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C1q/tumor necrosis factor-related protein-6 attenuates TNF-α-induced apoptosis in salivary acinar cells via AMPK/SIRT1-modulated miR-34a-5p expression

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Abstract

C1q/tumor necrosis factor-related protein-6 (CTRP6) is a newly identified adipokine involved in diverse biological processes. However, its role in salivary glands remains unknown. Here, we demonstrated that CTRP6 was mainly distributed in the nuclei, apicolateral membranes, and cytoplasm of human submandibular glands (SMGs), serous cells of parotid glands, and ducts and apicolateral membranes of serous cells in rats and mice. CTRP6 inhibited the apoptosis rate and reversed the increased levels of cleaved caspase 3, caspase 8, caspase 9, and cytochrome C and the decreased Bcl-2 expression induced by tumor necrosis factor (TNF)-α in both SMG-C6 cells and cultured human SMG tissues. Microarray analysis identified 43 differentially expressed microRNAs (miRNAs) in the SMGs of nonobese diabetic mice. miR-34a-5p was selected due to its upregulation by TNF-α, which was abolished by CTRP6. The miR-34a-5p inhibitor promoted whereas the miR-34a-5p mimic suppressed the effects of CTRP6 on TNF-α-induced apoptosis. CTRP6 increased AMP-activated protein kinase (AMPK) phosphorylation and reversed TNF-α-induced SIRT1 downregulation in salivary cells. AraA, an AMPK inhibitor, reversed the effects of CTRP6 on TNF-α-induced alterations in the levels of SIRT1, miR-34a-5p, Bcl-2, and cleaved caspase 3 in vitro and ex vivo, whereas activating AMPK by AICAR reversed the decrease in SIRT1 expression and increase in miR-34a-5p expression induced by TNF-α. Inhibition of SIRT1 by EX527 suppressed the effects of CTRP6 on TNF-α-induced changes in miR-34a-5p and apoptosis-related proteins. Our findings indicate that salivary glands are novel sites for CTRP6 synthesis and secretion. CTRP6 protects acinar cells against TNF-α-induced apoptosis via AMPK/SIRT1-modulated miR-34a-5p expression.

KEYWORDS

AMP-activated protein kinase, apoptosis, C1q/tumor necrosis factor-related protein-6, miR-34a-5p, salivary gland, sirtuin 1

Ling-Han Qu and Xia Hong contributed equally to this study.

1 | INTRODUCTION

The complement C1q/tumor necrosis factor-related protein (CTRP) superfamily, comprising 15 members (CTRP1–CTRP15), is a newly identified and highly conserved family of adiponectin paralogs, which participates in a variety of biological processes such as energy metabolism and inflammation (Wong et al., 2008). CTRP6 is widely expressed in multiple tissues and organs, including the adipose tissue, heart, brain, and placenta, and it exerts various effects on different physiological or pathological processes (Wong et al., 2008). CTRP6 promotes the oxidation of fatty acids in skeletal muscle cells, regulates lipogenesis in myocytes (Lee et al., 2010), promotes the production of interleukin-10 in macrophages, and exerts anti-inflammatory effects in arthritis and atherosclerosis (Kim et al., 2010; Murayama et al., 2015). We previously reported that CTRP6 attenuates post-infarct cardiac fibrosis (Lei et al., 2015). CTRP6 inhibits the transforming growth factor-β1-induced mesangial cell proliferation, extracellular matrix protein expression (Wang, Sun, et al., 2018), and platelet-derived growth factor-BB-induced vascular smooth muscle cell migration and proliferation (Dong et al., 2018). Furthermore, CTRP6 alleviates cellular damage in Angiotensin II-induced hypertension and vascular endothelial dysfunction in doxorubicin-induced cardiac injury and gentamicin-induced acute kidney injury (Chi et al., 2017; Li et al., 2016; Zheng et al., 2019). These results suggest that CTRP6 plays an important role in cellular protection and functional preservation in multiple pathological conditions.

Salivary glands play critical roles in oral health, and a decrease in their secretory capacity is common in many disorders such as Sjögren's syndrome and postirradiation salivary gland damage, which can lead to rampant caries, oral candida infection, and dysfunction of the stomatognathic system. Despite the involvement of multiple mechanisms in hyposalivation, apoptosis, a process of cellular self-destruction, is the common manifestation of these diseases leading to a decrease in the number of acinar cells (Lau et al., 2017; Nakamura et al., 2018). Therefore, protecting parenchymal cells from apoptosis is a potential method for preserving salivary secretion. Although CTRP6 has gained interest as a potential antiapoptotic factor (Chi et al., 2017; Zheng et al., 2019), its expression and functions in the salivary gland have not been elucidated to date.

Therefore, the purpose of our study was to investigate the expression and distribution of CTRP6 in salivary glands, to explore its effect on tumor necrosis factor (TNF)-α-induced apoptosis of submandibular cells, and to identify the underlying mechanism.

2 | MATERIALS AND METHODS

2.1 | Ethical approval

The study was approved by the Ethics Committee of Peking University School and Hospital of Stomatology (PKUSSIRB-201523064 for human research and LA2015088 for animal experiments) and Ethics Committee of Peking University Shenzhen Hospital (PKUSZEC(R)2019007). All participants signed an informed consent form before sample collection.

2.2 | Human and animal samples

Human submandibular glands (SMGs) of patients who underwent neck dissection as a part of surgical treatment for oral malignant tumors and human parotid glands (PGs) of patients who underwent superficial parotidectomy for benign PG tumors were collected. None of the patients had any systemic disease nor did they receive irradiation or chemotherapy before surgery. All SMG and PG tissues were confirmed to be histologically normal.

Human serum and saliva samples were collected from 114 healthy volunteers (aged 18–26 years, 56 males). As a part of routine investigations, nonstimulated whole saliva samples were collected between 9:00 and 11:00 a.m. (Wang et al., 2015). The subjects were asked not to drink, eat, or smoke before saliva collection. After rinsing their mouths, the subjects bent their heads forward for 10 min, which allowed saliva to flow into the cups. The saliva samples were centrifuged at 1500 rpm at 4°C for 15 min, and the supernatants were collected for CTRP6 detection.

SMGs and PGs were isolated from male Sprague-Dawley rats weighing 180–220 g, and mouse salivary glands were collected from 12-week-old female BALB/c and nonobese diabetic (NOD) mice, an animal model of Sjögren's syndrome.

2.3 | Cell culture and treatment

The rat submandibular acinar cell line SMG-C6 was cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1 mixture) containing 5 μg/ml transferrin, 2 nM triiodothyronine, 0.1 μM retinoic acid, 1.1 μM hydrocortisone, 80 ng/ml epidermal growth factor, 5 μg/ml insulin, 5 mM glutamine, 50 μg/ml gentamicin sulfate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5% fetal bovine serum (FBS) at 37°C with 5% CO_2 using a humidified incubator. All reagents used in cell culture experiments were obtained from Sigma-Aldrich Co.

