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CTRP3 promotes TNF-α-induced apoptosis and barrier dysfunction in salivary epithelial cells

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ABSTRACT

Background: C1q/tumour necrosis factor-related protein 3 (CTRP3) plays important roles in metabolism and inflammatory responses in various cells and tissues. However, the expression and function of CTRP3 in salivary glands have not been explored.
Methods: The expression and distribution of CTRP3 were detected by western blot, polymerase chain reaction, immunohistochemical and immunofluorescence staining. The effects of CTRP3 on tumour necrosis factor (TNF)-α-induced apoptosis and barrier dysfunction were detected by flow cytometry, western blot, co-immunoprecipitation, and measurement of transepithelial resistance and paracellular tracer flux.
Results: CTRP3 was distributed in both acinar and ductal cells of human submandibular gland (SMG) and was primarily located in the ducts of rat and mouse SMGs. TNF-α increased the apoptotic rate, elevated expression of cleaved caspase 3 and cytochrome C, and reduced B cell lymphoma-2 (Bcl-2) levels in cultured human SMG tissue and SMG-C6 cells, and CTRP3 further enhanced TNF-α-induced apoptosis response. Additionally, CTRP3 aggravated TNF-α-increased paracellular permeability. Mechanistically, CTRP3 promoted TNF-α-enhanced TNF type I receptor (TNFR1) expression, inhibited the expression of cellular Fas-associated death domain (FADD)-like interleukin-1β converting enzyme inhibitory protein (c-FLIP), and increased the recruitment of FADD with receptor-interacting protein kinase 1 and caspase 8. Moreover, CTRP3 was significantly increased in the labial gland of Sjögren's syndrome patients and in the serum and SMG of nonobese diabetic mice.
Conclusions: These findings suggest that the salivary glands are a novel source of CTRP3 synthesis and secretion. CTRP3 might promote TNF-α-induced cell apoptosis through the TNFR1-mediated complex II pathway.

1. Introduction

C1q/tumour necrosis factor-related protein (CTRP) 3 was initially described in 2001 as a secretory protein expressed in the cartilage and kidney of adult mice and has been identified as a member of the CTRP superfamily adipokines [1]. CTRP3 is widely expressed in cartilage, adipose tissue, muscle, testis, heart, kidney and liver and is involved in the modulation of metabolism, host defence and inflammation [2]. As an adiponectin paralogue, CTRP3 regulates triglyceride metabolism in hepatic cells [3], promotes proliferation in endothelial and vascular

Abbreviations: CTRP, C1q/tumour necrosis factor-related protein; TNF, tumour necrosis factor; TNFR1, TNF type I receptor; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; SMG, human submandibular gland; PG, parotid gland; LG, labial gland; NOD, nonobese diabetic; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; FADD, fas-associated death domain; Cln-1, claudin-1; Cln-3, claudin-3; Cln-4, claudin-4; Bcl-2, B cell lymphoma-2; RIPK1, receptor-interacting protein kinase 1; c-FLIP, cellular FADD-like interleukin-1β converting enzyme inhibitory protein; NF-κB, nuclear factor-κB; p-p65, phospho-NF-κB p65; t-p65, total-NF-κB p65; PBS, phosphate buffer saline; TER, transepithelial resistance.

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smooth muscle cells, and elevates adipokine secretion in adipocytes [4–6]. However, CTRP3 may exert distinct functions in different pathological processes. CTRP3 suppresses cardiomyocyte apoptosis and promotes angiogenesis after cardiac infarction [7], whereas it aggravates β-glycerophosphate-induced vascular calcification [8]. Recent studies have identified several adipokines, such as adiponectin and leptin, that are expressed in salivary glands [9,10], and they function in the regulation of saliva secretion, inflammation and apoptosis [11,12]. Changes in leptin and adiponectin levels are associated with the progression of salivary gland tumours [13,14]. However, whether CTRP3 is synthesized and secreted by salivary glands has not been determined.

The major types of cell death include necrosis, apoptosis, autophagy, pyroptosis and oncosis. Apoptosis is characterized by distinctive morphological changes in the cellular structure and a series of biochemical enzyme-dependent processes that help to remove cells from the body with minimal damage to neighbouring tissues [15]. Inappropriate apoptosis is involved in many pathological conditions, including ischemic damage, neurological disorders, cancer and autoimmune diseases, such as Crohn's disease, rheumatoid arthritis, and Sjögren's syndrome [16–19]. As a chronic inflammatory autoimmune disease, Sjögren's syndrome induces hyposecretion in exocrine glands [20], especially in salivary glands, and it is primarily a kind of salivary epithelitis [21]. Among the upregulated proinflammatory cytokines in Sjögren's syndrome, tumour necrosis factor (TNF)-α is a classically important inducer of apoptosis, and it is also involved in regulating differentiation, growth, and barrier function [22]. In the salivary glands of Sjögren's syndrome patients, upregulated TNF-α induces cell apoptosis and barrier dysfunction [23], which is a vital mechanism for hyposalivation and glandular atrophy [16,24]. TNF-α disrupts barrier function [25], which is mainly controlled by tight junction proteins, such as the claudin superfamily, and accumulated TNF-α causes cell apoptosis in salivary epithelial cells, directly reducing the saliva-secreting resource [26]. TNF-α-induced apoptosis occurs through the binding of TNF type I receptor (TNFR1), which contains a death domain that transmits apoptotic signals through caspase activation. Reactive oxygen species (ROS) have been implicated in the induction of inflammation and tissue damage in Sjögren's syndrome [27]. Hydrogen peroxide (H_2O_2), as the main ROS inducer, mediates apoptotic progression in Sjögren's syndrome [28].

Therefore, this study was designed to investigate the expression of CTRP3 in the salivary glands and to explore its role and underlying mechanism in the regulation of TNF-α-induced salivary epithelial apoptosis.

2. Materials and methods

2.1. Ethics

The research protocols for human salivary gland tissues were approved by the Ethics Committee for Human Experiments of Peking University School and Hospital of Stomatology and performed in keeping with the guidelines of the Declaration of Helsinki 2000. All participants provided informed consent. The animal experimental procedures were approved by the Ethics Committee of Animal Research, Peking University Health Science Center, and complied with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996).

2.2. Human and animal samples

Human submandibular glands (SMGs) were obtained from 15 patients (45–67 years old, 12 male) who underwent functional neck dissection for their primary squamous cell carcinoma in the oral and maxillofacial regions. Human parotid glands (PGs) were obtained from 6 patients (35–57 years old, 4 male) who underwent extended resection for their pleomorphic adenoma in PG. The patients with a history of radiotherapy or chemotherapy were excluded. All SMG and PG tissues used were confirmed to be histologically normal. Human labial glands (LGs) were obtained from the patients with Sjögren's syndrome, and LGs obtained from mucocele patients served as controls. Human serum and saliva samples were collected from 134 healthy controls (18 to 26 years old, 58 male) during routine health screening. The exclusion criteria were as follows: (1) having hypertension, diabetes, heart disease, hepatopathy, nephropathy, or other serious diseases, such as fractures and tumours; (2) having unfilled dental caries, periodontitis, and oral mucosal diseases; (3) having an infection; and (4) taking medications.

Rat SMGs and PGs were obtained from Sprague-Dawley rats (male, weight: 180–220 g), and mouse SMGs, PGs and serum were obtained from 8- and 12-week-old female BALB/c and nonobese diabetic (NOD) mice.

2.3. Cell culture

An immortalized rat SMG acinar cell line, SMG-C6 cell line was grown at 37 °C in a humidified 5% CO_2 atmosphere in DMEM/F12 (1:1 mixture) medium containing 2.5% fetal bovine serum, 5 mg/L transferrin, 1.1 μmol/L hydrocortisone, 0.1 μmol/L retinoic acid, 2 nmol/L thyronine T3, 5 mg/L insulin, 80 μg/L epidermal growth factor, 50 mg/L gentamicin sulfate, 5 mmol/L glutamine, 100 U/ml penicillin, and 100 mg/L streptomycin as described previously [29]. All constituents utilized in culturing SMG-C6 cells were obtained from Sigma-Aldrich Co.

2.4. Human SMG tissue culture

Human SMG tissues were isolated, placed into 4 °C Krebs-Ringer Hepes solution (120 mmol/L NaCl, 11.1 mmol/L glucose, 5.4 mmol/L KCl, 1 mmol/L $CaCl_2$, 0.8 mmol/L $MgCl_2$, 20 mmol/L HEPES, pH 7.4) and transported to the laboratory within 30 min after collection. The samples were cut into cubes (side length: 0.2 cm), immersed in DMEM/F12 (1:1 mixture) with 10% fetal bovine serum, and subsequently incubated with 5% CO_2 in a humidified incubator at 37 °C for the indicated time as described previously [30].

