN mRNA expression level is markedly higher than any CTRPs investigated (data not shown). To determine the levels of CTRPs in adult mouse heart, mRNA expression levels of eight CTRPs known to be expressed in rodent tissue concomitant with APN were determined. Interestingly, several CTRPs including CTRP1, CTRP4, CTRP7, and CTRP9 are cardiac expressed at levels significantly greater than APN (Fig. 1a). Most notably, the mRNA level of CTRP9, the closest paralog of APN, is 300-times less than APN in adipose tissue (Fig. 1b), but exceeds APN by >100-fold in cardiac tissue (Fig. 1c).

Cardiac CTRP9 expression level is only four-times less than that of adipose tissue, suggesting that the levels of locally produced CTRP9 in the heart may exceed the plasma levels of CTRP9 that are produced by remote organs and present in circulation. To directly test this possibility, we determined CTRP9 protein concentration in adipose tissue, plasma, and cardiac tissue. Because CTRP9 ELISA assay methods are currently unavailable, identical amounts of total proteins (80 lg) were loaded, and CTRP9 protein levels were analyzed by Western blot. As summarized in Fig. 1d, high levels of CTRP9 protein are detected in adipose tissue. However, plasma CTRP9 level is extremely low. Consistent with CTRP9 mRNA results presented in Fig. 1a, significant CTRP9 protein is detected in cardiac tissue reaching concentrations approximately tripled of plasma CTRP9 (Fig. 1d). Even after total protein normalization, cardiac levels of CTRP9 remain approximately 1.6-fold higher than plasma CTRP9 levels. These results differ significantly from APN, as plasma APN levels (7.46 ± 0.42 µg/ml) are the several orders of magnitude greater than APN locally produced in the heart (18.39 ± 1.68 ng/ml). Finally, both low and high molecular weight CTRP9 isoforms were clearly detected in medium collected 48 h after H9c2 cell culture (E, left lane, no-reducing SDS-PAGE). CTRP9 was also detected in adult cardiomyocyte culture medium (E, right lane). However, because adult mouse cardiomyocytes cannot be cultured for a prolonged period in vitro and culture medium was collected at an earlier time point (16 h), CTRP9 levels were lower in adult cardiomyocytes medium than that in H9c2 cell medium (collected 48 h after culture).

Reduced cardiac CTRP9 expression in type 2 diabetes

We recently reported that adipocyte CTRP9 expression is significantly reduced in HFD-induced diabetic mice [26]. Having demonstrated that CTRP9 is highly expressed in the adult heart, we next determined whether diabetes may alter cardiac CTRP9 expression. Both mRNA and protein levels of CTRP9 were determined in cardiac tissue of high-fat diet fed (HFD) mice and their age-matched normal diet fed (ND) mice. There was no significant difference between CTRP9 mRNA and protein levels from either adipose or cardiac tissue of animals fed ND up to 16 weeks (data not shown). Compared to age-matched mice fed ND,

CTRP9 mRNA levels were slightly increased 2 weeks after HFD, followed by significant reduction 8 and 16 weeks after HFD (Fig. 2a). Cardiac CTRP9 protein levels were increased in HFD-fed animals at 4 weeks ($p < 0.05$), and significantly decreased at 8 weeks ($p < 0.05$) and 16 weeks ($p < 0.01$) (Fig. 2b). These results demonstrate that cardiac CTRP9 is significantly inhibited when mice are fed HFD for 8 weeks or longer, when diet-induced type 2 diabetes develops [25].