Cells were treated with TNF-α (200 ng/ml; 400-14; Peprotech) for 36 h to induce apoptosis. Human recombinant CTRP6 (1–4 μg/ml; 00089-01-100; Aviscera Bioscience) was added to the incubation solution 30 min before TNF-α treatment. AraA (HY-B0277; MedChemExpress), AICAR (A9978; Sigma-Aldrich Co.) or EX527 (E7034; Sigma-Aldrich Co.) were used for some experiments.

2.4 | Human SMG tissue culture

Fresh human SMG samples were suspended in Krebs-Ringer HEPES solution (11.1 mM glucose, 120 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 20 mM HEPES, pH 7.4) at 4°C, and they were

transported to the laboratory within 30 min of obtaining the samples. SMG samples were cut into small pieces, placed in DMEM/F12 (1:1 mixture) containing 10% FBS, incubated in a humidified atmosphere with 5% CO_2 at 37°C for 30 min, and treated with TNF-α for 24 h.

2.5 | Immunohistochemical staining

Salivary gland specimens were fixed with 4% paraformaldehyde, embedded in paraffin wax, and serially cut into 5 μm thick sections. The sections were deparaffinized and rehydrated, and antigens were retrieved using citrate buffer (pH 6.0). Subsequently, they were incubated with 3% hydrogen peroxide, blocked with 5% bovine serum albumin, and incubated with primary anti-CTRP6 antibody (1:500; ab36900; Abcam) and horseradish peroxidase (HRP)-conjugated secondary antibody (Zhongshan Laboratories). Samples were developed with 3, 3′-diaminobenzidine and stained with hematoxylin. Morphological features were observed under a light microscope (Q550CW; Leica).

2.6 | Enzyme-linked immunosorbent assay

CTRP6 levels in the serum and saliva samples were measured using an enzyme-linked immunosorbent assay (ELISA) kit (SK00089-06; Aviscera Bioscience) according to manufacturer's protocol.

2.7 | Western blot analysis

Tissues and cells were harvested in radioimmunoprecipitation assay buffer (140 mM NaCl; 10 mM Tris-HCl, pH 8.0; 1 mM EDTA; 0.5 mM EGTA; 1% Triton X-100; 0.1% sodium dodecyl sulfate [SDS]; 0.1% sodium deoxycholate; and protease inhibitors), and they were ultrasonicated at 4°C for 15 s. Cell extracts were centrifuged at 12,000 *g* for 10 min, and protein concentrations were determined using a Bicinchoninic Acid Protein Assay Kit (MPK002; M&C Gene Technology). Equal amounts of proteins (20 μg) were separated using 12% SDS-PAGE, and they were transferred onto polyvinylidene difluoride membranes. The membranes were blocked with phosphate-buffered saline (PBS) containing 5% milk powder and 0.05% Tween 20, and subsequently, they were probed with primary antibodies at 4°C overnight. The primary antibodies included anti-CTRP6 (1:1000), anti-β-actin (1:2000; sc-58673; Santa Cruz Biotechnology), anti-caspase 12 (1:1000; sc-12396; Santa Cruz Biotechnology), anti-caspase 9 (1:1000; 9508; Cell Signaling Technology), anti-caspase 8 (1:1000; 4790; Cell Signaling Technology), anti-cytochrome C (1:1000; BS1689; Bioworld Technology), anti-cleaved caspase 3 (1:1000; 9664; Cell Signaling Technology), anti-Bcl-2 (1:1000; 3498; Cell Signaling Technology), anti-Bax (1:1000; 2772; Cell Signaling Technology), anti-adenosine 5′-monophosphate-activated protein kinase (AMPK; 1:1000; 2532; Cell Signaling Technology), anti-phospho-AMPK (p-AMPK; 1:1000; 2531; Cell Signaling Technology), and anti-sirtuin 1 (SIRT1; 1:1000; BS6494; Bioworld Technology) antibodies. Following this, membranes were incubated with HRP-conjugated secondary antibodies. The target protein bands were detected using an enhanced chemiluminescence reagent, and band densities were analyzed using the ImageJ software (National Institutes of Health).

2.8 | Semi-quantitative and quantitative PCR

Total RNA was extracted from SMG and PG tissues using TRIzol (Invitrogen), and it was used as a template for cDNA synthesis. The primer sequences are listed in the supplementary table. The semi-quantitative polymerase chain reaction (PCR) products were separated using 1.5% agarose gel electrophoresis, and DNA bands were stained using ethidium bromide for visualization. Quantitative PCR was performed using the DyNAmo™ ColorFlashProbe qPCR Kit (F456L; Thermo Fisher Scientific) with a PikoReal Real-Time PCR System (Thermo Fisher Scientific), and the average quantification cycle was calculated.

2.9 | Microarray analysis

Total RNA was extracted from the SMGs of 12-week-old female NOD mice and age- and sex-matched BALB/c mice, and microRNAs (miRNAs) were fluorescently labeled using the miRCURY™ Array Power Labeling kit (208032-A; Exiqon) according to manufacturer's instructions. Subsequently, the labeled samples were hybridized on the miRCURY™ Array (v.18.0, Exiqon) in a heat-shrank hybridization bag at 56°C overnight. After hybridization, the microarray was washed, and it was scanned using a GenePix 4000B microarray scanner (Axon Instruments). Scanned images were gridded with GenePix Pro 6.0 software (Axon Instruments). Replicated miRNAs were averaged, and probes with intensities more than or equal to 30 in all samples were selected for normalization. miRNAs with a fold change value greater than 1.5 and a p value less than .05 were regarded as differentially expressed.

2.10 | Transfection of miRNA mimic and inhibitor

The miRNA mimic, miRNA inhibitor, and negative control miRNA were supplied by RiboBio Co. Ltd., and they were used at 50 nM. SMG-C6 cells were cultured to approximately 70% confluency, and they were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. After the indicated incubation time, total RNA or proteins were extracted for further experiments.

2.11 | Analysis of cell apoptosis using flow cytometry

Apoptotic rate was calculated using an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Dojindo), which detects

phosphatidylserine on the cell plasma membrane using propidium iodide (PI). Cells were washed using PBS, resuspended in 300 µl of binding buffer, incubated with 5 µl of annexin V-FITC solution for 30 min, and then incubated with 2.5 µl of PI for 5 min. Apoptosis was assessed using flow cytometry (FACSCalibur; Becton Dickinson).