Human and rat SMG tissues and SMG-C6 cells were incubated with TNF-α (#400–14, Peprotech, Rocky Hill, NJ, USA) in the present or absent human recombinant globular CTRP3 (#00082–04-100, Aviscera Bioscience, Santa Clara, CA, USA) for the indicated doses and times. In the preliminary study, 200 ng/mL and 300 ng/mL TNF-α significantly increased the apoptosis rate and the level of cleaved caspase 3, but 100 ng/mL TNF-α did not induce obvious change (seen in supplementary fig. 1), then we used 200 ng/mL TNF-α in apoptosis experiment. H_2O_2 (100 μmol/L for 24 h) was used to induce apoptosis in SMG tissue and SMG-C6 cell line, which concentration was reported previously in rat parotid gland [31].

2.5. Enzyme-linked immunosorbent assay (ELISA)

Human serum and saliva CTRP3 levels were measured by a human CTRP3 ELISA kit (SK00082, Aviscera Bioscience, Santa Clara, CA, USA). The linear ranges of the assays were 5 to 320 ng/mL for CTRP3.

Mouse serum CTRP3 levels were measured by a mouse CTRP3 ELISA kit (JL46153-96 T, Jianglai Bio, Shanghai, China). The linear ranges of the assays were 0.625 to 20 μg/L for CTRP3.

2.6. Semiquantitative polymerase chain reaction (PCR) and quantitative PCR

Total RNA was isolated from cells and tissues using TRIzol (Invitrogen, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from reverse-transcribed RNA (1 μg) using the First-Strand cDNA Synthesis kit (Fermentas, Burlington, ON, Canada). The primer sequences were as follows: β-actin, forward TCGTGCGTGACATTAAAGAG, reverse ATTGCCGATAGTGATGACCT; CTRP3, forward

CGTGGAGGAAGTGTATGTG, reverse CTCAGATCCTAACAACCCT as described previously [8,32]. Semiquantitative PCR was performed by electrophoresis on a 1.5% agarose gel, and DNA bands were visualized by staining with ethidium bromide. For quantitative PCR, amplifications were performed for 40 cycles using an Opticon continuous fluorescence detection system and the DyNAmo™ ColorFlash Probe qPCR Kit (Thermo Fisher Scientific, MA, USA). The qPCR experiment was conducted independently for 5 times with triplicates. The average quantification cycle was calculated.

2.7. Western blot analysis

The samples were homogenized with lysis buffer (RIPA buffer, #89900, Thermo Fisher Scientific) and centrifuged. The supernatant was collected and the protein concentration was determined by the Bradford method (M&C Gene Technology Ltd). Equal amounts of proteins (20 μg) were separated by 12% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and subsequently incubated with primary and secondary antibodies. Immunoreactive bands were visualized with enhanced chemiluminescence (Thermo Scientific Pierce). The target protein band densities were quantified by positive surface area and grey scale value using Image J software (National Institutes of Health). β-actin was used as an internal control for protein loading. Antibodies against CTRP3 (#ab36870), Fas-associated death domain (FADD, #ab24533) and caspase 10 (#ab2012) were obtained from Abcam (Cambridge, MA, USA). Antibodies against β-actin (#sc-58,673) and caspase 12 (#sc-12,396) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against claudin-1 (Cln-1, #BS1063), claudin-3 (Cln-3, #BS1067), claudin-4 (Cln-4, #BS1068), cytochrome C (#BS1689) and TNFR1 (#BS1478) were purchased from Bioworld Technology (Minneapolis, MN, USA). Antibodies against cleaved caspase 3 (#9446), B cell lymphoma-2 (Bcl-2, #3498), Bax (#2772), caspase 8 (#4790), receptor-interacting protein kinase 1 (RIPK1, #3493), cellular FADD-like interleukin-1β converting enzyme inhibitory protein (c-FLIP, #8510), phospho-nuclear factor-κB (NF-κB) p65 (p-p65, #3033), and total-NF-κB p65 (t-p65, #8242) were obtained from Cell Signalling Technology (Beverly, MA, USA).

2.8. Histological, immunohistochemical and immunofluorescence staining

The salivary gland sections of humans, rats, and mice (5 μm thick) were fixed in 4% paraformaldehyde and stained with haematoxylin and eosin. For immunohistochemical staining, the sections were incubated with primary antibody against CTRP3 (#ab36870) followed by incubation with horseradish peroxidase-conjugated secondary antibody (Zhongshan Laboratories, Beijing, China). The reaction was developed with 3,3-diaminobenzidine. A semi-quantitative immunoreactivity score was analysed from five randomly chosen fields from each section by ImageJ software (NIH, Bethesda, MD, USA) [33]. For immunofluorescence, SMG-C6 cells were incubated with CTRP3 antibody and then with Alexa Fluor® 488-conjugated secondary antibody (Molecular Probes, OR, USA). The images were acquired on a confocal microscope (Leica TCS SP8, Wetzlar, Germany).

2.9. Flow cytometry

Apoptotic rates were detected with the Annexin V-FITC apoptosis detection kit (#AD10, Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Cells were washed in phosphate buffer saline (PBS), resuspended in 300 μL of binding buffer, incubated with 5 μL of Annexin V-FITC solution for 30 min, and followed with 2.5 μL of propidium iodide for 5 min. Cell apoptosis was assessed by flow cytometry (FACSCalibur; Becton Dickinson, NJ, USA).

To detect TNFR1 expressed at the cell surface, SMG-C6 cells were harvested and resuspended in PBS. FITC-labelled TNFR1 antibody (#sc-12,746 FITC, Santa Cruz Biotechnology, CA, USA) was added to the cells for 30 min on ice. After removal of unbound antibodies (3× wash with PBS), the cells were analysed by flow cytometry (FACSCalibur; Becton Dickinson, NJ, USA).

2.10. Measurement of transepithelial resistance and paracellular tracer flux

Transepithelial resistance (TER) was detected by using epithelial volt ohm meter (WPI, FL, USA). SMG-C6 cells were cultured on Corning Transwell™ filters (6.5-mm diameter, 0.4-μm pore size). The final TER values were subtracted from the blank value and expressed as $\Omega \bullet cm^2$. A 4-kDa or 40-kDa FITC-dextran flux assay was performed to detect paracellular permeability as described previously [30,34].

2.11. Immunoprecipitation

The immunoprecipitation assay was performed using the Pierce Co-Immunoprecipitation Kit (#26149, Thermo Fisher Scientific Inc., MA, USA) according to the manufacturer's protocol. For statistical analysis, the amounts of the same proteins in the input fraction with the immunoprecipitated antibodies were measured as controls.

2.12. Statistical analysis

GraphPad Prism 5.0 software was used to analyse the data. All data are presented as the mean ± SEM. Statistical analysis was conducted with unpaired Student's *t*-test for two groups and one-way analysis of variance followed by Bonferroni's test for multiple groups. *P*-value <0.05 was considered to be significant.

3. Results

3.1. Expression pattern of CTRP3 in salivary glands

RT-PCR and western blot analysis showed that CTRP3 mRNA and protein were expressed in human, rat and mouse SMG and PG tissues, as well as in SMG-C6 cells (Fig. 1a and b). Rat testicular tissues were used as positive controls. Immunohistochemical staining showed that CTRP3 was widely distributed in the cytoplasm of serous acinar and ductal cells from human SMG but stained only faintly in mucous acinar cells. In rat and mouse SMGs, CTRP3 was primarily located in granular convoluted tubules, which are unique secretory structures differentiated from striated ducts in rodent SMG, and was faintly stained in both serous and mucous acinar cells. In human and rat PGs, CTRP3 was predominantly distributed in the cytoplasm of serous acinar cells and only slightly stained in ductal cells. In mouse PG, CTRP3 was primarily located in granular convoluted tubules and was faintly expressed in acinar cells (Fig. 1c). Additionally, the immunofluorescence image showed that CTRP3 was distributed in the cytoplasm of SMG-C6 cells (Fig. 1d).

ELISA results showed that the level of CTRP3 was 128.24 ± 8.15 ng/mL in serum and 49.90 ± 8.06 ng/mL in saliva in 134 healthy volunteers, suggesting that CTRP3 synthesized in salivary gland cells was secreted into the saliva (Fig. 1e). There were no differences in serum and saliva CTRP3 concentrations between males and females (Fig. 1f).

