Original Research

CTRP3 protects against uric acid-induced endothelial injury by inhibiting inflammation and oxidase stress in rats

Junxia Zhang[1,2,3]*, Xue Lin[1,2]*, Jinxiu Xu[1,3], Feng Tang[1,3] and Lupin Tan[1,2]

[1]Department of Endocrinology, Central Theater Command General Hospital of the Chinese PLA, Wuhan 430070, China; [2]The First School of Clinical Medicine, Southern Medical University, Guangzhou 510515, China; [3]School of Medicine, Wuhan University of Science and Technology, Wuhan 430065, China

Corresponding author: Junxia Zhang. Email: zhangjx023@163.com

*These authors contributed equally to this article.

Impact statement

Uric acid could cause damage in vascular endothelium, which is involved in the development of various cardiovascular diseases (CVDs). The present study reported that C1q-tumor necrosis factor-related protein-3 (CTRP3) ameliorated thoracic aortic endothelium damage in rats with hyperuricemia and protected against uric acid-induced endothelial damage in human umbilical vein endothelial cells (HUVECs). Both reduced toll-like receptor 4 (TLR4)-mediated inflammation and improved oxidative stress are involved in the protective effect of CTRP3 on uric acid-induced endothelial dysfunction. Our data shed lights on a novel role of CTRP3 in the prevention and treatment of endothelial damage caused by uric acid.

Abstract

Hyperuricemia, which contributes to vascular endothelial damage, plays a key role in multiple cardiovascular diseases. This study was designed to investigate whether C1q/tumor necrosis factor (TNF)-related protein 3 (CTRP3) has a protective effect on endothelial damage induced by uric acid and its underlying mechanisms. Animal models of hyperuricemia were established in Sprague-Dawley (SD) rats through the consumption of 10% fructose water for 12 weeks. Then, the rats were given a single injection of Ad-CTRP3 or Ad-GFP. The animal experiments were ended two weeks later. *In vitro*, human umbilical vein endothelial cells (HUVECs) were first infected with Ad-CTRP3 or Ad-GFP. Then, the cells were stimulated with 10 mg/dL uric acid for 48 h after pretreatment with or without a Toll-like receptor 4 (TLR4)-specific inhibitor. Hyperuricemic rats showed disorganized intimal structures, increased endothelial apoptosis rates, increased inflammatory responses and oxidative stress, which were accompanied by reduced CTRP3 and elevated TLR4 protein levels in the thoracic aorta. In contrast, CTRP3 overexpression decreased TLR4 protein levels and ameliorated inflammatory responses and oxidative stress, thereby improving the morphology and apoptosis of the aortic endothelium in rats with hyperuricemia. Similarly, CTRP3 overexpression decreased TLR4-mediated inflammation, reduced oxidative stress, and rescued endothelial damage induced by uric acid in HUVECs. In conclusion, CTRP3 ameliorates uric acid-induced inflammation and oxidative stress, which in turn protects against endothelial injury, possibly by inhibiting TLR4-mediated inflammation and downregulating oxidative stress.

Keywords: C1q/tumor necrosis factor related protein-3, uric acid, endothelial cell, Toll-like receptor 4, inflammation, oxidative stress

***Experimental Biology and Medicine* 2022; 247: 174–183. DOI: 10.1177/15353702211047183**

Introduction

It is well known that hyperuricemia is involved in various cardiovascular diseases (CVDs), such as hypertension, coronary artery disease, and ischemic stroke.[1–3] A well-functioning vascular endothelium plays a key role in the protection of the cardiovascular system. Multiple studies have suggested that high levels of uric acid could cause damage to the vascular endothelium. A cross-sectional survey reported that hyperuricemia was a strong predictor of an increased risk of CVDs in women.[4] Levels of serum nitrites and nitrates were decreased in rats with hyperuricemia induced by oxonic acid, and these levels were reversed by the short-term effect of allopurinol.[5] Similarly, benzbromarone prevented a reduction in nitric oxide (NO) in human umbilical vein endothelial cells (HUVECs) stimulated by uric acid.[6] However, a method

to improve uric acid-induced damage in the vascular endothelium has not been well described.

Adipose tissue is the largest endocrine organ in our body and can secrete a great variety of bioactive peptides known as adipokines. C1q-tumour necrosis factor-related protein-3 (CTRP3), a novel adipokine, is considered a paralogue in a highly conserved family of adiponectins.[7] It has been demonstrated that CTRP3 can improve insulin sensitivity and regulate glycolipid metabolism.[8] More interestingly, CTRP3 may have a potential protective effect against CVDs. An observational study reported that exercise training could increase serum CTRP3 levels, which was positively associated with reduced arterial stiffness in middle-aged and older adults.[9] CTRP3 overexpression or recombinant CTRP3 replenishment could inhibit cardiac fibrosis, promote angiogenesis, improve cardiac function, and increase the survival rates of mice with myocardial infarction.[10,11] In addition, rats with diabetic cardiomyopathy showed decreased myocyte death and restored cardiac function with CTRP3-specific overexpression in cardiomyocytes.[12] However, the role of CTRP3 in uric acid-induced endothelial damage remains elusive.

It is well established that both inflammation and oxidative stress are involved in endothelial dysfunction. In our previous study, uric acid caused toll-like 4 (TLR4)-related inflammatory responses in the adipose tissue of rats with hypertension.[13] We also found that uric acid stimulated oxidative stress in adipocytes.[14] CTRP3 has been reported to have anti-inflammatory and antioxidant effects.[12] Therefore, our study was designed to determine whether CTRP3 could protect against uric acid-induced endothelial injury using hyperuricemic rats or HUVECs stimulated with uric acid as models. We also tried to determine the potential mechanisms, focusing on inflammation and oxidative stress.

Materials and methods

Animal model and experimental design

Animals. All of the animal experimental protocols were approved by the Ethics Committee of Central Theater Command General Hospital of the Chinese PLA and followed the guidelines of the Care and Use of Laboratory Animals of the National Institutes of Health. Twenty male Sprague-Dawley (SD) rats (six to eight weeks old, 180 to 220 g) were purchased from Hunan SJA Laboratory Animal Co., Ltd (Changsha, Hunan, China) and housed under standard conditions. The rats were fed adaptively for one week before the experiments.

Animal models. In recent years, the epidemic of hyperuricemia parallels the increased consumption of foods that are rich in fructose.[15] Therefore, in the present study, 10% (w/v) fructose (Juntai Biotech Co., Ltd, F8100, Wuhan, China) water was used to establish an animal model of hyperuricemia. Serum uric acid levels greater than 1.5 mg/dL were considered to indicate hyperuricemia in rats. The rats that drank water without fructose served as controls. All rats were fed a standard chow diet (HFK Bioscience, D12450B, Beijing, China). At 12 weeks, the rats were given a single caudal vein injection of adenoviruses expressing CTRP3 (Ad-CTRP3, Vigene Bioscience, 20180727005, Shandong, China) or adenoviruses expressing green fluorescent protein (Ad-GFP, Vigene Bioscience, 20180912004, Shandong, China) at a dose of 2×10^9 p.f.u. per rat. Then, the rats were divided into four groups ($n = 5$ each group): ① In the normal control (NC) group, the rats drank water without fructose and were infected with Ad-GFP; ② in the CTRP3 group, the rats drank water without fructose and were infected with Ad-CTRP3; ③ in the high uric acid (HUA) group, the rats drank 10% fructose water and were infected with Ad-GFP; and ④ in the HUA +CTRP3 group, the rats drank 10% fructose water and were infected with Ad-CTRP3. The experiment was ended at 14 weeks.