2.12 | Statistical analysis

Data are presented as mean ± *SEM*. Statistical analysis was performed using an un-paired Student's *t*-test for two groups and a one-way analysis of variance, followed by Bonferroni's test for multiple group analysis using Prism 5.0 software (GraphPad). $p < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Expression and distribution of CTRP6 in salivary glands

To examine the expression of CTRP6 in salivary glands, RT-PCR and western blot analysis were performed on SMG-C6 cells and SMGs and PGs of different species. CTRP6 messenger RNA (mRNA) and protein expression were observed in the salivary glands of humans, rats, and mice (Figure 1a,b). ELISA results revealed that the concentrations of CTRP6 in the serum and saliva of healthy volunteers were 273.21 ± 18.25 ng/ml and 378.28 ± 28.50 ng/ml, respectively (Figure 1c). CTRP6 levels in the saliva and serum of males and females were not significantly different (Figure 1d). These results suggest that salivary cells can synthesize and secrete CTRP6.

The distribution of CTRP6 in SMGs and PGs of the three species is shown in Figure 2. CTRP6 staining was obvious in the nucleus, apicolateral membranes, and was also positive in the cytoplasm of serous cells, but was rarely observed in mucous acini of human SMGs. In rat and mouse SMGs, strong CTRP6 immunostaining was observed in the apicolateral membranes of mucous acini and cytoplasm of granular convoluted tubules, which are the characteristic structures of SMG ducts in all rodents (Gresik, 1994). Moreover, CTRP6 exhibited an intensely dispersed staining in the nuclei, membranes, and cytoplasm of serous acini and ducts of human PGs whereas CTRP6 was mainly localized to the apicolateral membranes of rodent PG serous acini and mouse ducts.

3.2 | CTRP6 inhibits TNF-α-induced apoptosis in SMG-C6 cells and cultured human SMG tissues

To explore the function of CTRP6 in salivary glands, we investigated the effect of CTRP6 on TNF-α-induced apoptosis in vitro. Annexin V-FITC/PI double staining revealed that incubating SMG-C6 cells with TNF-α at 200 ng/ml for 36 h significantly

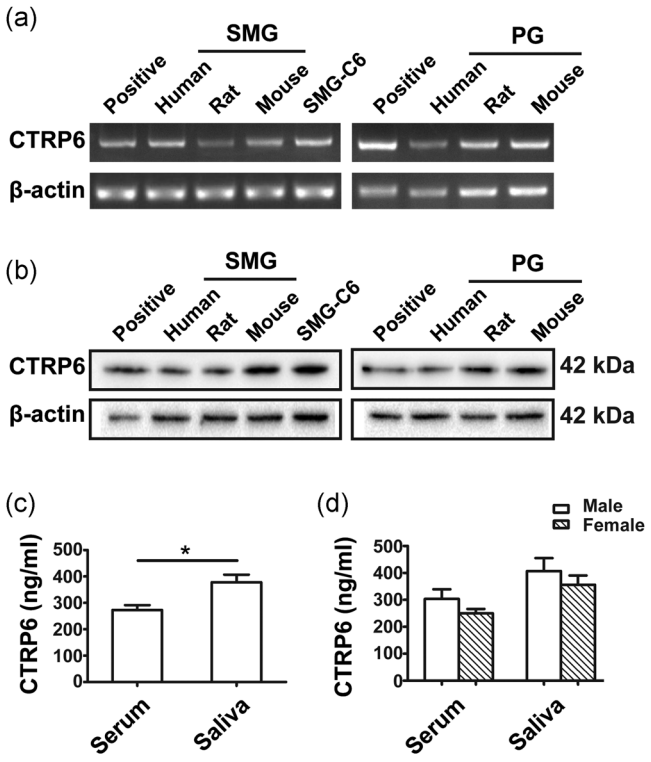

FIGURE 1 Expression of CTRP6 in salivary glands, serum, and saliva. (a) The CTRP6 mRNA expression in the submandibular glands (SMGs) and parotid glands (PGs) of humans, rats, mice, and a rat submandibular acinar cell line, SMG-C6, was analyzed using semi-quantitative PCR. Rat testicular tissue was used as the positive control. (b) The expression of CTRP6 protein in SMG and PG tissues and SMG-C6 cells was analyzed using western blot analysis. Rat testicular tissue was used as the positive control. (c) and (d) The serum and saliva CTRP6 concentrations in healthy participants were measured using ELISA. Samples were collected from 114 healthy volunteers including 56 males. Data are expressed as mean ± SEM. *$p < .05$. CTRP, C1q/tumor necrosis factor-related protein; ELISA, enzyme-linked immunosorbent assay; mRNA, messenger RNA; PCR, polymerase chain reaction

increased the apoptotic rate. Pre-incubation with CTRP6 at 2 µg/ml or 4 µg/ml for 30 min markedly inhibited TNF-α-induced apoptosis whereas CTRP6 alone did not affect the apoptotic rate (Figure 3a,b). Caspase 3 is considered to be the most important executioner caspase in apoptosis (Lossi et al., 2018). CTRP6 (2 µg/ml) inhibited the TNF-α-induced increase in the levels of cleaved caspase 3 (Figure 3c,d). Caspase 8 initiates the death-receptor pathway of apoptosis (Fritsch et al., 2019). TNF-α significantly increased the levels of caspase 8 whereas CTRP6 reversed the effects of TNF-α (Figures 3c and 3e). Caspase 9 is a key player in the mitochondrial pathway, which is involved in various stimuli (Li et al., 2017). Pre-incubating with CTRP6 suppressed the TNF-α-induced increase of caspase 9 (Figures 3c and 3f). Caspase 12 mediates the endoplasmic reticulum-related apoptotic pathway (Zhang et al., 2016). TNF-α did not affect the expression of caspase 12 in the presence or absence of CTRP6 (Figures 3c and 3g). Cytochrome C, released from injured