3.2. CTRP3 promotes TNF-α-induced apoptosis in rat SMG cells and human SMGs

To explore the role of CTRP3 in salivary glands, annexin V-FITC/propidium iodide double staining was performed. Incubation of SMG-C6 cells with TNF-α (200 ng/mL) for 36 h significantly increased the apoptotic rate. Notably, preincubation with CTRP3 (2 μg/mL) for 30 min significantly promoted the TNF-α-induced cell apoptotic rate compared with that of TNF-α alone group (Fig. 2a and b). To confirm the role of CTPR3 in cell apoptosis, we detected changes in apoptosis-related proteins. Cytochrome C released from mitochondria into the cytoplasm

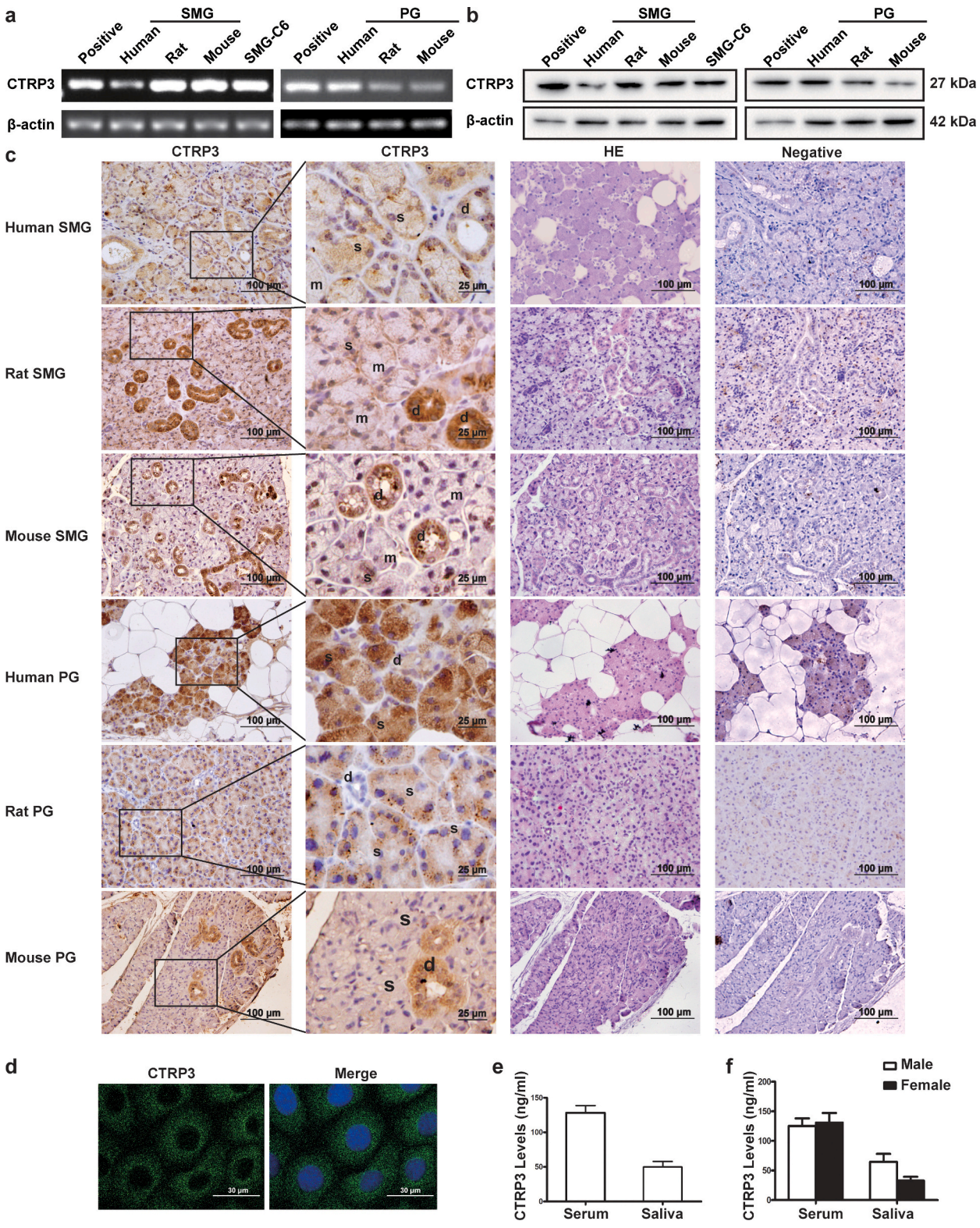

Fig. 1. Expression and distribution of CTRP3 in the salivary glands. (a) The mRNA expression of CTRP3 in the submandibular gland (SMG) and parotid gland (PG) of humans, rats and mice. (b) The protein expression of CTRP3 in the SMG and PG. (c) Immunohistochemical staining of CTRP3 in the SMG and PG, s: serous acinus, m: mucous acinus, d: duct. (d) Immunofluorescence staining of CTRP3 in SMG-C6 cells. (e) The concentrations of CTRP3 in serum and saliva. Values are the mean ± SEM from 134 healthy volunteers. (f) The concentrations of CTRP3 in the serum and saliva of males ($N = 58$) and females ($N = 76$).

triggers the apoptosis process, and cleaved caspase-3 is essential for the execution of apoptosis [35]. TNF-α increased, and CTRP3 further enhanced, the expression levels of cleaved caspase 3 (Fig. 2c) and cytochrome C (Fig. 2d). The Bcl-2 family regulates cytochrome C release from mitochondria to the cytoplasm, and Bcl-2 is known as an anti-apoptotic protein [35]. TNF-α decreased Bcl-2 expression, and CTRP3 promoted TNF-α-induced Bcl-2 reduction (Fig. 2e). Neither TNF-α nor CTRP3 induced notable changes in Bax expression (Fig. 2f). Caspase 12

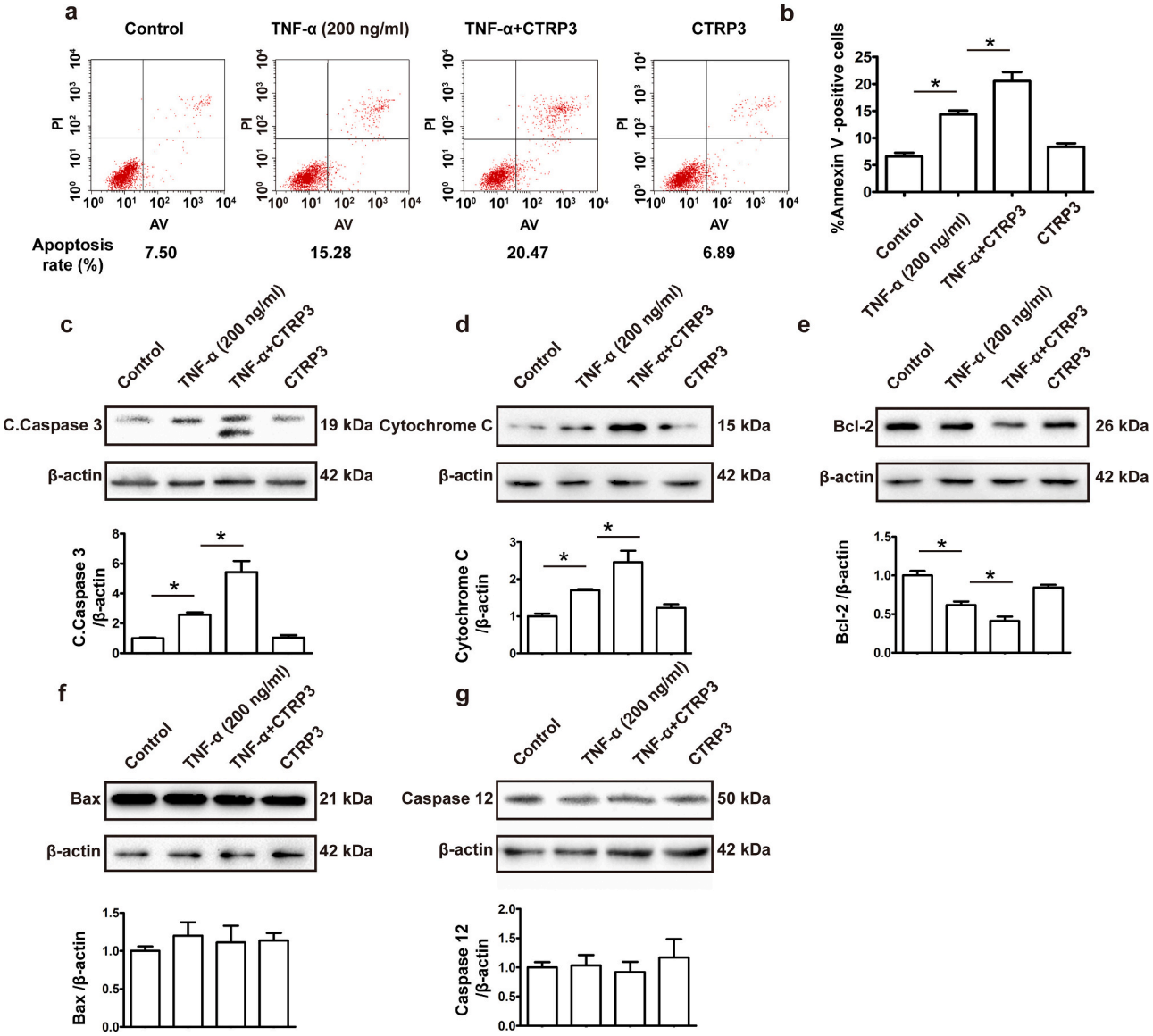

Fig. 2. Effect of CTRP3 on TNF-α-induced apoptosis in SMG-C6 cells. SMG-C6 cells were incubated with TNF-α (200 ng/mL) for 36 h in the absence or presence of CTRP3 (2 μg/mL) preincubation for 30 min, and then, cell apoptosis was evaluated by Annexin V-FITC/propidium iodide double staining using flow cytometry assay (a). Statistical analysis is shown in (b). The protein expression levels of cleaved caspase 3 (C. Caspase 3) (c), cytochrome C (d), Bcl-2 (e), Bax (f), and caspase 12 (g) were detected by western blot analysis in SMG-C6 cells. $^{*}P < 0.05$.