Measurements. At the end of the experiment, the rats were anesthetized by intraperitoneal injection of 10% chloral hydrate. Blood samples were collected from the jugular vein and centrifuged immediately at 1000 r/min for 20 min. Then, serum samples were stored at −80°C to measure NO, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), hydroxyl radical scavenging activity, and malondialdehyde (MDA). The thoracic aorta was isolated after the perivascular adipose tissue was removed completely. After being washed with prechilled PBS, thoracic aorta segments were obtained and sampled. Aorta inflammation levels were assessed with TNF-α and IL-6 mRNA as well as TLR-4 protein expression. Aorta oxidative stress levels were analyzed with MDA and reactive oxygen species (ROS). Hematoxylin-eosin (HE) and TUNEL staining were used to assess the aorta morphology changes.

Cell culture and adenovirus transfection

HUVECs were purchased from Shanghai Institute of Biochemistry and Cell Biology, CAS (China). The cells were cultured in DMEM (Gibco, 1677149, USA) containing 10% fetal bovine serum (Gibco, 10099–141, USA) and 1% penicillin-streptomycin (HyClone, DY14011, USA). HUVECs at the exponential growth stage were seeded onto six-well culture plates at a density of 5×10^5 cells per well. After 24 h, endothelial cells were infected with Ad-CTRP3 or Ad-GFP at a dose of 2.56×10^8 p.f.u. per well. Then, 48 h later, infected cells were stimulated with 10 mg/dL uric acid (Ultrapure, Sigma, U2625, USA) after pretreatment with TAK242 (a TLR4-specific inhibitor, MCE, HY-11109, USA) for 48 h, and these cells were divided into the HUA+ TAK242 group or HUA+ CRPP3+TAK242 group. Cells stimulated with uric acid without TAK242 pretreatment were divided into the HUA group or HUA+CTRP3 group. Infected cells without uric acid stimulation served as the NC group and CTRP3 control group. At the end of the experiments, cells and supernatant were collected for further measurements.

Measurement of biochemical indexes, hydroxyl radical scavenging activity, and ROS

The thoracic aorta segments were mixed with PBS at a ratio of 1:9 and ground with a glass homogenizer. Then, the homogenate was centrifuged at 5000 r/min for 10 min to

obtain the tissue supernatant. The levels of uric acid in rat thoracic aorta were measured with a commercial kit at an absorbance of 690 nm (Jiancheng Bioengineering Institute, C012-1, Nanjing, China). Serum uric acid levels were also assessed with the same kit. The concentrations of NO in rat serum and in the supernatants of cultured HUVECs were measured at an absorbance of 550 nm (Jiancheng Bioengineering Institute, A013-2, Nanjing, China). MDA levels in rat serum and the thoracic aorta, as well as cultured HUVECs, were measured at an absorbance of 532 nm (Jiancheng Bioengineering Institute, A003-1, Nanjing, China). In addition, hydroxyl radical scavenging activity in rat serum was measured at 550 nm (Jiancheng Bioengineering Institute, A018, Nanjing, China). All of target parameters were measured using a Thermo Scientific instrument (Multiscan MK3, USA) according to the manufacturer's instructions.

To determine ROS levels in the thoracic aorta, tissue homogenate was incubated with 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, Jiancheng Bioengineering Institute, E004, Nanjing, China), a fluorescent probe, for 30 min at 37°C. Then, the fluorescence intensity was analyzed with a multiscan spectrum (Molecular Devices, Flexstation3, USA) at an excitation wavelength of 485 nm and an emission wavelength of 525 nm. Similarly, ROS levels in cultured HUVECs were measured with a commercial kit (Beyotime Institute of Biotechnology, S0063, Shanghai, China). Briefly, cells were incubated with 5 μM dihydroethidium for 20 min at 37°C. The fluorescence intensity was measured using a flow cytometer (cytoFLEX, Beckman Coulter).

Aortic intima morphotype and vascular endothelial apoptosis

HE staining was conducted to observe changes in the intima morphotype in the thoracic aorta using a standardized protocol. To determine the apoptosis rate of the endothelium, thoracic aorta tissue were fixed with Carnoy solution, embedded in paraffin, and then sectioned at a thickness of 5 μm. Based on the manufacturer's protocols, aortic endothelium apoptosis was assessed with a commercial kit (Roche Applied Science, 11684817910, Switzerland) by a fluorescence microscope (BX-53, Olympus). Similarly, HUVECs cultured *in vitro* were fixed with 4% paraformaldehyde for 25 min. Endothelial apoptosis was analyzed in the same way. The apoptosis rate of TUNEL-positive cells relative to the total number of cells was calculated (5 visual fields taken from each sample, 80–200 cells analyzed per image). TUNEL-positive cells showed red fluorescence as compared with normal cells with blue fluorescence.

Quantitative real-time reverse transcriptase–polymerase chain reaction

Total RNA was extracted from thoracic aorta segments or cell lysates using TRIzol reagent (Ambion, 15596026, USA). First-strand cDNA was synthesized from 2 μg of total RNA with a RevertAid First Strand cDNA Synthesis Kit (Vazyme, R101-01/02, China). Target genes and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified with SYBR Green Master Mix (Vazyme, Q111-02, China) using a real-time PCR system (ABI QuantStudio 6, USA). The reaction conditions were as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 30 s. All primers used in the present study are shown in Table 1. The mRNA expression of these target genes was normalized to GAPDH.

Enzyme-linked immunosorbent assay and Western blotting

The serum samples were centrifuged at 1000 r/min for 20 min and carefully transferred to labeled EP tubes for analysis. The levels of TNF-α (Elabscience Biotechnology Co., E-EL-R0019c, Wuhan, China) and IL-6 (Elabscience Biotechnology Co., E-EL-R0015c, Wuhan, China) in rat serum were determined by commercial ELISA kits at an absorbance of 450 nm using a Thermo Scientific instrument (Multiscan MK3, USA). The protein expression levels of CTRP3 and toll-like receptor 4 (TLR4) were measured using Western blotting with the following primary antibodies: rabbit anti-CTRP3 at a 1:1000 dilution (Abcam, ab36870, UK), rabbit anti-TLR4 at a 1:1000 dilution (Abcam, ab217274, UK), and rabbit anti-GAPDH at a 1:3000 dilution (Abcam, ab9485, UK). Secondary antibodies were labeled with HRP (1:50,000, Boster Biological Technology, BA1054, Wuhan, China). Protein bands were measured with BandScan software and normalized to the levels of GAPDH.