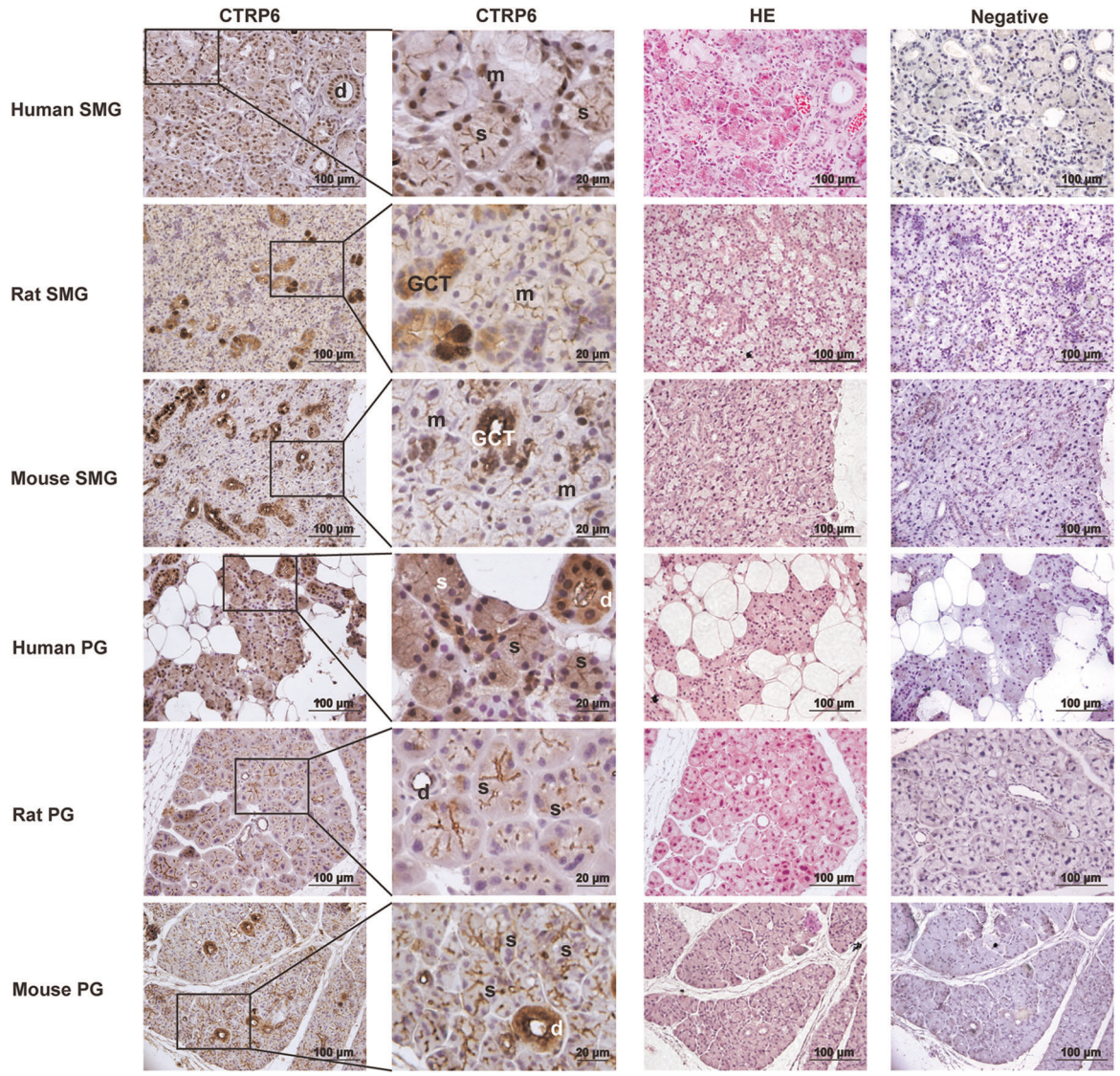

FIGURE 2 Expression and distribution of CTRP6 in SMGs and PGs of humans, rats, and mice. CTRP, C1q/tumor necrosis factor-related protein; d, duct; GCT, granular convoluted tubule; m, mucous acinus; PGs, parotid glands; s, serous acinus; SMGs, submandibular glands

mitochondria, plays a key role in inducing apoptosis (Cao et al., 2015). CTRP6 decreased the cytoplasmic cytochrome C levels, which were increased by TNF-α (Figures 3c and 3h). Bcl-2 and Bax are apoptosis-related proteins that participate in the cross-talk between the death-receptor pathway and the mitochondrial pathway (Edlich, 2018). TNF-α reduced Bcl-2 expression, but it did not alter Bax levels (Figures 3c and 3i,j). CTRP6 pretreatment reversed the effects of TNF-α on Bcl-2 expression. In addition, CTRP6 alone did not affect the levels of these apoptosis-related proteins. These findings indicate that CTRP6 inhibits TNF-α-induced apoptosis of SMG-C6 cells.

Next, we explored the effect of CTRP6 on TNF-α-induced apoptosis in cultured fresh human SMG tissues. CTRP6 (2 μg/ml) significantly inhibited the TNF-α-induced increase of cleaved caspase 3, caspase 8, caspase 9, and cytochrome C levels, and reversed the TNF-α-induced decrease in Bcl-2 expression (Figure 3k–q). These results confirm that CTRP6 plays an anti-apoptotic role in salivary glands.

3.3 | CTRP6 inhibits the increase in the expression levels of miR-34a-5p and miR-34a-3p induced by TNF-α

miRNAs regulate the expression of target genes at the post-transcriptional level, and many miRNAs participate in the regulation of apoptosis (Anvarnia et al., 2019). We identified the possible miRNAs involved in the antiapoptotic effects of CTRP6 in salivary glands. miRNA microarray analysis revealed that 22 miRNAs were significantly upregulated and 21 miRNAs were downregulated in the SMGs of NOD mice (Figure 4a), which is an animal model of Sjögren's syndrome characterized by salivary gland damage and dysfunction. Based on their expression profiles, we selected three miRNAs, namely miR-34a-5p, let-7a-5p, and miR-181b-5p, whose target gene was predicted as Bcl-2 using the miRBase 21.0 database (http://www.mirbase.org/). miR-34a-3p was added as a control. We further validated the