is known to regulate mitochondria-mediated apoptosis [35], while TNF-α and CTRP3 did not alter caspase 12 expression (Fig. 2g). CTRP3 alone did not affect the cell apoptosis rate and the expression of apoptosis-related proteins in SMG-C6 cells. These results suggested that CTRP3 enhanced TNF-α-induced cell apoptosis in SMG-C6 cells.

Since previous studies have reported that CTRP3 plays an anti-apoptotic role in various tissues and cells [7,36,37], we further evaluated the facilitating apoptotic effect of CTRP3 on SMG cells treated with low doses of TNF-α. As shown in Fig. 3a and b, treatment cells with 100 ng/mL TNF-α for 36 h alone did not affect the expression of cleaved caspase 3, Bcl-2 and Bax, suggesting that this relatively low dose of TNF-α did not induce salivary epithelial apoptosis. However, when SMG-C6 cells were preincubated with 2 μg/mL CTRP3 for 30 min prior to 100 ng/mL TNF-α treatment, the expression of cleaved caspase 3 was significantly increased, and Bcl-2 was considerably decreased (Fig. 3a and b). These findings further indicated that CTRP3 enhanced TNF-α-mediated cell apoptosis in SMG-C6 cells.

To clarify the effect of CTRP3 on TNF-α-induced apoptosis in different species, we performed the experiments in cultured human SMG tissue. Consistent with the results obtained in SMG-C6 cells, TNF-α (200 ng/mL) significantly increased the expression of cleaved caspase 3, cytochrome C, and caspase 10, whereas decreased Bcl-2 expression (Fig. 3c and d). Furthermore, CTRP3 preincubation further promoted TNF-α-induced changes of apoptosis-related proteins compared with TNF-α treatment alone. These data confirmed that CTRP3 promoted TNF-α-induced cell apoptosis in human SMGs.

3.3. *CTRP3 does not affect H_2O_2-induced apoptosis in rat SMG cells and human SMGs*

H_2O_2, which is a free radical that belongs to ROS, conventionally induces oxidative stress and apoptosis responses [38]. We detected the effect of CTRP3 on H_2O_2-induced apoptosis in SMG-C6 cells. As shown in Fig. 4a and b, treatment with H_2O_2 (100 μmol/L for 24 h) significantly increased the expression of cleaved caspase 3 and cytochrome C and decreased the expression of Bcl-2 in SMG-C6 cells. Notably, CTRP3 did

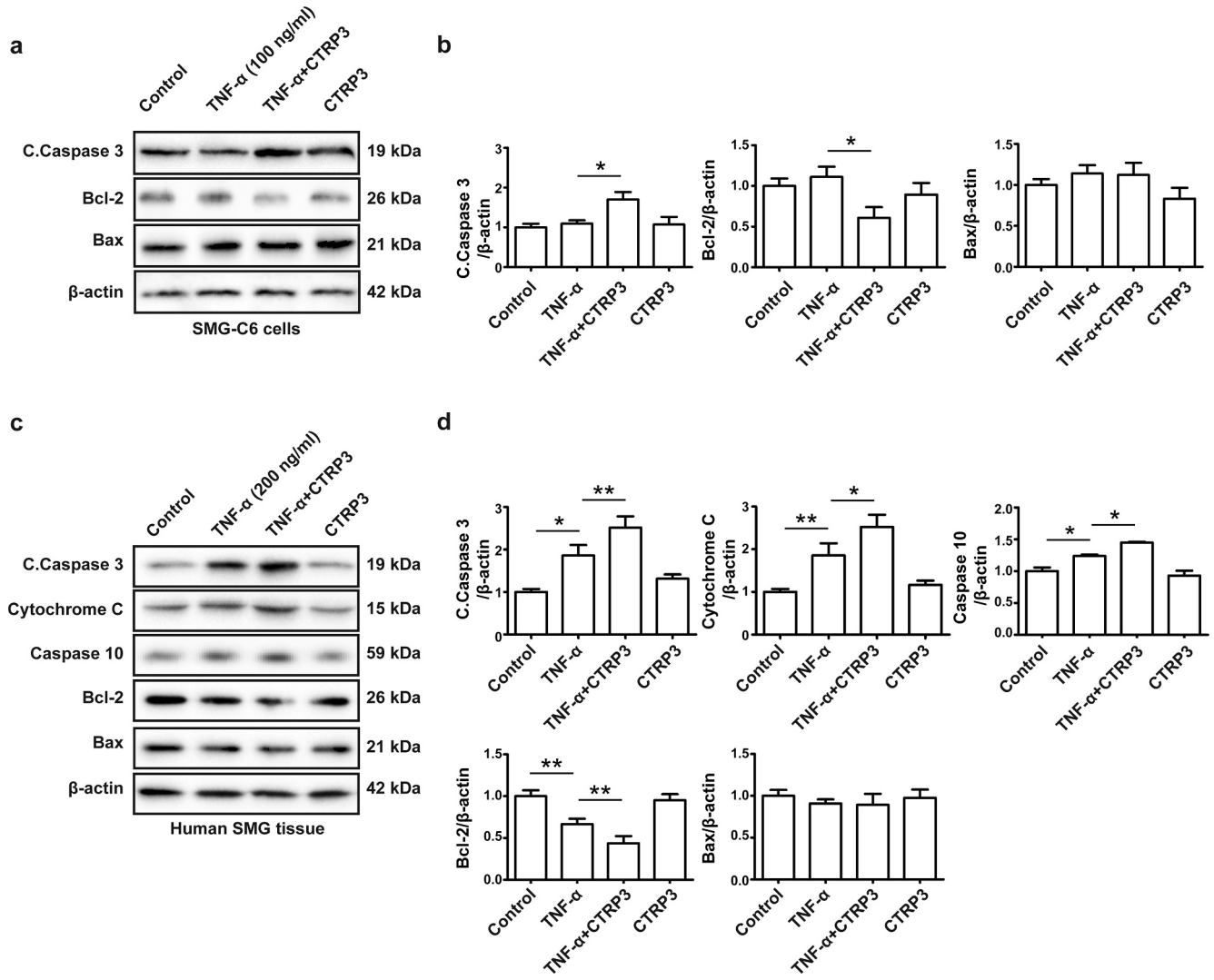

Fig. 3. Effect of CTRP3 on TNF-α-treated SMG-C6 cells and human SMG tissues. SMG-C6 cells were incubated with TNF-α (100 ng/mL) for 36 h in the absence or presence of CTRP3 (2 μg/mL) pre-treatment. Expression of cleaved caspase 3 (C. Caspase 3), Bcl-2 and Bax was detected by western blot analysis (a), and statistical analysis is shown in (b). Human SMG tissues were incubated with TNF-α (200 ng/mL) for 36 h in the absence or presence of CTRP3 (2 μg/mL) pre-treatment for 30 min, and the protein expression of C. caspase 3, cytochrome C, caspase 10, Bcl-2 and Bax was detected in incubated human SMGs (c), and statistical analysis is shown in (d). Values are the mean ± SEM from 4 to 6 independent experiments. *$P < 0.05$ and **$P < 0.01$.

not affect the H_2O_2-induced changes in apoptosis-related proteins. Consistent with the results obtained in SMG-C6 cells, CTRP3 did not affect the H_2O_2-induced alterations, including cleaved caspase 3, cytochrome C, caspase 10, Bcl-2, and Bax in human SMG tissues (Fig. 4c and d). These results suggested that CTRP3 was involved in TNF-α-induced, but not H_2O_2-induced, salivary acinar apoptosis.