TNF- α significantly inhibits CTRP9 expression

It is well accepted that obesity and diabetes constitute high plasma glucose/lipid, low-level inflammatory states, accompanied by elevated cytokines, particularly TNF- α [12]. To identify the molecules most responsible for inhibiting cardiac CTRP9 expression in the diabetic condition, H9c2 cells were treated with TNF- α , high glucose (HG), or high lipid (HL) for 24 h, and consequent effect upon CTRP9 expression (mRNA and protein) was determined. Incubation of H9c2 cells with HG or HL for 24 h only slightly inhibited CTRP9 expression (Fig. 3). However, incubation with TNF- α markedly inhibited CTRP9 expression at both the mRNA (Fig. 3a, >70 % reduction vs. control) and protein (Fig. 3b, >60 % reduction vs. control) levels.

Antioxidant treatment reverses CTRP9 inhibitory effect of TNF- α

Oxidative stress plays a critical role in TNF- α biological signaling [17]. To determine whether the observed CTRP9 inhibition by TNF- α is mediated by increased oxidative stress, we utilized tiron, a cell permeable superoxide scavenger. As expected, treatment with TNF- α significantly upregulated gp91^{phox} expression and caused a 2.47-fold increase in superoxide production. More importantly, co-treatment with tiron (10 mM) completely restored (mRNA level, Fig. 4a) or markedly reversed (protein level, Fig. 4b) the TNF- α inhibitory effect upon CTRP9 expression. These results suggest that TNF- α inhibits CTRP9 expression primarily via increasing superoxide production.

PPAR γ regulates cardiac CTRP9 expression and is inhibited by TNF- α

PPAR γ , a transcriptional factor, is crucial for adipocyte differentiation and APN gene expression [4]. After demonstrating CTRP9 downregulation by TNF- α via superoxide overproduction, we next investigated whether cardiac CTRP9 expression is regulated by PPAR γ , and whether PPAR γ expression is altered by TNF- α . As summarized in Fig. 4c, TNF- α administration to H9c2 cells significantly decreased PPAR γ levels compared to control ($p < 0.01$). Co-treatment with tiron significantly, although not completely, restored PPAR γ expression ($p < 0.01$). These results indicating PPAR γ followed a similar pattern as that of CTRP9 after TNF- α and tiron treatments suggest that cardiac CTRP9 expression might be regulated by PPAR γ which was inhibited by TNF- α via oxidative stress.

To obtain direct evidence to support this notion, the effect of PPAR γ agonist rosiglitazone upon CTRP9 expression was determined. As summarized in Fig. 5, rosiglitazone (10 μ M, 24 h) significantly increase CTRP9 expression in the absence of TNF- α , and reversed TNF- α -attenuated CTRP9 mRNA and protein expression. Taken together, these results demonstrate that TNF- α inhibits CTRP9 transcript and protein expression via oxidative stress mediating PPAR γ suppression.

CTRP9 knockdown significantly increases SI/R injury

Our aforementioned results demonstrated that CTRP9 is abundantly expressed in the adult heart, and is inhibited by diabetes likely via TNF- α -superoxide-PPAR γ signaling. To determine the pathological significance of diabetic inhibition of cardiac CTRP9, we employed siRNA-mediated knockdown of CTRP9 in H9c2 cells and determined its effect on simulated ischemia/reperfusion (SI/R)-induced apoptotic cell death. Pilot experiments demonstrated that altering the concentration of siRNA against CTRP9 reduced CTRP9 expression to varying degree (23–78 %). Since CTRP9 expression was reduced by approximately 30 % in diabetic heart, the concentration of siRNA employed was adjusted to reduce H9c2 cell CTRP9 expression to a level comparable to that observed in the diabetic heart (Fig. 6a). As summarized in Fig. 6b, knockdown of CTRP9 significantly increased SI/R-induced apoptotic cell death, which was completely reversed when exogenous CTRP9 (3 μ g/ml) was administered. Moreover, knockdown of CTRP9 further enhanced SI/R-induced superoxide production (Fig. 6c). Finally, overexpression of CTRP9 (pCMV-Tag4A encoding gCTRP9) significantly reduced SI/R-induced apoptotic cell death (Fig. 6d).