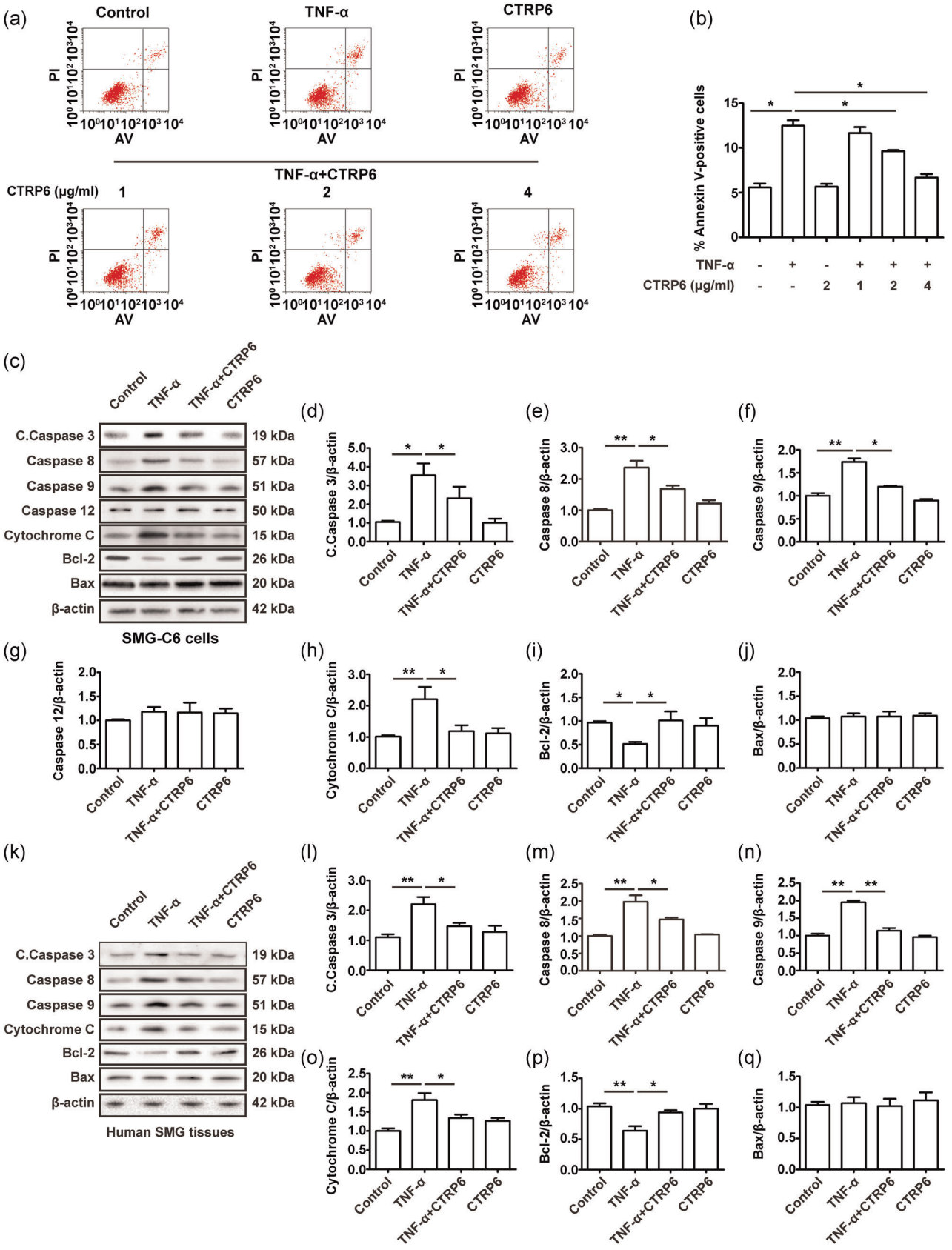

FIGURE 3 (See caption on next page)