3.4. CTRP3 accelerates TNF-α-induced barrier dysfunction in SMG-C6 cells

To determine the effect of CTRP3 on epithelial paracellular permeability, TER and the paracellular flux of FITC-dextran with different molecular weights were detected. SMG-C6 cells were cultured for 6 days on Transwell inserts to form a polarized monolayer. TNF-α (10 ng/mL) significantly reduced the TER value (Fig. 5a) and increased the paracellular permeability of 4 kDa and 40 kDa FITC-dextran flux (Fig. 5b and c) in SMG-C6 cells at 24 h. These responses induced by TNF-α were enhanced by CTRP3 (2 μg/mL) preincubation. Claudins are important transmembrane proteins of tight junctions. TNF-α (10 ng/mL) alone decreased Cln-3 expression but did not affect Cln-1 and Cln-4 levels at 24 h (Fig. 5d-5f). However, CTRP3 preincubation with 10 ng/mL TNF-α further decreased the levels of Cln-1, Cln-3 and Cln-4. These results indicated that CTRP3 accelerated TNF-α-induced epithelial barrier dysfunction in salivary gland cells.

3.5. CTRP3 further increases TNF type I receptor expression and complex II formation induced by TNF-α

TNF-α induces epithelial dysfunction via activation of its receptor TNFR1. The intracellular death domain of TNFR1 recruits the TNFR1-associated death domain protein and RIPK1 to form complex I and then to recruit FADD and caspase 8 precursor to form complex II, which mediates classical apoptotic signal transduction [39]. Treatment with TNF-α (200 ng/mL for 6 h) significantly increased TNFR1 expression in SMG-C6 cells, and CTRP3 preincubation with TNF-α further upregulated TNFR1 expression compared with the control and TNF-α groups, and CTRP3 alone slightly increased the expression of TNFR1 (Fig. 6a). Flow cytometry results showed that the expression of TNFR1 at the cell surface was increased in both CTRP3 and TNF-α groups and was further elevated in CTRP3 + TNF-α group at 6 h (Fig. 6b). Using an immunoprecipitation method, we measured the recruitment of FADD with RIPK1 and caspase 8 in SMG-C6 cells, which are the primary protein

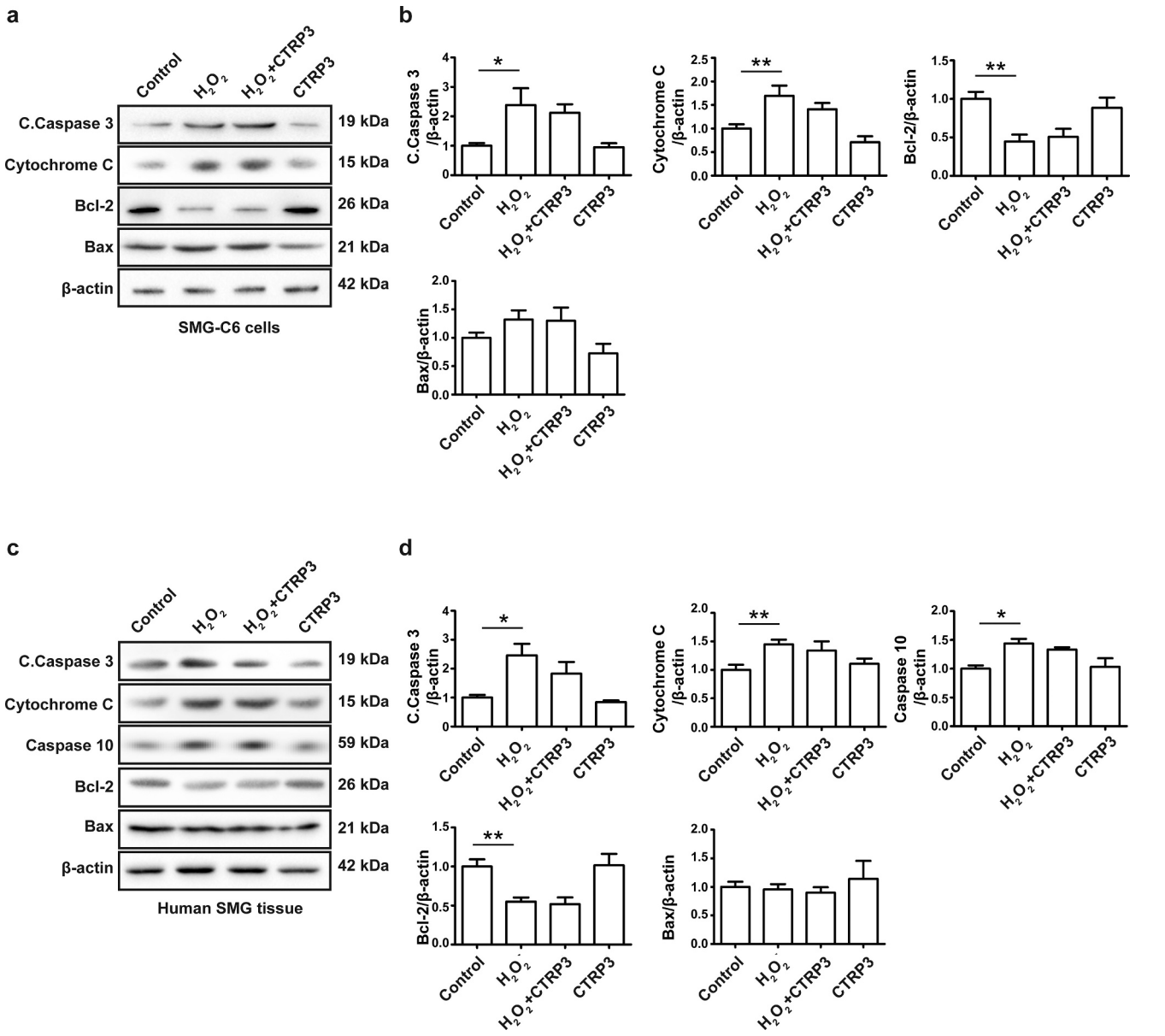

Fig. 4. Effect of CTRP3 on hydrogen peroxide (H_2O_2)-induced apoptosis. SMG-C6 cells were incubated with H_2O_2 (100 μmol/L) for 24 h in the absence or presence of CTRP3 (2 μg/mL) pre-treatment for 30 min. Expression of apoptosis-related proteins was detected by western blot analysis (a), and statistical analysis is shown in (b). Fresh human SMG tissue was incubated with H_2O_2 (100 μmol/L) for 24 h in the absence or presence of CTRP3 (2 μg/mL) pre-treatment. The expression of apoptosis-related proteins was detected by western blot analysis (c), and statistical analysis is shown in (d). C. Caspase 3: cleaved caspase 3. Values are the mean ± SEM from 4 independent experiments. $^*P < 0.05$ and $^{**}P < 0.01$.

components of complex II [39]. TNF-α significantly increased the recruitment of FADD with RIPK1 and caspase 8, and CTRP3 pre-incubation further enhanced the TNF-α-induced combination of these proteins (Fig. 6c), suggesting that CTRP3 promoted TNF-α-induced pro-apoptotic complex II formation. The anti-apoptotic protein c-FLIP plays a key role in inhibiting TNFR1-related apoptotic complex II formation [40]. The time-course of c-FLIP expression showed that TNF-α significantly increased c-FLIP expression at 3–6 h, whereas the response declined at 24 h and 36 h in SMG-C6 cells (Fig. 6d). We then explored the effect of CTRP3 on TNF-α-induced c-FLIP expression. TNF-α enhanced the expression of c-FLIP at 6 h, whereas CTRP3 inhibited TNF-α-induced c-FLIP upregulation (Fig. 6e). Moreover, TNF-α slightly decreased the expression of c-FLIP at 36 h, and CTRP3 further facilitated TNF-α-induced c-FLIP reduction (Fig. 6f). Activation of NF-κB is a key downstream event in TNF-α-induced survival signalling, which mediates the transcriptional regulation of c-FLIP. TNF-α significantly increased the level of p-p65 at 30 min, whereas CTRP3 preincubation reduced p-p65 expression in SMG-C6 cells (Fig. 6g).