CTRP9 reduces apoptosis, decreases infarct size, and improves cardiac function recovery post-MI/R

As a final step to prove our concept that CTRP9 is a cell survival molecule, we investigated the effect of exogenous gCTRP9 supplementation upon MI/R injury in diabetic mice. All mice survived for 3 h (for apoptosis) or 24 h (for other assays), and results from all mice were included. No significant difference was observed in area-at-risk between vehicle and gCTRP9 treated mice (46.7 ± 2.8 % vs. 47.4 ± 3.1 %). Consistent with previous reports, HFD-fed mice subjected to MI/R exhibited greater apoptosis, larger infarct size, and poorer cardiac function recovery after MI/R (data not shown). Exogenous CTRP9 supplementation reduced apoptosis (TUNEL: 40.4 % reduction; caspase 3 activation: 44.7 % reduction; Fig. 7a, b), decreased infarct size (41.3 % reduction; Fig. 7c), and augmented cardiac functional recovery compared to vehicle, as demonstrated by increased LVEF ($p < 0.01$, Fig. 8a), decreased LVEDP ($p < 0.01$, Fig. 8b), and increased maximum LV $dP/dt_{\max}$ ($p < 0.01$, Fig. 8c).

CTRP9 decreases myocardial oxidative stress during MI/R

Overproduction of reactive oxygen species (ROS) is a central cause of reperfusion-induced myocardial injury. Furthermore, increased NOX2 (gp91^{phox}) expression contributes to NADPH oxidase activation, a key enzyme involved in MI/R-induced oxidant species generation [1]. To determine whether CTRP9 may protect heart via an unreported anti-oxidative property, we determined the effect of CTRP9 upon superoxide production and gp91^{phox} expression. Consistent with previous reports, gp91^{phox} expression and superoxide production in the ischemic/reperfused heart are significantly increased in HFD-fed mice compared to ND-fed mice (data not shown). Importantly, gCTRP9 supplementation significantly decreased gp91^{phox} expression and attenuated superoxide production (Fig. 9). Together, these data demonstrate augmented gp91^{phox} expression and superoxide anion generation in diabetic hearts subjected to MI/R, and CTRP9 administration significantly attenuates oxidative stress during MI/R.

Discussion

We have made several important observations in the present study. Firstly, we demonstrate that several CTRPs including CTRP1, CTRP4, CTRP7, and CTRP9 are expressed in the heart at levels significantly greater than APN. Of those investigated, CTRP9 is the most abundantly expressed adipokine in the adult mouse heart, exceeding APN expression by more than 100-fold. In addition, using Western blot as a semi-quantitative assay we demonstrate that locally produced CTRP9 levels significantly exceed those in plasma circulation, suggesting that cardiac-produced CTRP9 may have significant regulatory role in cardiac physiology and its alteration may contribute to cardiac pathophysiology. CTRP9 is the closest APN paralog, and both share functions such as aging and gender regulation [22]. Moreover, when co-transfected into mammalian cells, CTRP9 forms hetero-oligomers with APN, as APN/CTRP9 complexes are readily found in the serum of APN and CTRP9 transgenic mice [22]. However, our current study demonstrates a significant difference in tissue distribution profile between these two closely related adipokines. Although CTRP9 mRNA is 300 times less abundant than APN mRNA levels in adipose tissue, the former exceeds the latter by >100-fold in the heart suggesting that although CTRP9 may have less significant systemic function compared to APN, this adipokine may have significant local regulatory role in the heart.