Table 1. Primer sequences.

Gene name	Forward primer	Reverse primer
Rat-eNOS	GGTATTTGATGCTCGGGACTGC	GTGATGGCTGAACGAAGATTGC
Rat-TNF-α	CCAGGTTCTCTTCAAGGGACAA	GGTATGAAATGGCAAATCGGCT
Rat-IL-6	AGGATACCACCCACAACAGACC	TTGCCATTGCACAACTCTTTTC
Rat-GAPDH	CTGGAGAAACCTGCCAAGTATG	GGTGGAAGAATGGGAGTTGCT
Hum-eNOS	CCTGACAACCCCAAGACCTAC	TAACATCGCCGCAGACAAAC
Hum-TNF-α	CTCGAACCCCGAGTGACAAG	TGAGGTACAGGCCCTCTGAT
Hum-IL-6	GGTCCAGTTGCCTTCTCCC	GTGCCTCTTTGCTGCTTTC
Hum-GAPDH	TCAAGAAGGTGGTGAAGCAGG	TCAAAGGTGGAGGAGTGGGT

eNOS: endothelial nitric oxide synthase; TNF-α: tumor necrosis factor-α; IL-6: interleukin-6; GADPH: glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis

All values in this study are expressed as the means ± standard deviation and were analyzed with SPSS software (version 21.0; SPSS, IBM, Armonk, NY). The data were analyzed with one-way ANOVA after the normal distribution tests. Then, a Bonferroni's *post hoc* test was performed. A P value less than 0.05 was defined as a statistically significant difference.

Results

Effect of CTRP3 on uric acid levels and the aortic endothelium in rats with hyperuricemia

CTRP3 protein expression in the thoracic aorta was measured to verify the efficacy of adenoviral infection (Figure 1 (a)). We found that CTRP3 protein levels were obviously decreased in the thoracic aortas of hyperuricemic rats. Ad-CTRP3 infection significantly increased CTRP3 protein expression in the CTRP3 control group and HUA+ CTRP3 group (Figure 1(a)). Moreover, both uric acid in the thoracic aorta and serum uric acid were increased at the end of the experiment in rats that drank 10% fructose water (Figure 1(b) and (c)). Interestingly, CTRP3 overexpression significantly reduced uric acid levels in the serum and thoracic aortas of rats with hyperuricemia (Figure 1(b) and (c)). However, there was no difference in body weight, average food intake, or water consumption in hyperuricemic rats with or without CTRP3 overexpression (data not shown).

Next, we observed the effect of CTRP3 overexpression on intimal structure and endothelial apoptosis in the thoracic aorta. HE staining showed that, in rats with hyperuricemia, the intima of thoracic aorta was disorganized and discontinuous, some of which was partially ruptured and detached (Figure 2(a)). According to the TUNEL assay, the rats in the HUA group had higher endothelial apoptosis rates in the thoracic aorta than rats in the NC group (Figure 2(a) and (b)). Additionally, the rats that drank 10% fructose water showed dramatically decreased endothelial nitric oxide synthase (eNOS) mRNA (Figure 2 (c)) expression in the thoracic aorta and slightly reduced serum NO concentrations (Figure 2(d)) compared with those of rats in the NC group. In contrast, CTRP3 overexpression significantly improved intimal structure, reduced the endothelial apoptosis rate, and increased eNOS mRNA in rats with hyperuricemia (Figure 2(a) to (c)). In addition, serum NO concentrations showed an increasing trend in hyperuricemic rats infected with Ad-CTRP3 (Figure 2(d)). These data indicate that CTRP3 protects rats from aortic endothelial damage induced by high uric acid.

CTRP3 improves inflammation and oxidative stress in the thoracic aorta in rats with hyperuricemia

Then, we investigated the effect of CTRP3 overexpression on inflammation in the thoracic aortas of hyperuricemic rats. Rats in the HUA group showed higher levels of serum TNF-α (Figure 3(a)) and IL-6 (Figure 3(b)) than normal controls. Similarly, there was a significant increase in the mRNA expression of TNF-α (Figure 3(d)) and IL-6 (Figure 3(e)) in the thoracic aortas of rats with hyperuricemia. However, hyperuricemic rats infected with Ad-CTRP3 showed significant reductions in the inflammatory response (Figure 3(a), (b), (d) and (e)). No difference was observed between normal controls and CTRP3 controls. Additionally, we observed changes in TLR4 protein expression in the thoracic aortas of rats. TLR4 protein levels were increased in rats with hyperuricemia compared with normal controls (Figure 3(c)). In contrast, CTRP3 overexpression significantly reduced TLR4 protein levels in both the CTRP3 group and HUA+CTRP3 group (Figure 3(c)). These data suggest that CTRP3 improves uric acid-induced inflammation possibly by inhibiting the TLR4 signaling pathway.

Moreover, we assessed oxidative stress in the thoracic aortas of rats. Compared with normal controls, rats in the HUA group showed lower abilities to scavenge serum hydroxyl radicals (Figure 4(a)) and higher serum MDA concentrations (Figure 4(b)). In accordance, there was a

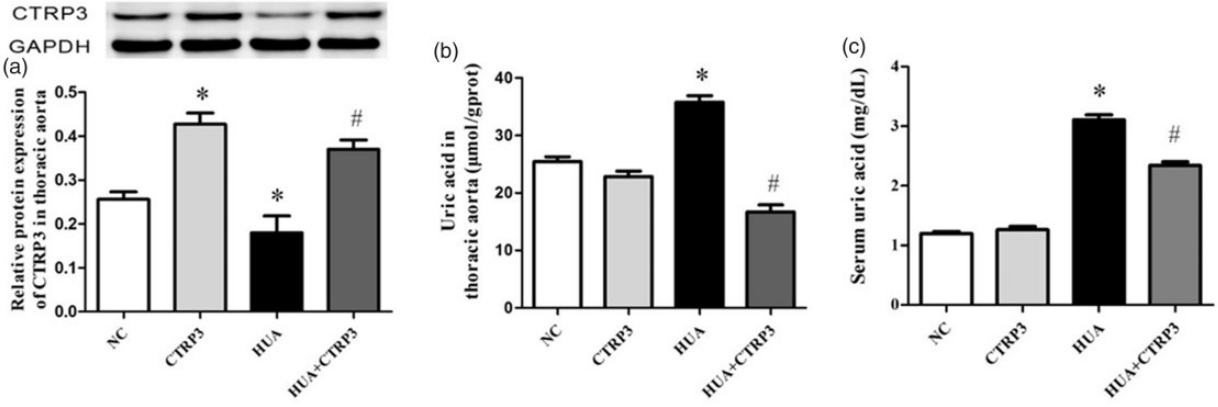

Figure 1. Effect of CTRP3 on uric acid levels in serum and thoracic aorta of rats with hyperuricemia. The SD rats drinking 10% fructose water for 12 weeks were given a single injection with Ad-CTRP3 or Ad-GFP. After two weeks, the experiment was ended. (a) Western blot of CTRP3, $n = 3$; (b) serum uric acid, $n = 5$; (c) uric acid in thoracic aorta, $n = 5$. *$P < 0.05$ compared with the control group; #$P < 0.05$ compared with the HUA group.
NC: normal control; CTRP3: C1q-tumor necrosis factor-related protein-3; HUA: high uric acid; GADPH: glyceraldehyde 3-phosphate dehydrogenase.