3.6. Expression of CTRP3 is elevated in the salivary glands of NOD mice and Sjögren's syndrome patients

To explore the possible role played by CTRP3 in Sjögren's syndrome, we detected the levels of CTRP3 in both serum and SMG of NOD mice, which can develop autoimmune sialoadenitis and exhibit Sjögren's syndrome-like exocrinopathy [41]. ELISA results showed that circulating CTRP3 was significantly higher in 12-week-old NOD mice than in age-matched BALB/c mice, whereas serum CTRP3 levels were almost the same in 8-week-old NOD mice and age-matched BALB/c mice (Fig. 7a). The levels of CTRP3 mRNA and protein in SMGs were increased in 12-week-old NOD mice compared with age-matched BALB/c mice (Fig. 7b and c), whereas there was no difference in CTRP3 mRNA

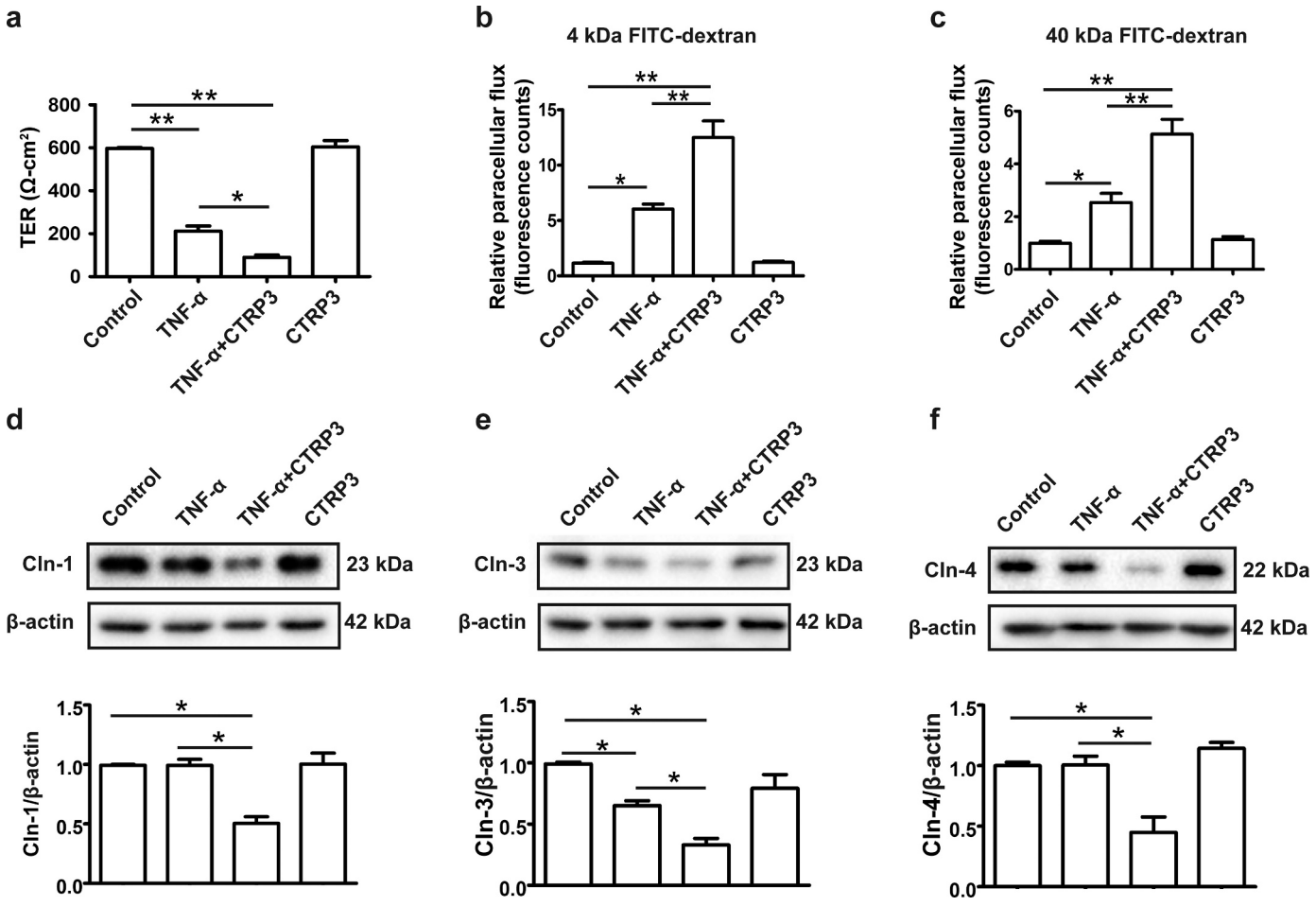

Fig. 5. Effect of CTRP3 on TNF-α-induced barrier dysfunction in submandibular cells. SMG-C6 cells were incubated with TNF-α (10 ng/mL) for 24 h in the absence or presence of CTRP3 (2 μg/mL) pre-treatment for 30 min. Transepithelial resistance (TER) was measured by using an Epithelial Volt Ohm Meter (a). The paracellular tracer flux assay was performed by using 4 kDa (b) or 40 kDa FITC-dextran (c). Expression of claudin-1 (Cln-1) (d), Cln-3 (e) and Cln-4 (f) was detected by western blot analysis. Values are the mean ± SEM from 3 to 6 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$.

expression between 8-week-old NOD and BALB/c mice (Fig. 7b). The results of immunohistochemistry showed that the staining intensity of CTRP3 was significantly enhanced in ducts of 12-week-old NOD mouse SMGs compared with those of age-matched BALB/c mice, both in the proximal and distal areas of lymphocytic infiltration (Fig. 7d). Moreover, we detected the distribution of CTRP3 in the LGs of the patients with Sjögren's syndrome and control individuals. CTRP3 was primarily expressed in the ducts and was only faintly expressed in mucous acinar cells in the LG of the control. The staining intensities in the areas both proximal and distal to the lymphocytic infiltration in LGs of the patients were markedly increased (Fig. 7e). The semi-quantitative results further suggested that CTRP3 was significantly elevated in the SMGs of NOD mice (Fig. 7f) and in the LGs of the patients (Fig. 7g).

4. Discussion

In this study, we determined the expression and distribution characteristics of CTRP3 in salivary glands from different species, including human, rat and mouse. We revealed that CTRP3 enhanced TNF-α-induced apoptosis and barrier dysfunction in the SMG epithelium. The mechanism by which CTRP3 promotes TNF-α-induced apoptosis might involve the enhanced expression and activity of TNFR1. Moreover, the expression of CTRP3 was significantly elevated in the LGs of patients with Sjögren's syndrome, as well as in the SMGs and serum of NOD mice. These results help to elucidate the important role of CTRP3 in the salivary epithelium.

Adipokines have recently attracted increased attention because of their expression and function in salivary glands. Adipokines, such as leptin, ghrelin and adiponectin, are expressed in acinar or/and ductal cells of salivary glands and are involved in the regulation of saliva secretion, energy metabolism and inflammatory responses [11,42–45]. In this study, we provided the first evidence that CTRP3 was expressed in the salivary glands of multiple species. The PG is primarily composed of serous acinus, while the SMG is a combined gland with seromucous acinus [46]. CTRP3 was widespread in the cytoplasm of serous acinus of PG and SMG in humans and rats, whereas it was primarily located in mouse granular convoluted tubules, suggesting that PG and SMG were new sources of CTRP3 synthesis and secretion.

Apoptosis of the epithelial cells in salivary glands has been proposed to be involved in the impairment of secretory function in inflammatory salivary gland diseases, such as Sjögren's syndrome. The accumulated proinflammatory cytokines, such as TNF-α and interferon-γ, eventually trigger apoptosis in Sjögren's syndrome. CTRP3 protects mesenchymal stem cells against hypoxia/serum deprivation-triggered apoptosis [36], inhibits spontaneous apoptosis in prostate cells [37], and suppresses myocardial infarction-induced apoptosis in ischaemic mouse hearts [7]. CTRP3 attenuates high glucose-induced apoptosis and oxidative stress in retinal pigment epithelial cells [47] and inhibits inflammation and apoptosis in lipopolysaccharide-exposed microglial cells by targeting the p38 MAPK and JNK signalling pathways [48]. Our previous studies showed that CTRP3 alleviates cardiac fibrosis by inhibiting AMPK-induced Smad3 activation and myofibroblast differentiation [32],