Secondly, we demonstrate that cardiac CTRP9 expression decreases significantly in high-fat diet induced type 2 diabetic mice. In obesity and type 2 diabetes, increased adiposity results in a chronic low-grade inflammatory state accompanied by elevated serum inflammatory cytokines, such as TNF- α , IL-6, and IL-18 [2, 7, 9, 13]. The regulatory role of TNF- α upon APN expression and biological function has been extensively investigated in the past decade. Plasma APN levels are inversely correlated with TNF- α concentration. TNF- α decreases APN expression and secretion by suppressing transcriptional factors promoting APN expression (e.g., PPAR γ , C/EBP, SREBP, DsbA-L, and retinoid X receptor- α), and promoting transcriptional factors suppressing APN transcription (e.g., IGFBP-3). Our current study demonstrates that antioxidant administration reduced superoxide content, and reversed the inhibitory effect of TNF- α upon PPAR γ and CTRP9 expression. In addition, PPAR γ agonist rosiglitazone restored TNF- α -induced attenuated CTRP9 mRNA and protein levels. To the best of our knowledge, these are the first direct evidence that TNF- α inhibits cardiac CTRP9 expression via oxidative stress mediated inhibition of transcription factor PPAR γ . These results suggest that TNF- α -mediated augmented oxidative stress with resultant PPAR γ attenuation is a possible mechanism explaining CTRP9 deficiency in the diabetic state.

Finally, we have demonstrated that locally produced CTRP9 is a cell survival molecule, and that exogenously supplemented CTRP9 exerts cardioprotective effect against MI/R injury via a previously unrecognized antioxidant function. The physiological functions regulated by CTRP9 remain under investigation. However, evidence suggests an important metabolic role for CTRP9, as Wong et al. [22] demonstrated that CTRP9 overexpression not only reduces serum glucose levels, but also insulin levels without affecting body weight or the amount of food consumed in ob/ob mice. Our previous study demonstrated that CTRP9 administration induced potent endothelium-dependent and nitric-oxide (NO)-mediated vasorelaxation [26].

Moreover, while this manuscript is in final preparation, a study by Kambara et al. [6] reported that in non-diabetic mice systemic administration of CTRP9 protects against myocardial injury following ischemia–reperfusion through activation of AMPK. In the current experiment, we demonstrated that in vivo CTRP9 supplementation in diabetic mice reduced oxidative stress, decreased infarct size and apoptotic index, and augmented cardiac function post-MI/R. More importantly, we have demonstrated for the first time that knockdown of CTRP9 with siRNA in H9c2 cells to a comparable extent as that observed in the diabetic heart significantly increased apoptotic cell death after SI/R, indicating that physiologically expressed CTRP9 in cardiomyocytes is required for cell survival against ischemic insult. Such data further confirms the pathologic significance of CTRP9 deficiency, and attributes potential therapeutic value to exogenous CTRP9 administration.

In conclusion, we demonstrate for the first time that abundantly expressed CTRP9 in the heart is a novel cell survival molecule, and its downregulation by TNF- α -initiated oxidative PPAR γ suppression contributes to exacerbated cardiac injury in diabetic heart. Preservation of CTRP9 expression or augmentation of CTRP9 initiated signaling mechanisms may be the potential avenues for ameliorating ischemic diabetic cardiac injury.

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Fig. 1.

CTRP9 mRNA and protein are highly expressed in adult mouse heart. **a** The mRNA levels of APN and CTRPs in adult mouse heart. **b** The mRNA level of CTRP9 and APN in adipose tissue. **c** The mRNA level of CTRP9 and APN in cardiac tissue. **d** CTRP9 protein in adipose tissue, plasma and cardiac tissue determined by representative Western blots. **e** Detection of CTRP9 in culture medium of H9c2 cells (24 h) and adult cardiomyocytes (12 h). $n = 5-8$ per group

Fig. 2.