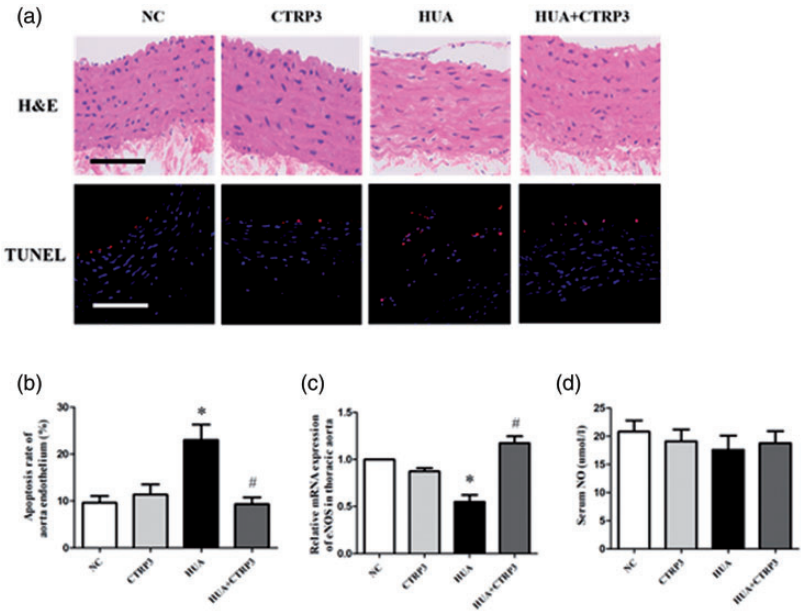

Figure 2. Effect of CTRP3 on endothelium in thoracic aorta of rats with hyperuricemia. The SD rats drinking 10% fructose water for 12 weeks were given a single injection with Ad-CTRP3 or Ad-GFP. After two weeks, the experiment was ended. (a) Representative images for HE staining and TUNEL assay in thoracic aorta; (b) apoptosis rate of aorta endothelium, five images for each sample, 80–200 cells per image, $n = 5$; (c) eNOS mRNA levels, $n = 3$; (d) serum NO levels, $n = 5$. Scale bar = 50 μm, $^*P < 0.05$ compared with the control group; $\#P < 0.05$ compared with the HUA group. (A color version of this figure is available in the online journal.)
NC: normal control; CTRP3: C1q-tumor necrosis factor-related protein-3; HUA: high uric acid.

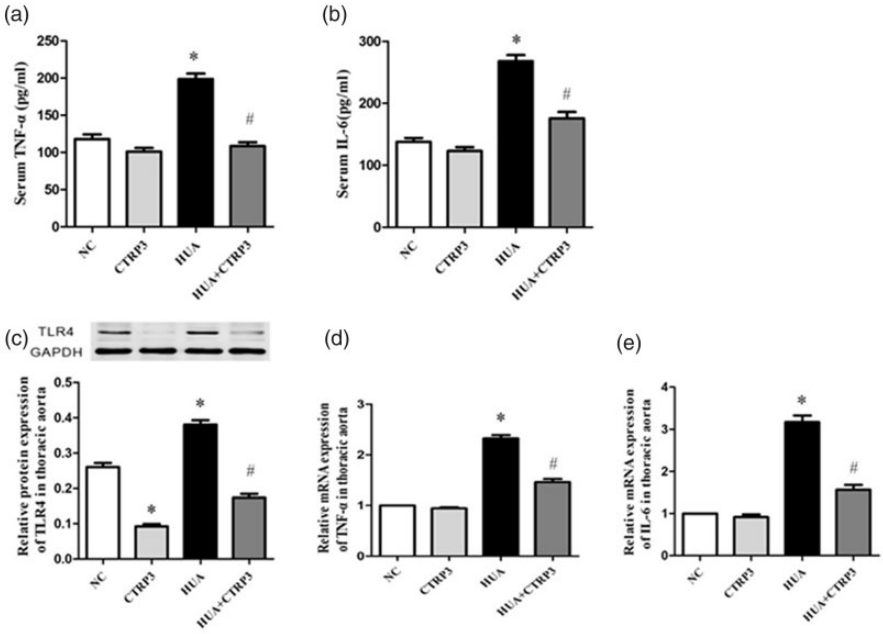

Figure 3. Effect of CTRP3 on inflammation of aorta endothelium in rats with hyperuricemia. The SD rats drinking 10% fructose water for 12 weeks were given a single injection with Ad-CTRP3 or Ad-GFP. After two weeks, the experiment was ended. (a) Serum TNF-α levels, $n = 5$; (b) serum IL-6 levels, $n = 5$; (c) Western blot of TLR4, $n = 3$; (d) TNF-α mRNA levels, $n = 3$; (e) IL-6 mRNA levels, $n = 3$. $^*P < 0.05$ compared with the control group; $\#P < 0.05$ compared with the HUA group.
NC: normal control; CTRP3: C1q-tumor necrosis factor-related protein-3; IL-6: interleukin-6; TNF-α: tumor necrosis factor-α; HUA: high uric acid.

significant increase in ROS and MDA concentrations in the thoracic aortas of rats with hyperuricemia (Figure 4(c) and (d)). However, CTRP3 overexpression significantly rescued oxidative stress induced by high uric acid in the thoracic aortas of rats (Figure 3(c) and (d)). These parameters did not show any differences in the normal control group and the CTRP3 group.