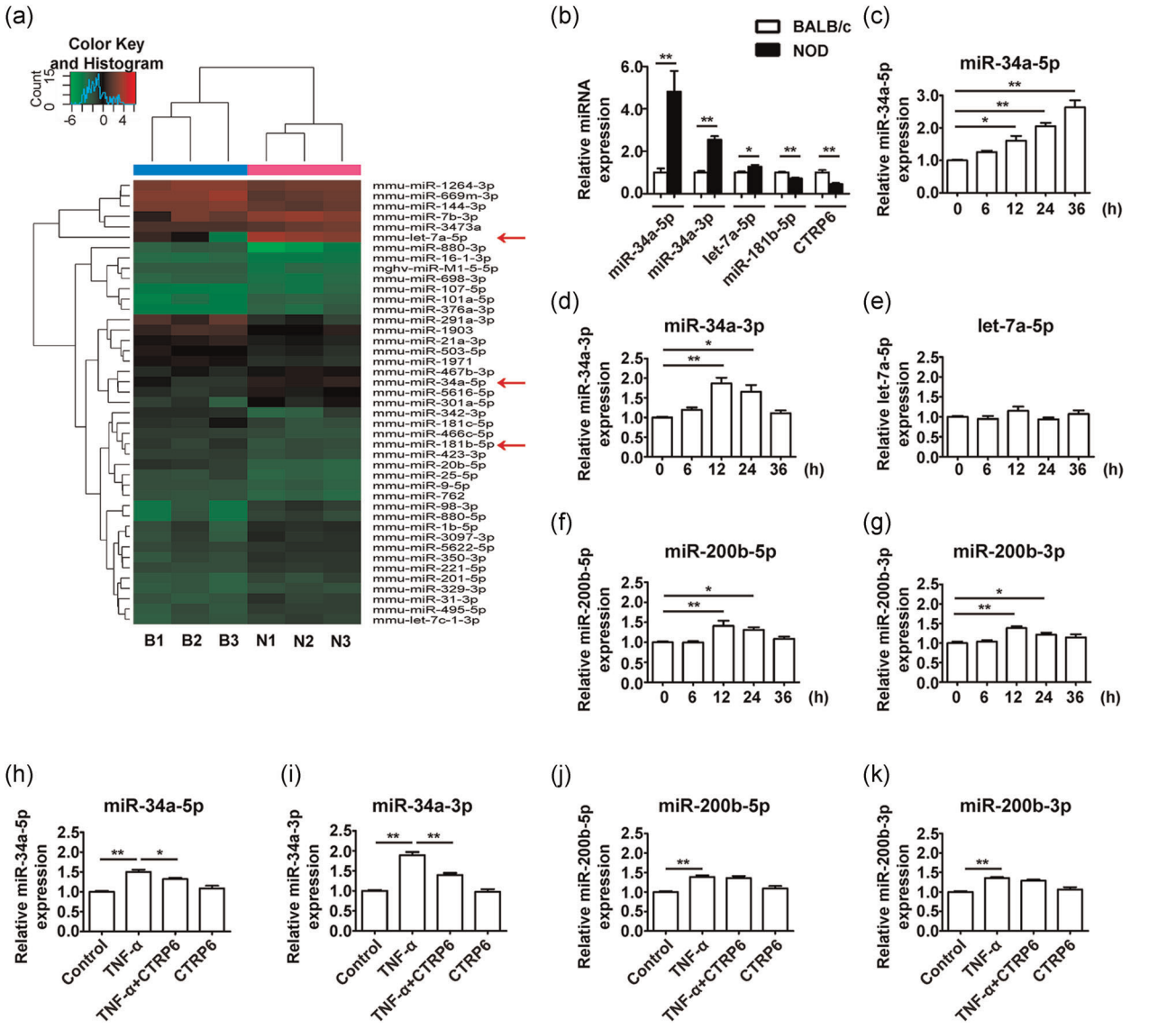

FIGURE 4 CTRP6 inhibits the TNF-α-induced increase in the levels of miR-34a-5p and miR-34a-3p. (a) The heatmap of differentially expressed miRNAs in the SMGs of three NOD mice (N1, N2, and N3) as compared with that of three BALB/c mice (B1, B2, and B3). (b) The expression levels of miR-34a-5p, miR-34a-5p, let-7a-5p, miR-181b-5p, and CTRP6 in NOD and BALB/c mice validated by real-time PCR; $n = 8$. (c–g) SMG-C6 cells were incubated with TNF-α (200 ng/ml) for the indicated time. The expression levels of the selected miRNAs, including miR-34a-5p, miR-34a-3p, let-7a-5p, miR-200b-5p, and miR-200b-3p, were determined using real-time PCR; $n = 6$. (h–k) Treatment of SMG-C6 cells with TNF-α (200 ng/ml) for 24 h with or without CTRP6 (2 μg/ml) pre-incubation. The expression levels of miR-34a-5p, miR-34a-3p, miR-200b-5p, and miR-200b-3p were determined using real-time PCR; $n = 6$. Data are expressed as mean ± *SEM* from 6 to 8 independent experiments. $^{*}p < .05$ and $^{**}p < .01$. CTRP, C1q/tumor necrosis factor-related protein; miR, microRNA; NOD, nonobese diabetic; PCR, polymerase chain reaction; SMGs, submandibular glands; TNF-α, tumor necrosis factor-α

FIGURE 3 Effect of CTRP6 on TNF-α-induced apoptosis of submandibular cells. (a) and (b) SMG-C6 cells were incubated with TNF-α (200 ng/ml) for 36 h, and they were pretreated with CTRP6 at different concentrations for 30 min. Apoptosis was evaluated using annexin V-FITC/PI double staining, which was detected by flow cytometry (a), and it was quantitatively analyzed (b). (c–j) SMG-C6 cells were incubated with TNF-α (200 ng/ml) for 36 h with or without CTRP6 (2 μg/ml) pretreatment for 30 min. The protein levels of cleaved caspase 3 (C. Caspase 3), caspase 8, caspase 9, caspase 12, cytochrome C, Bcl-2, and Bax were determined using western blot analysis (c), and they were quantified (d–j). (k–q) Fresh human SMG tissues were incubated with TNF-α (200 ng/ml) for 24 h with or without CTRP6 (2 μg/ml) pretreatment. Expression levels of apoptosis-related proteins were determined using western blot analysis (k), and they were quantified (l–q). Data are expressed as mean ± *SEM* from 4–6 independent experiments. $^{*}p < .05$ and $^{**}p < .01$. CTRP, C1q/tumor necrosis factor-related protein; FITC, fluorescein isothiocyanate; PI, propidium iodide; SMG, submandibular gland; TNF-α, tumor necrosis factor-α

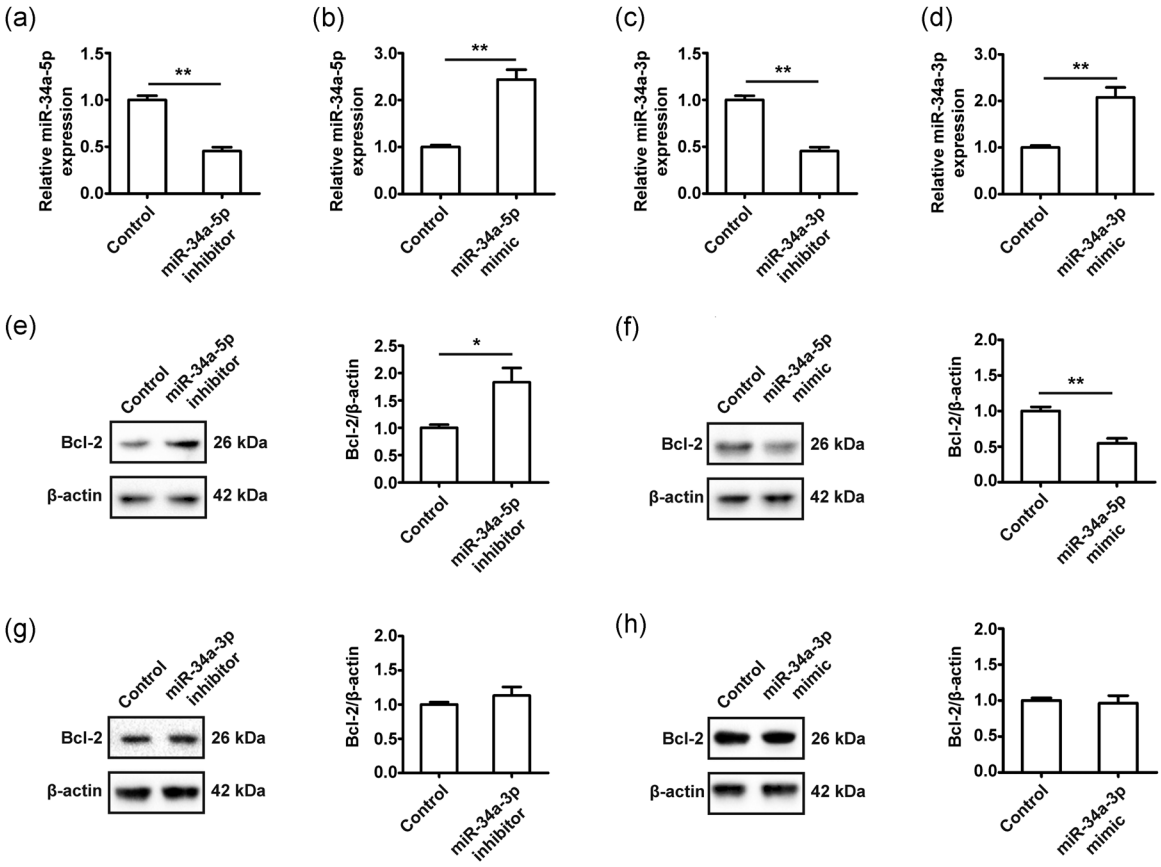

FIGURE 5 miR-34a-5p regulates Bcl-2 expression in SMG-C6 cells. SMG-C6 cells were transfected with miR-34a-5p and miR-34a-3p inhibitors and mimics. The transfection efficiencies were determined using real-time PCR (a–d). The levels of Bcl-2 protein were detected using western blot analysis (e–h). Data are expressed as mean ± *SEM* from 4 to 6 independent experiments. $^*p < .05$. miR, microRNA; PCR, polymerase chain reaction; SMG, submandibular gland

miRNA hits in SMGs collected from NOD and BALB/c mice and confirmed the increased levels of miR-34a-5p, miR-34a-3p, and let-7a-5p as well as the decreased levels of miR-181-5p and CTRP6 mRNA in NOD SMGs (Figure 4b). In addition, miR-200b-5p and miR-200b-3p were selected due to their upregulation in minor salivary glands of Sjögren's syndrome patients and their potential role as proapoptotic factors in human hypertrophic scar fibroblasts (Kapsogeorgou et al., 2011; Li et al., 2014; Reale et al., 2018). To determine the roles of these miRNAs in TNF-α-induced apoptosis of acinar cells, we measured their expression levels using real-time PCR after incubating the cells with TNF-α for indicated time. After treating with TNF-α for 12 and 24 h, the levels of miR-34a-5p, miR-34a-3p, miR-200b-5p, and miR-200b-3p significantly increased whereas the expression of let-7a-5p remained unchanged (Figure 4c–g).