CTR9 expression is reduced in cardiac tissue from high-fat diet (HFD)-induced diabetic mice. **a** Fasting plasma glucose, **b** fasting plasma insulin and **c** HOMA Score before (0) and after 8 weeks normal (ND) or high-fat diet (HFD). **d** CTR9 mRNA levels in cardiac tissue from HFD-fed groups. **e** CTR9 protein levels of cardiac tissue from HFD-fed groups determined by representative Western blots. $n = 5-8$ heart per group. * $p < 0.05$, ** $p < 0.01$ versus HFD 0 week group

Fig. 3. TNF- α significantly downregulates CTRP9 expression. **a** CTRP9 mRNA in H9c2 cells treated with TNF- α (10 ng/ml), high glucose (HG, 33 mM), and high lipid (HL, palmitate, 300 μ M). The relative expression levels of CTRP9 transcript were normalized to the control group. **b** CTRP9 protein in H9c2 cells treated with TNF- α , HG, and HL. $n = 8-10$ per group. * $p < 0.05$, ** $p < 0.01$ versus control group

Fig. 4. Antioxidant treatment reverses inhibitory effect of TNF- α on CTRP9 and PPAR γ expression. **a** TNF- α inhibited CTRP9 mRNA expression via oxidative stress in H9c2 cells. **b** TNF- α inhibited CTRP9 protein via augmented oxidative stress. **c** TNF- α inhibited PPAR γ expression via oxidative stress. $n = 8-10$ per group. * $p < 0.05$, ** $p < 0.01$ versus control group

Fig. 5.

TNF- α inhibits cardiac CTRP9 expression via decreased PPAR γ . **a** PPAR γ agonist rosiglitazone (RSG, 10 μ M) reversed the effect of TNF- α upon CTRP9 mRNA levels in H9c2 cells. **b** RSG reversed the effect of TNF- α upon CTRP9 protein expression in H9c2 cells. $n = 8-10$ per group. * $p < 0.05$, ** $p < 0.01$ versus control group. ## $p < 0.01$ versus TNF- α group

Fig. 6. siRNA-mediated CTRP9 knockdown significantly increases simulated ischemia/reperfusion (SI/R) injury in H9c2 cells. **a** The effect of CTRP9-siRNA upon CTRP9 protein levels in H9c2 cells. **b** The effect of CTRP9 knockdown upon caspase-3 activity in SI/R H9c2 cells. **c** The effect of CTRP9 knockdown upon superoxide production in SI/R H9c2 cells. **d** The effect of CTRP9 overexpression upon caspase-3 activity in SI/R H9c2 cells. $n = 8-10$ per group. $**p < 0.01$ vs. sham group. $##p < 0.01$ versus SI/R scramble group (**b**) or versus SI/R group (**c**, **d**)

Fig. 7. CTRP9 reduces myocardial ischemia/reperfusion (MI/R) injury in high-fat diet (HFD)-induced diabetic mice. CTRP9 reduced cardiomyocyte apoptosis of HFD mice subjected to MI/R. Cardiomyocyte apoptosis determined by **a** terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and **b** Caspase-3 activity. **c** CTRP9 reduced infarct size determined by Evans blue/2,3,5-triphenyl tetrazolium chloride double-staining technique in HFD mice subjected to MI/R. $n = 5-8$ hearts per group. ** $p < 0.01$ vs. sham. ## $p < 0.01$ versus vehicle group

Fig. 8.

CTRP9 improves cardiac function in HFD-induced diabetic mice post myocardial ischemia/reperfusion (MI/R). **a** Left ventricular ejection fraction (LVEF), determined by echocardiography. **b** Left ventricular end-diastolic pressure (LVEDP), determined by hemodynamic measurements. **c** Maximum rate of left ventricular pressure change (LV dP/dt_{max}), determined by hemodynamic measurements. $n = 5-8$ hearts per group. * $p < 0.05$, ** $p < 0.01$ versus sham group, ## $p < 0.01$ versus vehicle group

Fig. 9.

CTRP9 reduces oxidative stress in ischemic/reperfused heart. **a** CTRP9 reduced gp91^{phox} determined by representative Western blots in HFD mice hearts subjected to MI/R. **b** CTRP9 reduced superoxide anion production of HFD mice subjected to MI/R. $n = 5-8$ hearts per group. ** $p < 0.01$ versus sham. ## $p < 0.01$ versus vehicle group