Reduced TLR4-mediated inflammation is involved in the protective effect of CTRP3 on uric acid-induced endothelial dysfunction in HUVECs

In vivo, we found that CTRP3 overexpression rescued inflammation and protected the aortic endothelium in rats with hyperuricemia. *In vitro*, using HUVECs cultured in the presence of uric acid as a model, we tried to examine

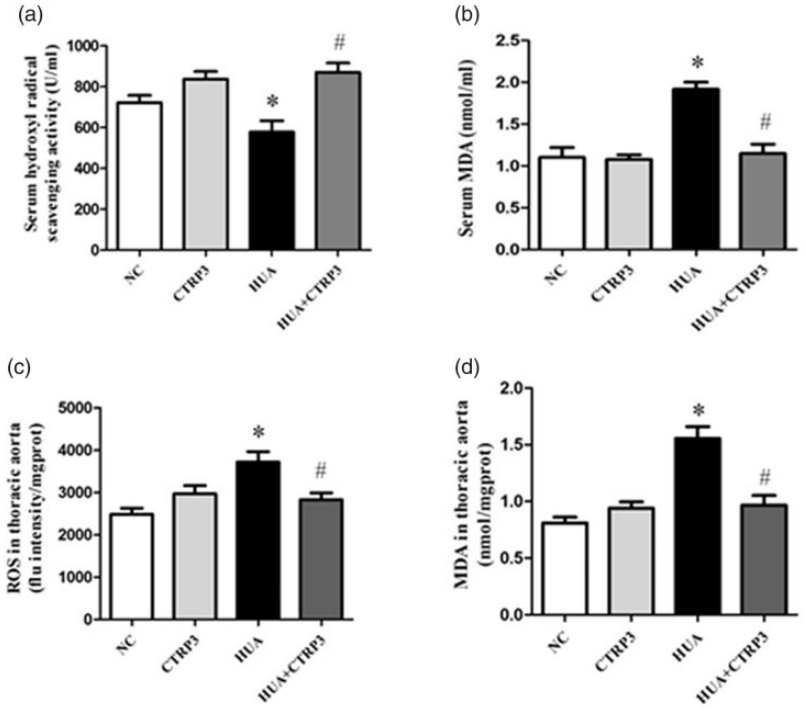

Figure 4. Effect of CTRP3 on oxidative stress of aorta endothelium in rats with hyperuricemia. The SD rats drinking 10% fructose water for 12 weeks were given a single injection with Ad-CTRP3 or Ad-GFP. After two weeks, the experiment was ended. (a) Serum hydroxyl radical scavenging activity, $n = 5$; (b) serum MDA levels, $n = 5$; (c) ROS levels in thoracic aorta, $n = 5$; (d) MDA levels in thoracic aorta, $n = 5$. *$P < 0.05$ compared with the control group; #$P < 0.05$ compared with the HUA group.
NC: normal control; CTRP3: C1q-tumor necrosis factor-related protein-3; MDA: malondialdehyde; ROS: reactive oxygen species; HUA: high uric acid.

the direct effect of CTRP3 on inflammatory cytokine expression and endothelial function. In particular, we focused on the role of TLR4 in this process. HUVECs were infected with Ad-CTRP3 or Ad-GFP. Next, the infected cells were incubated with 10 mg/dL uric acid for 48 h. Some cells were pretreated with 1 μM TAK242 for 1 h and then stimulated with 10 mg/dL uric acid. The infected cells incubated without uric acid stimulation served as normal controls or CTRP3 controls.

First, we measured CTRP3 protein expression levels in HUVECs. As shown in Figure 5(a), HUVECs incubated with uric acid showed lower CTRP3 protein expression than normal controls, and this level could be augmented when cells were infected with Ad-CTRP3. For cells in the HUA group, TAK242 pretreatment also led to increased CTRP3 protein levels. Interestingly, there was a further increase in CTRP3 protein expression when cells infected with Ad-CTRP3 were pretreated with TAK242.

Then, we examined endothelial function and apoptosis in HUVECs cultured in vitro. As shown in Figure 5(b) to (d), HUVECs stimulated with uric acid had increased apoptosis rates, reduced eNOS mRNA expression, and reduced NO concentrations in the supernatant. These effects could be partially abrogated by CTRP3 overexpression. Similarly, TLR4 inhibition also decreased apoptosis and improved endothelial function in cells incubated with uric acid. Moreover, CTRP3 overexpression in combination with TLR4 inhibition caused a greater improvement in endothelial damage induced by uric acid than CTRP3 overexpression alone.

Next, we assessed the protective effect of CTRP3 on TLR4-mediated inflammation induced by uric acid. As shown in Figure 6(a), Ad-CTRP3 infection obviously inhibited TLR4 protein expression in cells in the CTRP3 control group and HUA+CTRP3 group. TAK242 caused a decrease in TLR4 protein expression in cells incubated with uric acid. In addition, cells pretreated with TAK242 and infected with Ad-CTRP3 showed a further reduction in TLR4 expression compared with that of cells infected with Ad-CTRP3 alone. These data demonstrated that CTRP3 could suppress the expression of TLR4. Accordingly, both CTRP3 overexpression and TLR4 inhibition reduced TNF-α and IL-6 mRNA levels in uric acid-stimulated HUVECs (Figure 6(b) to (c)). Additionally, CTRP3 amplified the anti-inflammatory effect of TAK242 on HUVECs administered uric acid (Figure 6(b) and (c)). Taken together, these data suggest that TLR4-mediated inflammation suppression is involved in the protective effect of CTRP3 on uric acid-induced endothelial dysfunction in HUVECs.

CTRP3 mitigates oxidative stress induced by uric acid in HUVECs

In vivo, oxidative stress in the thoracic aorta was prevented by CTRP3 overexpression in rats with hyperuricemia. We then tried to confirm these findings in HUVECs cultured *in vitro*. HUVECs were transfected with or without Ad-CTRP3 and treated with 10 mg/dL uric acid for 48 h. Ad-GFP- or Ad-CTRP3-infected cells without any stimulation served as controls. As shown in Figure 6(d) and (e), HUVECs in the

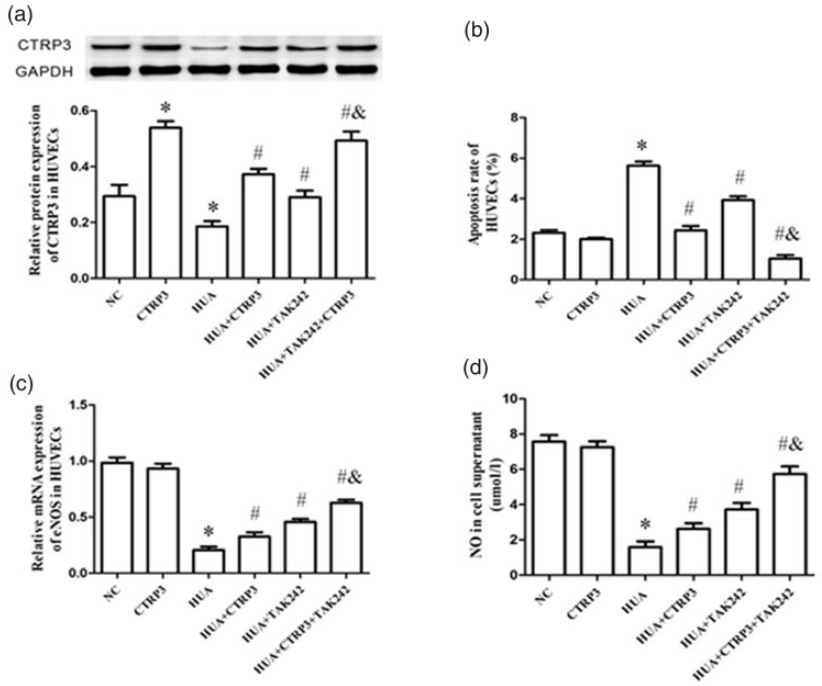

Figure 5. Effect of CTRP3 on HUVECs treated with high uric acid. HUVECs were infected with Ad-CTRP3 or Ad-GFP firstly. Then, the cells were stimulated with 10 mg/dL uric acid for 48 h after pretreated with or without TLR4-specific inhibitor for 30 min. (a) Western blot of CTRP3; (b) apoptosis rate of HUVECs, five images for each sample, 80–200 cells per image, $n = 3$; (c) eNOS mRNA levels; (d) NO concentrations in the supernate. *$P < 0.05$ compared with the control group; #$P < 0.05$ compared with the HUA group; &$P < 0.05$ compared with the HUA+CTRP3 group.
NC: normal control; CTRP3: C1q-tumor necrosis factor-related protein-3; HUA: high uric acid; GADPH: glyceraldehyde 3-phosphate dehydrogenase; HUVEC: human umbilical vein endothelial cells.