We further explored the potential roles of these miRNAs in the antiapoptotic effects of CTRP6. Pre-incubation with CTRP6 significantly inhibited the increase in the levels of miR-34a-5p and miR-34a-3p, which was induced by TNF-α, but it did not affect the expression levels of miR-200b-5p and miR-200b-3p (Figure 4h–k). These results suggest that the inhibition of miR-34a-5p and miR-34a-3p may be involved in the inhibitory effects of CTRP6 against TNF-α-induced apoptosis.

3.4 | miR-34a-5p is involved in the inhibitory effects of CTRP6 against TNF-α-induced apoptosis by regulating Bcl-2

Bcl-2 plays a critical role in regulating apoptosis, and based on the above results, it may be a downstream effector in the inhibitory effects of CTRP6 against TNF-α-induced apoptosis. We next explored the roles of miR-34a-5p and miR-34a-3p, focusing on the regulation of Bcl-2 in SMG-C6 cells. miR-34a-5p and miR-34a-3p inhibitors and mimics were transfected into SMG-C6 cells (Figure 5a–d) and Bcl-2 expression was determined. The miR-34a-5p inhibitor significantly upregulated Bcl-2 levels whereas the mimic reduced Bcl-2 levels (Figure 5e,f). However, no significant effect of the miR-34a-3p inhibitor or mimic was observed on Bcl-2 expression (Figure 5g,h). These results suggest that miR-34a-5p, but not miR-34a-3p, modulates the expression of Bcl-2 in SMG-C6 cells. To further verify the involvement of miR-34a-5p in the antiapoptotic effects of CTRP6, the cells transfected with the miR-34a-5p inhibitor or mimic were incubated with CTRP6 and TNF-α. The miR-34a-5p inhibitor reversed the decreased Bcl-2 expression, increased cleaved caspase 3 levels and elevated apoptotic rate elevation induced by TNF-α, and enhanced the antiapoptotic effects of CTRP6 (Figure 6a–e). Moreover, the miR-34a-5p mimic reversed the

protective effects of CTRP6 on the apoptotic rate and expression of Bcl-2 and cleaved caspase 3 in TNF-α-treated cells (Figure 6f–j). These results suggest that miR-34a-5p is involved in the protective effects of CTRP6 against TNF-α-induced apoptosis.

3.5 | Activation of AMPK is required for the protective effects of CTRP6 against TNF-α-induced apoptosis

We next identified the signaling molecule involved in the anti-apoptotic effects of CTRP6. AMPK is an important intracellular protein kinase that mediates the increase in the oxidation of fatty acids induced by CTRP6 in skeletal muscle cells (Lee et al., 2010). After an in vitro and ex vivo incubation with CTRP6 for 30 or 60 min, a significant increase in p-AMPK levels was observed (Figure 7a–d). When SMG-C6 cells or cultured human SMG tissues were pretreated with AraA (1 mM), an AMPK inhibitor, the effects of CTRP6 on TNF-α-induced alterations in the expression levels of Bcl-2 and cleaved caspase 3 were reversed (Figure 7e–j), suggesting that the activation of AMPK was necessary for the protective roles of CTRP6 against TNF-α-induced apoptosis. Moreover, AraA treatment reversed the effects of CTRP6 on miR-34a-5p expression, and it further enhanced the effect of TNF-α on the upregulation of miR-34a-5p (Figure 7k,l). However, directly activating AMPK by AICAR (1 mM), a selective AMPK activator, decreased the expression of miR-34a-5p and reversed the TNF-α-induced elevation of miR-34a-5p (Figure 7m), suggesting that miR-34a-5p was the downstream target of AMPK. These results indicate that AMPK mediates the protective effects of CTRP6 against TNF-α-induced apoptosis.

3.6 | SIRT1 is the key mediator in CTRP6-modulated cellular protection

Recently, the cross-talk between miR-34a and SIRT1 has been reported (Ding et al., 2015; Simão et al., 2019), and the activation of AMPK/SIRT1 signaling pathway plays an important role in energy metabolism and inflammation (Potenza et al., 2019). Thus, we explored the role of SIRT1 in the antiapoptotic effects of CTRP6. TNF-α decreased the levels of SIRT1 whereas pre-incubation with CTRP6 reversed the effects of TNF-α on SIRT1 expression. CTRP6 alone increased the expression of SIRT1 in both SMG–C6 cells and human SMG tissues (Figure 8a–d), suggesting that SIRT1 might be involved in the antiapoptotic effects of CTRP6. AraA downregulated the expression of SIRT1 in vitro and ex vivo, and simultaneously, it reversed the effects of CTRP6 against TNF-α-induced SIRT1 reduction (Figure 8e–h). Moreover, AICAR increased the expression of SIRT1 in SMG-C6 cells with or without TNF-α treatment (Figure 8i,j). To find out whether SIRT1 mediates the regulation of miR-34a-5p expression via AMPK as well as to further explore the importance of SIRT1 in the protective functions of CTRP6, EX527 (10 μM), a SIRT1 inhibitor, was used. EX527 reversed the effects of CTRP6 on TNF-α-induced changes in the expression levels of miR-34a-5p, cleaved caspase 3, caspase 8, caspase 9, and Bcl-2 (Figure 8k–p). These results demonstrate that the AMPK/SIRT1 signaling pathway is required for the inhibition of miR-34a-5p and the antiapoptotic effect of CTRP6 against TNF-α.

4 | DISCUSSION

In the present study, we demonstrated that the salivary gland is a novel site for CTRP6 synthesis and secretion. CTRP6 significantly inhibited TNF-α-induced apoptosis in both SMG-C6 cells and human SMG tissues. AMPK/SIRT1-modulated miR-34a-5p expression was responsible for the protective effects of CTRP6 against TNF-α-induced apoptosis. These results reveal a new antiapoptotic mechanism of CTRP6 in salivary glands.