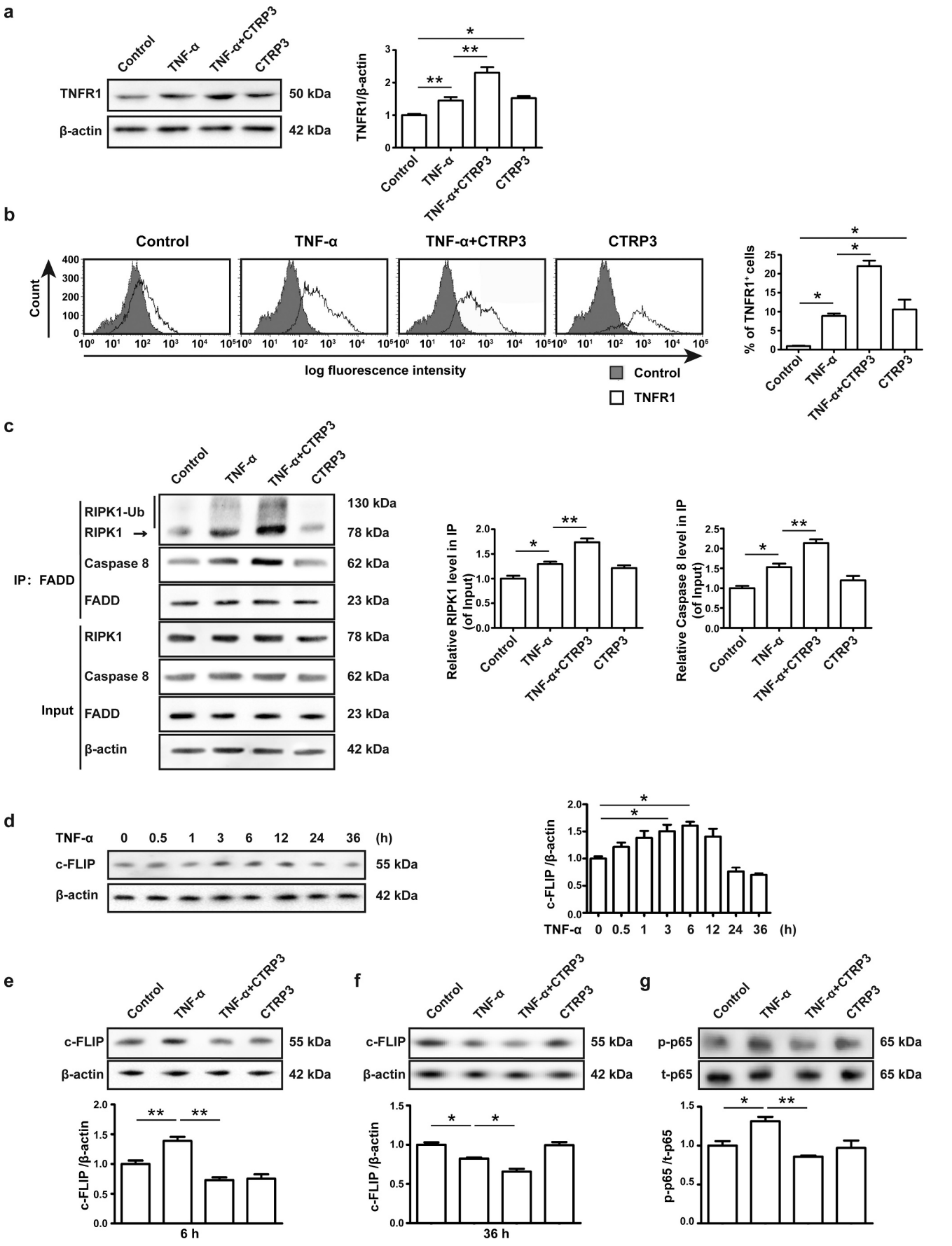

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Fig. 6. Effect of CTRP3 on the TNF-α-induced TNFR1 signalling pathway. SMG-C6 cells were incubated with TNF-α (200 ng/mL) for 6 h in the absence or presence of CTRP3 (2 μg/mL) pre-treatment for 30 min, and then TNFR1 expression was detected by western blot analysis (a). (b) TNFR1 expression at the cell surface after 6 h TNF-α/CTRP3 treatment was detected by flow cytometry. (c) The combinations of FADD with RIPK1 and caspase 8 were measured by using an immunoprecipitation assay. (d) The time-course of c-FLIP expression induced by TNF-α was detected by western blot analysis. C-FLIP expressions after TNF-α/CTRP3 treatment for 6 h (e) and 36 h (f) were detected by western blot analysis. (g) SMG-C6 cells were incubated with TNF-α (200 ng/mL) for 30 min in the absence or presence of CTRP3 (2 μg/mL), and phospho-NF-κB p65 expression/total-NF-κB p65 expression were detected. Values are the mean ± SEM from 4 to 6 independent experiments. RIPK1-Ub: ubiquitinated RIPK1. $^{*}P < 0.05$ and $^{**}P < 0.01$.

however, CTRP3 promotes β-glycerophosphate-triggered vascular smooth muscle cell calcification via ROS-ERK1/2-Runx2 activation [8]. In the present study, CTRP3 enhanced TNF-α-induced apoptosis characterized by an increased apoptotic rate and changed apoptosis-related protein expression in human and rat SMGs, but CTRP3 alone did not induce apoptosis. Moreover, H_2O_2 induced apoptosis in both SMG-C6 cell line and SMG tissues, consistently with previous studies which reported that H_2O_2 mediated apoptosis in human SMG cells and SV-40 transformed human salivary gland acinar cells [49,50]. However, our results showed that CTRP3 did not accelerate H_2O_2-induced apoptosis, suggesting that the biological effect of CTRP3 may vary depending on the cell type, the physiological and pathological stimuli.

Disruption of the epithelial barrier is linked to the development of a wide variety of chronic inflammatory epithelial diseases, such as cystic fibrosis, Crohn's disease and Sjögren's syndrome. Previous studies indicated that in the salivary glands of Sjögren's syndrome, the accumulated proinflammatory cytokines, such as TNF-α and IFN-γ, disrupted the epithelial barrier. Leptin alone induces barrier dysfunction in intestinal Caco-2 cells by increasing paracellular permeability and decreasing the expression of ZO-3 and Cln-5 [51], and resistin increases paracellular permeability and reduces ZO-1 and occludin expression in human coronary artery endothelial cells. Our previous study showed that TNF-α increased paracellular permeability and decreased the expression of Cln-3, but not Cln-1 and Cln-4, in SMG-C6 cells [30]. In this study, we found that CTRP3 further enhanced TNF-α-increased paracellular permeability and changed tight junction protein expression, suggesting that CTRP3 may mediate in TNF-α-induced salivary barrier dysfunction.

TNF-α activates its receptor TNFR1 to induce two distinct signalling pathways. One is cascade activation downstream of apoptotic complex II, which leads to apoptosis, and the other pathway is NF-κB activation induced by complex I, which leads to inflammation, survival, and anti-apoptotic transcriptional activation [52]. To reveal the mechanism by which CTRP3 promotes TNF-α-induced apoptosis, we detected TNFR1 expression and the combination of FADD with RIPK1 and caspase 8 to evaluate the formation of complex II. We found that CTRP3 further enhanced TNF-α-induced TNFR1 expression, especially at the cell surface, and promoted complex II formation by facilitating the combination of FADD with RIPK1 and caspase 8, suggesting that CTRP3 sensitized the TNF-α-mediated apoptotic complex II signalling pathway.

Previous studies have reported that CTRP3 inhibits the lipopolysaccharide-induced inflammatory response by suppressing NF-κB activation in the myocardium of mouse [53]. Our results indicated that CTRP3 significantly inhibited TNF-α-induced NF-κB activation, suggesting that CTRP3 is a potent inhibitor of NF-κB activation and inflammation. c-FLIP, a major anti-apoptotic protein that could be regulated by NF-κB activation triggered by complex I, plays a crucial role in inhibiting the TNFR1-induced apoptotic event. High levels of c-FLIP result in a predominance of FLIP/procaspase-8 heterodimers, thereby inhibiting the TNFR1-related apoptotic pathway by suppressing complex II formation, whereas low levels of c-FLIP result in the predominance of procaspase-8 homodimers, thereby promoting apoptosis induction by enhancing complex II formation [40]. TNF-α increases c-FLIP expression in Huh-7.5 cells [54] and TK-10 cells [55], however, it decreases the level of c-FLIP in podocytes of kidney [56]. We found that TNF-α could modulate the expression of c-FLIP in a time-dependent manner, increased c-FLIP expression at 3–6 h and slightly decreased c-FLIP expression from 24 h incubation. Notably, CTRP3 inhibited TNF-α-mediated c-FLIP expression both at 6 h and 36 h. These results suggest that CTRP3 may enhance TNF-α-induced apoptotic complex II pathway at least in part by inhibiting c-FLIP expression. TNF-α-induced pathological conditions depend on the imbalance between NF-κB-regulated inflammation and survival and caspase-mediated apoptosis signals [57]. CTRP3 enhanced TNF-α-induced apoptosis by promoting complex II formation and inhibiting c-FLIP expression. The exact roles and mechanisms of complex I and complex II balance, as modulated by TNF-α and CTRP3, in salivary cells need to be further investigated.

Notably, CTRP3 promoted TNF-α-induced apoptosis but not H_2O_2-induced apoptosis. Moreover, CTRP3 alone was not able to induce apoptosis in SMGs. One possible explanation was that the combination of TNF-α with TNFR1 at the cell surface is an essential component of the activation of the apoptotic complex II pathway. Although CTRP3 alone increased TNFR1 expression at the cell surface, CTRP3 could not activate TNFR1 and further affect complex II formation in the absence of TNF-α. These results indicated that the enhancing role of CTRP3 in apoptosis was dependent on TNF-α coexistence.