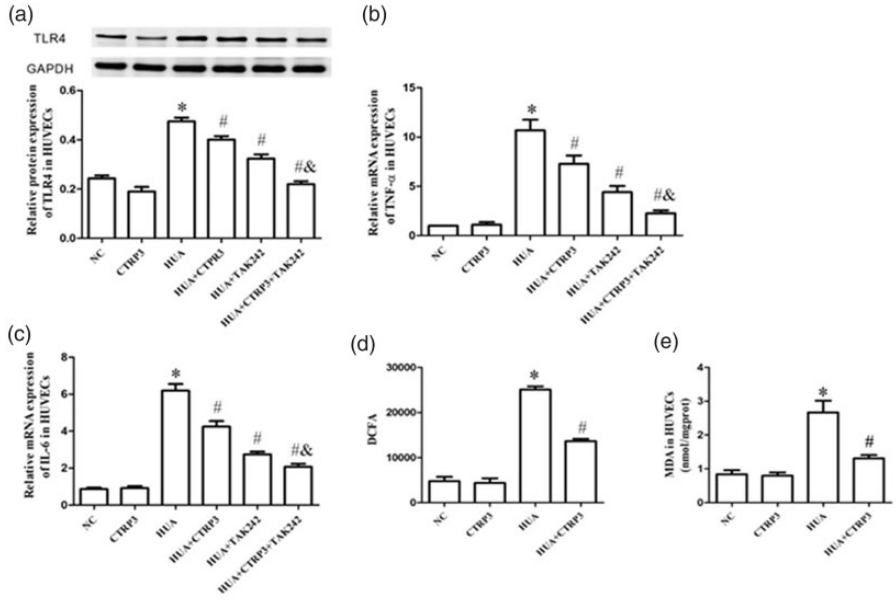

Figure 6. Effect of CTRP3 on inflammation and oxidative stress in HUVECs treated with high uric acid. HUVECs were infected with Ad-CTRP3 or Ad-GFP firstly. Then, the cells were stimulated with 10 mg/dL uric acid for 48 h after pretreated with or without TLR4-specific inhibitor for 30 min. (a) Western blot of TLR4; B. TNF-α mRNA levels; (c) IL-6 mRNA levels; (d) ROS DCFA value; (e) intracellular MDA levels. $n = 3$ in each group; *$P < 0.05$ compared with the control group; #$P < 0.05$ compared with the HUA group; &$P < 0.05$ compared with the HUA + CTRP3 group.
NC: normal control; CTRP3: C1q-tumor necrosis factor-related protein-3; HUA: high uric acid; GADPH: glyceraldehyde 3-phosphate dehydrogenase; HUVEC: human umbilical vein endothelial cells.

HUA group showed higher ROS and MDA concentrations than normal controls. Conversely, CTRP3 overexpression dramatically inhibited oxidative stress induced by uric acid in HUVECs. There was no significant difference in the level of ROS and MDA between the normal group and the CTRP3 group. These data suggest another mechanism by which CTRP3 protects against uric acid-induced endothelial dysfunction *in vitro*.

Discussion

In vivo, we found that CTRP3 overexpression in the thoracic aorta could abolish the inflammatory response and rescue oxidative stress. Thus, rats with hyperuricemia showed improvements in aortic endothelial injury after CTRP3 infection. *In vitro*, HUVECs stimulated with uric acid showed increased levels of proinflammatory cytokines, augmented oxidative stress, and impaired endothelial function, and these changes were partially restored by CTRP3 overexpression. We also found that CTRP3 inhibited TLR4-related inflammation both *in vivo* and *in vitro*. Here, we demonstrated for the first time that CTRP3 protected against endothelial injury induced by uric acid, possibly by inhibiting TLR4-mediated inflammation and downregulating oxidative stress.

CTRP3 is widely expressed in various tissues, such as adipose tissue,[16] the liver,[17] and the heart.[10] In the present study, CTRP3 was found to be expressed in rat thoracic aorta. The specific localization of CTRP3 in the vessel remains unexplored and warrants further investigation in the future. The protective effects of CTRP3 on the vascular endothelium have been demonstrated in several experimental studies. Akiyama *et al.* showed that CTRP3 accelerated proliferation and migration in mouse endothelial MSS31 cells.[18] Another study by Chen *et al.* found that CTRP3 significantly increased viability in mouse aortic endothelial cells.[19] In addition, there were several studies indicating the antiapoptotic effects of CTRP3 on HUVECs and mouse aortic endothelial cells stimulated with oxidized low-density lipoprotein (ox-LDL)[19] or high glucose.[20] However, there are little data regarding the role of CTRP3 in endothelial injury induced by high uric acid. Our study showed that, for rats with hyperuricemia, CTRP3 increased eNOS mRNA expression in the thoracic aortas. Serum NO concentrations showed an increasing trend without statistical difference, which might be attributed to the presence of some confounding factors in vivo. Additionally, CTRP3 reduced the aortic endothelial apoptosis rate and improved intimal structure in hyperuricemic rats. Similarly, CTRP3 increased NO concentrations in the supernatant, and caused increased eNOS mRNA levels coupled with a reduced apoptosis rate in HUVECs stimulated by uric acid. These data suggest that CTRP3 can improve uric acid-induced endothelial injury.

Hyperuricemia has been reported to promote the inflammatory response, thereby contributing to the occurrence and development of CVD.[21] Aroor *et al.* reported that mice with hyperuricemia had higher levels of proinflammatory cytokines and increased vascular stiffness, which could be attenuated by uric acid-lowering treatment with allopurinol.[22] Moreover, pound mice, a model of hereditary hyperuricemia, showed reduced inflammation in adipose tissue and decreased blood pressure when administered allopurinol.[23] In contrast, CTRP3 plays an anti-inflammatory role in a number of pathophysiologic settings. CTRP3 inhibited proinflammatory cytokine production in rats with diabetic cardiomyopathy.[12] CTRP3 also attenuated the inflammatory response induced by lipopolysaccharide (LPS) or a high-fat diet in mice.[24,25] In addition, CTRP3 was found to reduce vascular cell adhesion molecule-1 production in cultured human retinal microvascular endothelial cells stimulated by high levels of glucose and lipids.[26] In line with these findings, we found that CTRP3 decreased the expression of TNF-α and IL-6 in both hyperuricaemic rats and HUVECs in the presence of 10 mg/dL uric acid.

TLR4 is closely associated with various chronic low-grade inflammatory diseases. It is interesting to examine the interaction between CTRP3 and TLR4. Kopp *et al.* reported that CTRP3 blocked the binding of LPS to its receptor, TLR4/MD-2, in monocytes and adipocytes,[27] indicating a negative regulatory role of CTRP3 in TLR4-related inflammation. In the present study, we investigated the direct effect of CTRP3 on TLR4 regulation in endothelial cells. Our data showed that CTRP3 downregulated the protein expression of TLR4 in the thoracic aortas of rats with hyperuricemia. An *in vitro* study demonstrated that in HUVECs with high uric acid stimulation, TLR4 protein levels were further decreased under the joint action of Ad-CTRP3 infection and a specific inhibitor of TLR4. These data indicate that the suppression of TLR4-related inflammation induced by uric acid contributes to the protective effect of CTRP3 on the endothelium. However, it was surprising that the protein expression of CTRP3 was increased in the presence of a TLR4 inhibitor, suggesting potential feedback regulation between CTRP3 and TLR4.

Uric acid is commonly considered a powerful antioxidant in humans.[28] However, in some cases, uric acid can be converted into a pro-oxidant and subsequently contribute to the development of CVD.[29,30] Previous works from our group and Sautin *et al.* reported that uric acid could promote the imbalance in prooxidative and antioxidant enzymes[31] and increase ROS production in adipose tissue.[32] Here, we also found that high levels of uric acid led to oxidative stress in rats and endothelial cells cultured in vitro. In recent years, multiple lines of evidence have suggested that the beneficial effects of CTRP3 may be in part due to its antioxidant action. CTRP3 knockdown increased ROS levels in neonatal rat cardiomyocytes,[12] while CTRP3 overexpression obviously reduced ROS and MDA in retinal pigment epithelial cells in the presence of high glucose.[33] A very recent study verified the effect of CTRP3 against oxidative stress in a rat model of brain injury after intracerebral hemorrhage.[34] Likewise, our data also showed that CTRP3 abolished uric acid-induced oxidative stress in rats and HUVECs.

In the present study, we also observed the relationship between CTRP3 and serum uric acid levels for the first time. The rats with hyperuricemia had lower CTRP3 protein expression in the thoracic aorta than the control rats. In HUVECs stimulated with 10 mg/dL uric acid, CTRP3 protein expression significantly decreased compared with normal controls, indicating a directly inhibitory effect of high uric acid on CTRP3. The above phenomena suggest that uric acid may play a role in negative regulation of CTRP3, but the specific mechanism remains unclear. However, the uric acid levels in both the serum and thoracic aorta were markedly decreased after CTRP3

overexpression in hyperuricemic rats. It is well known that insulin resistance reduces urinary uric acid excretion from the kidney, which in turn causes elevated serum uric acid.[35] It was hypothesized that CTRP3 improved insulin sensitivity, which decreased uric acid levels in rats.

In conclusion, our data indicate that CTRP3 inhibits TLR4-related inflammation, attenuates oxidative stress, and in turn improves uric acid-induced endothelial dysfunction. CTRP3 may become a novel target for the prevention and treatment of CVDs complicated with hyperuricemia.

AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; JXZ designed the study and wrote the article, XL and JXX conducted the experiments, FT and LPT analyzed the data. JXZ and XL contributed equally to this article.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by the Hubei Province health and family planning scientific research project (No: WJ2018H0062), Natural Science Foundation of Hubei Province (No: 2020CFB574), Health Commission of Hubei Province scientific research project (No: WJ2021Z010), and China Diabetes Young Scientific Talent Research Project (No: 2020-N-01).