Sjögren's syndrome is a systemic disease with chronic inflammation in exocrine glands, primarily in lacrimary and salivary glands. The destruction of salivary glands of Sjögren's syndrome is routine along with the development of adipose tissue, which is the main secreting source of adipokines, and the normal gland tissues are replaced [58]. Resistin is upregulated in serum and salivary gland tissues of patients with Sjögren's syndrome, and its levels correspond to the intensity of lymphocytic inflammation in Sjögren's syndrome [59]. The levels of adiponectin in serum and minor salivary gland epithelial cells are higher in patients with Sjögren's syndrome than in controls [10,60], suggesting that adipokines might play important roles in Sjögren's syndrome. In this study, CTRP3 levels were significantly increased in serum and in the SMGs of NOD mice, as well as the LGs of Sjögren's syndrome patients. Our in vitro experiments indicated that CTRP3 enhanced TNF-α-induced apoptosis and barrier dysfunction in SMG-C6 cells and incubated tissue, suggesting that increased CTRP3 expression might be involved in the TNF-α-related pathological processes of Sjögren's syndrome. Apoptosis and fibrosis are involved in the pathogenesis of salivary gland damages and dysfunction, such as radiation-induced salivary gland injury [61,62]. Previous study has shown that another adipokine, adiponectin, protected fibroblast of mouse prostate from radiation-induced cell death and myofibroblast formation [63]. The effect of CTRP3 on radiation-induced salivary gland apoptosis and fibrosis is worthy of further study.

5. Conclusion

In conclusion, we demonstrated that salivary glands represent novel sources of CTRP3 synthesis and secretion. CTRP3 is a multifunctional adipokine whose role may vary depending on tissue and stimuli specificity. CTRP3 promotes TNF-α-induced apoptosis in the salivary glands. The possible mechanism of CTRP3 promoting TNF-α-induced acinar apoptosis included increasing TNFR1 expression, then enhancing complex II formation and altering expression of downstream apoptotic proteins such as caspase 8, caspase 10, Bcl-2, cytochrome C and cleaved caspase 3 (Fig. 8). Moreover, CTRP3 might enhance TNF-α-induced complex II formation by inhibiting NF-κB-mediated antiapoptotic c-FLIP expression (Fig. 8). Our findings may help to elucidate the physiological and pathological roles of CTRP3 in salivary glands.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2021.110042.

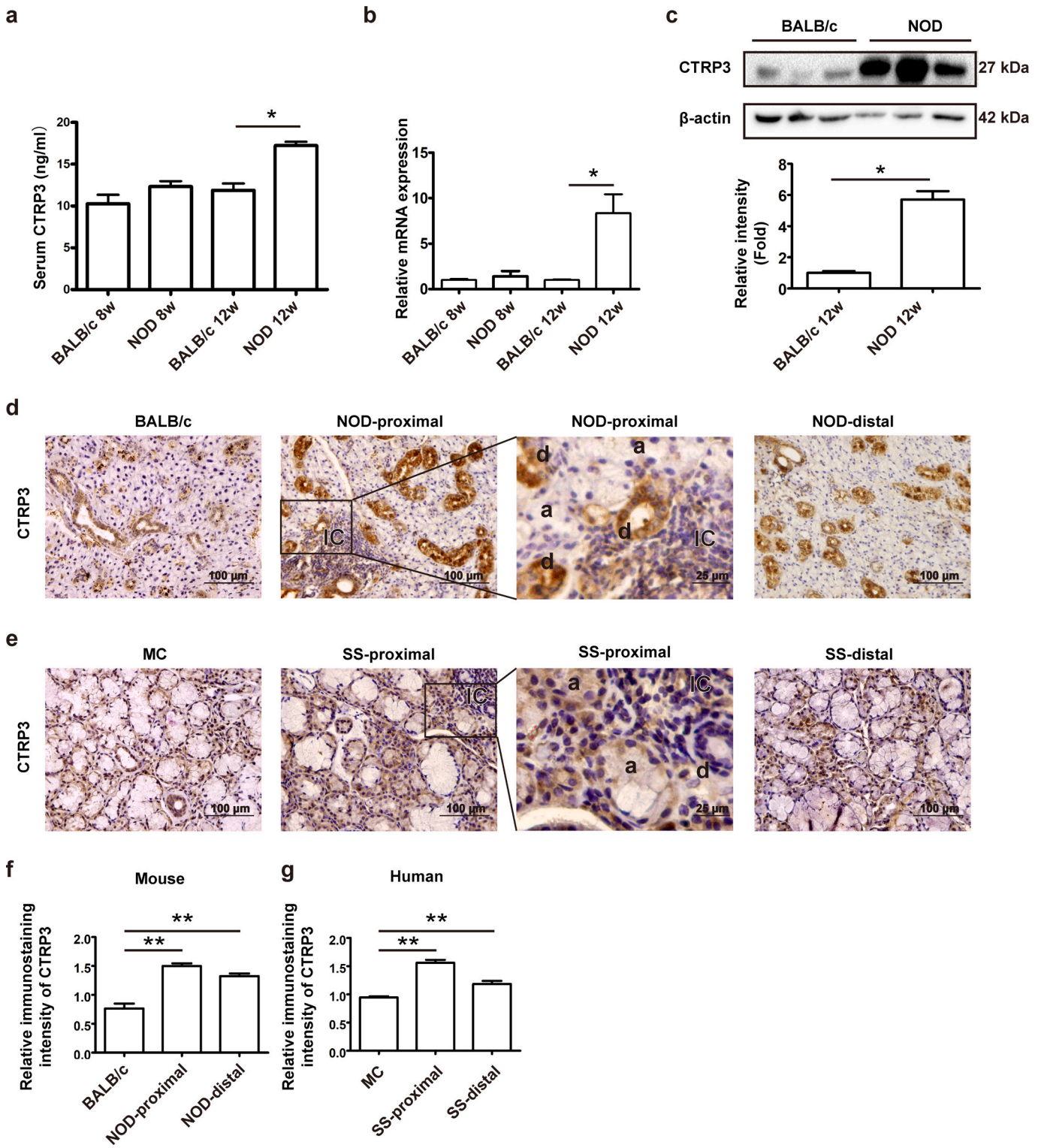

Fig. 7. Expression of CTRP3 in NOD mice and Sjögren's syndrome patients. (a) Serum CTRP3 levels in BALB/c and NOD mice. (b) The mRNA expression of CTRP3 in BALB/c and NOD mice. (c) The protein expression of CTRP3 in BALB/c and NOD mice. (d) Immunohistochemical staining of CTRP3 was detected in the areas proximal and distal to lymphocytic infiltration in the SMG of NOD mice. (e) Immunohistochemical staining of CTRP3 in the labial glands of Sjögren's syndrome patients and controls. Quantitative analysis of CTRP3 staining intensities in mice (f) and in Sjögren's syndrome patients (g). IC, infiltrating cells. a: acinus, d: duct, MC: mucous cyst, SS: Sjögren's syndrome. Values are the mean ± SEM from 6 to 12 mice or people in each group. $*P < 0.05$ and $**P < 0.01$.

CRediT authorship contribution statement

Mei Mei: Conceptualization, Investigation, Methodology, Writing - original draft. **Ling-Han Qu:** Software, Writing - original draft. **Xin Cong:** Methodology, Investigation. **Yan Zhang:** Methodology, Validation. **Ruo-Lan Xiang:** Data curation. **Guang-Yan Yu:** Writing - review & editing. **Jia-Zeng Su:** Supervision, Writing - review & editing. **Li-Ling Wu:** Conceptualization, Writing - review & editing.

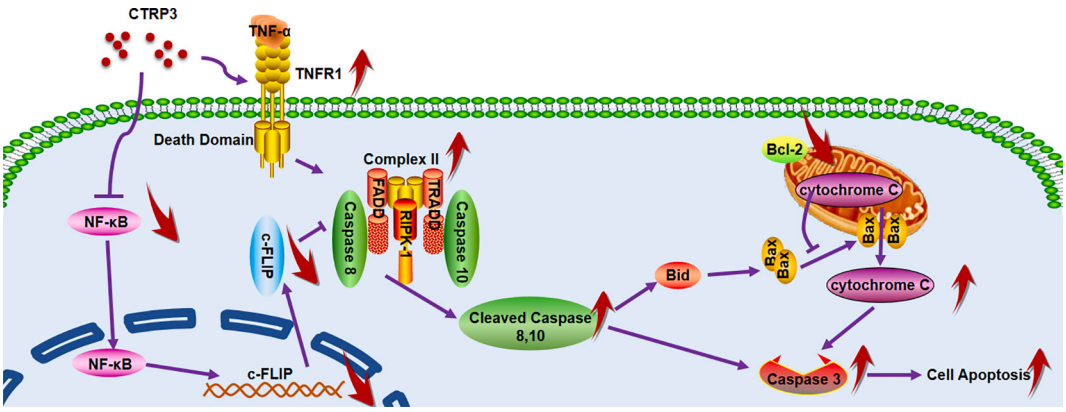

Fig. 8. A proposed model illustrating the molecular mechanism of CTRP3 promoting TNF-α-induced salivary epithelial cells apoptosis.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Acknowledgements

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