ORCID iD

Junxia Zhang https://orcid.org/0000-0003-1350-9001

REFERENCES

1. Gunawardhana L, McLean L, Punzi HA, Hunt B, Palmer RN, Whelton A, Feig DI. Effect of febuxostat on ambulatory blood pressure in subjects with hyperuricemia and hypertension: a phase 2 randomized placebo-controlled study. *J Am Heart Assoc* 2017;**6**:e006683
2. Lim DH, Lee Y, Park GM, Choi SW, Kim YG, Lee SW, Kim YH, Yang DH, Kang JW, Lim TH, Kim HK, Choe J, Hong S, Kim YG, Lee CK, Yoo B. Serum uric acid level and subclinical coronary atherosclerosis in asymptomatic individuals: an observational cohort study. *Atherosclerosis* 2019;**288**:112–7
3. Arévalo-Lorido JC, Carretero-Gómez J, Pérez-Monteoliva NRR. Association between serum uric acid and carotid disease in patients with atherosclerotic acute ischemic stroke. *Vascular* 2019;**27**:19–26
4. Taher R, Sara JD, Prasad M, Kolluri N, Toya T, Lerman LO, Lerman A. Elevated serum uric acid is associated with peripheral endothelial dysfunction in women. *Atherosclerosis* 2019;**290**:37–43
5. Khosla UM, Zharikov S, Finch JL, Nakagawa T, Roncal C, Mu W, Krotova K, Block ER, Prabhakar S, Johnson RJ. Hyperuricemia induces endothelial dysfunction. *Kidney Int* 2005;**67**:1739–42
6. Mishima M, Hamada T, Maharani N, Ikeda N, Onohara T, Notsu T, Ninomiya H, Miyazaki S, Mizuta E, Sugihara S, Kato M, Ogino K, Kuwabara M, Hirota Y, Yoshida A, Otani N, Anzai N, Hisatome I. Effects of uric acid on the NO production of HUVECs and its restoration by urate lowering agents. *Drug Res (Stuttg)* 2016;**66**:270–4
7. Schäffler A, Weigert J, Neumeier M, Schölmerich J, Buechler C. Regulation and function of collagenous repeat containing sequence of 26-kDa protein gene product "cartonectin". *Obesity (Silver Spring)* 2007;**15**:303–13
8. Yang Y, Li Y, Ma Z, Jiang S, Fan C, Hu W, Wang D, Di S, Sun Y, Yi W. A brief glimpse at CTRP3 and CTRP9 in lipid metabolism and cardiovascular protection. *Prog Lipid Res* 2016;**64**:170–7
9. Hasegawa N, Fujie S, Horii N, Uchida M, Kurihara T, Sanada K, Hamaoka T, Iemitsu M. Aerobic exercise training-induced changes in serum C1q/TNF-related protein levels are associated with reduced arterial stiffness in middle-aged and older adults. *Am J Physiology-Regulatory Integr Comp Physiology* 2018;**314**:R94–R101
10. Yi W, Sun Y, Yuan Y, Lau WB, Zheng Q, Wang X, Wang Y, Shang X, Gao E, Koch WJ, Ma X-L. C1q/tumor necrosis factor-related protein-3, a newly identified adipokine, is a novel antiapoptotic, proangiogenic, and cardioprotective molecule in the ischemic mouse heart. *Circulation* 2012;**125**:3159–69
11. Wu D, Lei H, Wang JY, Zhang CL, Feng H, Fu FY, Li L, Wu LL. CTRP3 attenuates post-infarct cardiac fibrosis by targeting Smad3 activation and inhibiting myofibroblast differentiation. *J Mol Med (Berl)* 2015;**93**:1311–25
12. Ma ZG, Yuan YP, Xu SC, Wei WY, Xu CR, Zhang X, Wu QQ, Liao HH, Ni J, Tang QZ. CTRP3 attenuates cardiac dysfunction, inflammation, oxidative stress and cell death in diabetic cardiomyopathy in rats. *Diabetologia* 2017;**60**:1126–37
13. Zhang J, Diao B, Lin X, Xu J, Tang F. TLR2 and TLR4 mediate an activation of adipose tissue renin-angiotensin system induced by uric acid. *Biochimie* 2019;**162**:125–33
14. Zhang JX, Zhang YP, Wu QN, Chen B. Uric acid induces oxidative stress via an activation of the renin–angiotensin system in 3T3-L1 adipocytes. *Endocrine* 2015;**48**:135–42
15. Jamnik J, Rehman S, Blanco Mejia S, de Souza RJ, Khan TA, Leiter LA, Wolever TM, Kendall CW, Jenkins DJ, Sievenpiper JL. Fructose intake and risk of gout and hyperuricemia: a systematic review and meta-analysis of prospective cohort studies. *BMJ Open* 2016;**6**:e013191
16. Wong GW, Krawczyk SA, Kitidis-Mitrokostas C, Revett T, Gimeno R, Lodish HF. Molecular, biochemical and functional characterizations of C1q/TNF family members: adipose-tissue-selective expression patterns, regulation by PPAR-γ agonist, cysteine-mediated oligomerizations, combinatorial associations and metabolic functions. *Biochem J* 2008;**416**:161–77
17. Peterson JM, Wei Z, Wong GW. C1q/TNF-related protein-3 (CTRP3), a novel adipokine that regulates hepatic glucose output. *J Biol Chem* 2010;**285**:39691–701
18. Akiyama H, Furukawa S, Wakisaka S, Maeda T. CTRP3/cartducin promotes proliferation and migration of endothelial cells. *Mol Cell Biochem* 2007;**304**:243–8
19. Chen L, Qin L, Liu X, Meng X. CTRP3 alleviates ox-LDL–induced inflammatory response and endothelial dysfunction in mouse aortic endothelial cells by activating the PI3K/akt/eNOS pathway. *Inflammation* 2019;**42**:1350–9
20. Wang F, Zhao L, Shan Y, Li R, Qin G. CTRP3 protects against high glucose-induced cell injury in human umbilical vein endothelial cells. *Anal Cell Pathol* 2019;**2019**:1–7
21. Oyabambi AO, Areola ED, Olatunji LA, Soladoye AO. Uric acid is a key player in salt-induced endothelial dysfunction: the therapeutic role of stigma maydis (corn silk) extract. *Appl Physiol Nutr Metab* 2020;**45**:67–71
22. Aroor AR, Jia G, Habibi J, Sun Z, Ramirez-Perez FI, Brady B, Chen D, Martinez-Lemus LA, Manrique C, Nistala R, Whaley-Connell AT, Demarco VG, Meininger GA, Sowers JR. Uric acid promotes vascular stiffness, maladaptive inflammatory responses and proteinuria in Western diet fed mice. *Metabolism* 2017;**74**:32–40
23. Baldwin W, McRae S, Marek G, Wymer D, Pannu V, Baylis C, Johnson RJ, Sautin YY. Hyperuricemia as a mediator of the proinflammatory endocrine imbalance in the adipose tissue in a murine model of the metabolic syndrome. *Diabetes* 2011;**60**:1258–69

24. Schmid A, Kopp A, Hanses F, Karrasch T, Schäffler A. C1q/TNF-related protein-3 (CTRP-3) attenuates lipopolysaccharide (LPS)-induced systemic inflammation and adipose tissue erk-1/-2 phosphorylation in mice in vivo. *Biochem Biophys Res Commun* 2014;**452**:8–13
25. Petersen PS, Wolf RM, Lei X, Peterson JM, Wong GW. Immunomodulatory roles of CTRP3 in endotoxemia and metabolic stress. *Physiol Rep* 2016;**4**:e12735
26. Yan Z, Zhao J, Gan L, Zhang Y, Guo R, Cao X, Lau WB, Ma X, Wang Y. CTRP3 is a novel biomarker for diabetic retinopathy and inhibits HGHL-induced VCAM-1 expression in an AMPK-dependent manner. *Plos One* 2017;**12**:e0178253
27. Kopp A, Bala M, Buechler C, Falk W, Gross P, Neumeier M, SchöLmerich J, SchäFfler A, C1q/TNF-related protein-3 represents a novel and endogenous lipopolysaccharide antagonist of the adipose tissue. *Endocrinology* 2010;**151**:5267–78
28. Ndrepepa G. Uric acid and cardiovascular disease. *Clin Chim Acta* 2018;**484**:150–63
29. Kuzkaya N, Weissmann N, Harrison DG, Dikalov S. Interactions of peroxynitrite with uric acid in the presence of ascorbate and thiols: implications for uncoupling endothelial nitric oxide synthase. *Biochem Pharmacol* 2005;**70**:343–54
30. Muraoka S, Miura T. Inhibition by uric acid of free radicals that damage biological molecules. *Pharmacol Toxicol* 2003;**93**:284–9
31. Zhang JX, Lin X, Xu JX, Tang F. Apelin-13 reduces oxidative stress induced by uric acid via downregulation of renin-angiotensin system in adipose tissue. *Toxicol Lett* 2019;**305**:51–7
32. Sautin YY, Nakagawa T, Zharikov S, Johnson RJ. Adverse effects of the classic antioxidant uric acid in adipocytes: NADPH oxidase-mediated oxidative/nitrosative stress. *Am J Physiol Cell Physiol* 2007;**293**: C584–C596
33. Zhang J, He J. CTRP3 inhibits high glucose-induced oxidative stress and apoptosis in retinal pigment epithelial cells. *Artif Cells Nanomed Biotechnol* 2019;**47**:3758–64
34. Yang B, Wang S, Yu S, Chen Y, Li L, Zhang H, Zhao Y. C1q/tumor necrosis factor-related protein 3 inhibits oxidative stress during intracerebral hemorrhage via PKA signaling. *Brain Res* 2017;**1657**:176–84
35. Galvan AQ, Natali A, Baldi S, Frascerra S, Sanna G, Ciociaro D, Ferrannini E. Effect of insulin on uric acid excretion in humans. *Am J Physiol-Endoc M* 1995;**268**:E1–5

(Received June 20, 2021, Accepted August 31, 2021)