Recently, the expression of certain adipokines in salivary glands has been reported. Ghrelin is expressed in the PG and SMG, and saliva and serum ghrelin levels increase with increasing body mass index (Li et al., 2011). Adiponectin and its receptor are expressed in salivary cells of humans and rodents, and they promote saliva secretion in SMGs (Ding et al., 2013; Mamali et al., 2012). Reduced adiponectin levels in the salivary gland contribute to glandular destruction during the progression of autoimmune sialadenitis by facilitating cytokine-induced apoptosis (Su et al., 2014). These results suggest that adipokines may be novel regulators of salivary gland functions. We demonstrated that CTRP6 was expressed in SMGs and PGs of humans, rats, and mice. CTRP6 protein was observed in all species, and it was predominantly distributed in the nuclei, apicolateral membranes, and cytoplasm of serous cells in human SMGs and PGs as well as in the ducts and apicolateral membranes of serous cells in rodents. The apicolateral membranes of acinar cells contain a large number of ion channels and transporters, which are active sites for secretory functions. Granular convoluted tubules, which contain numerous serous-type secretory granules that are repositories of a variety of bioactive substances and perform the endocrine functions of salivary glands, particularly exist in rodent salivary glands (Amano et al., 2012; Lomniczi et al., 2001). The different distribution patterns of CTRP6 in salivary glands among the species may be related to their histological structure and function, which modulates the metabolism, secretion, and absorption of salivary glands. In addition, we observed that the saliva CTRP6 levels were higher than the serum CTRP6 levels, which is similar to the trend of ghrelin (Li et al., 2011). This indicates that CTRP6 synthesized in salivary epithelia can be secreted with saliva. These results suggest that CTRP6 is a novel adipokine expressed and secreted in salivary glands.

Apoptosis is a process of programmed cell death that can directly lead to a loss of parenchymal cells and the consequent dysfunction (Lau et al., 2017; Nakamura et al., 2018). The inhibition of apoptosis may be a potential strategy for the treatment of many hypofunction-related diseases, such as Sjögren's syndrome, radioactive salivary gland injury, and SMG calculus (Choi et al., 2013; Lau et al., 2017; Nakamura et al., 2018). Several studies have reported

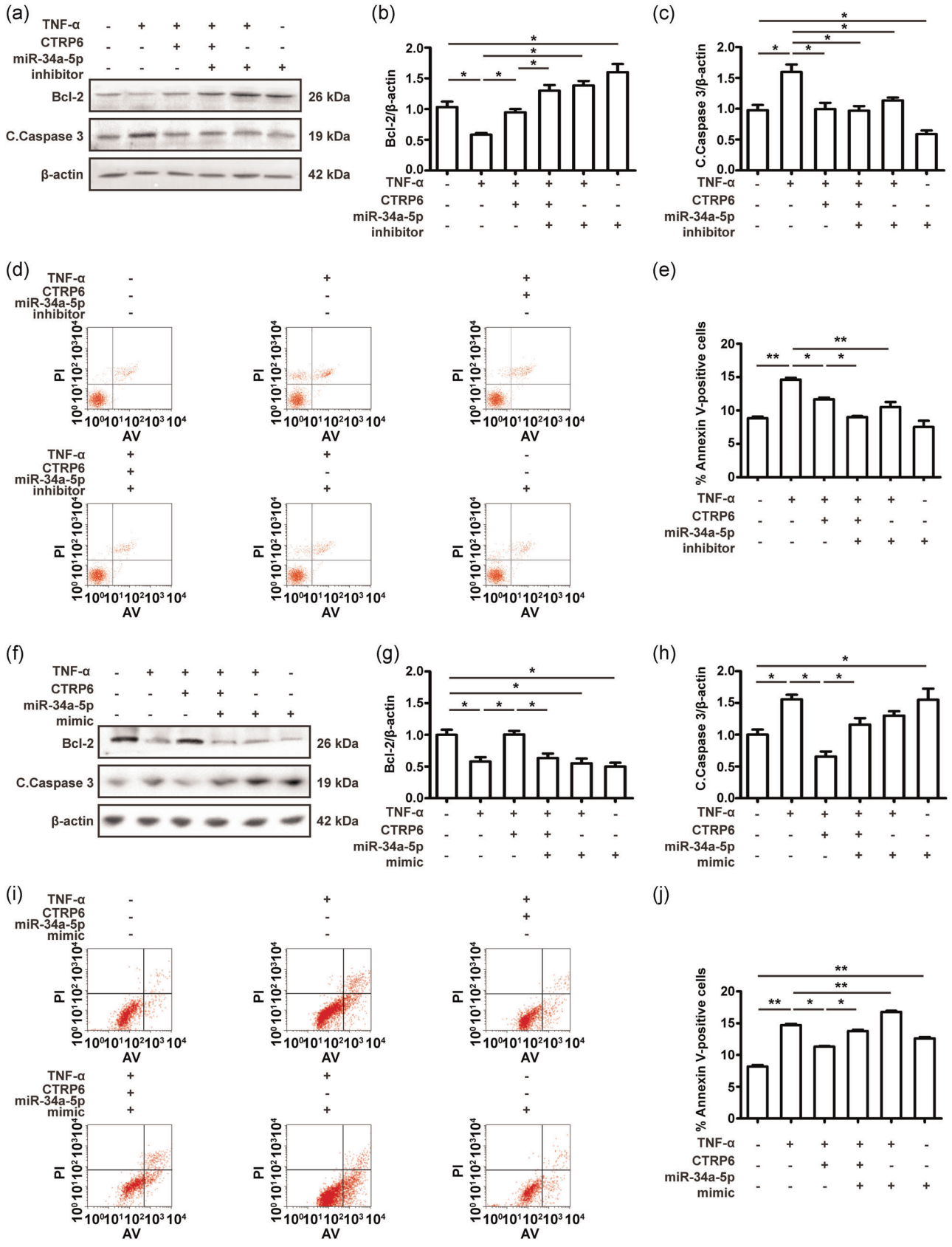

FIGURE 6 miR-34a-5p is involved in the inhibitory effects of CTRP6 against TNF-α-induced apoptosis. SMG-C6 cells were transfected with miR-34a-5p inhibitor (a–e) or mimic (f–j), and they were incubated with CTRP6 (2 μg/ml) and/or TNF-α (200 ng/ml) for 36 h. The levels of Bcl-2 and C. Caspase 3 were determined using western blot analysis, and they were quantitatively analyzed (a–c and f–h). The apoptotic rate was evaluated using annexin V-FITC/PI double staining, which was detected by flow cytometry (d), (e), (i), and (j). Data are expressed as mean ± *SEM* from 4 to 6 independent experiments. $^{*}p < .05$ and $^{**}p < .01$. CTRP, C1q/tumor necrosis factor-related protein; FITC, fluorescein isothiocyanate; miR, microRNA; PI, propidium iodide; SMG, submandibular gland; TNF-α, tumor necrosis factor-α

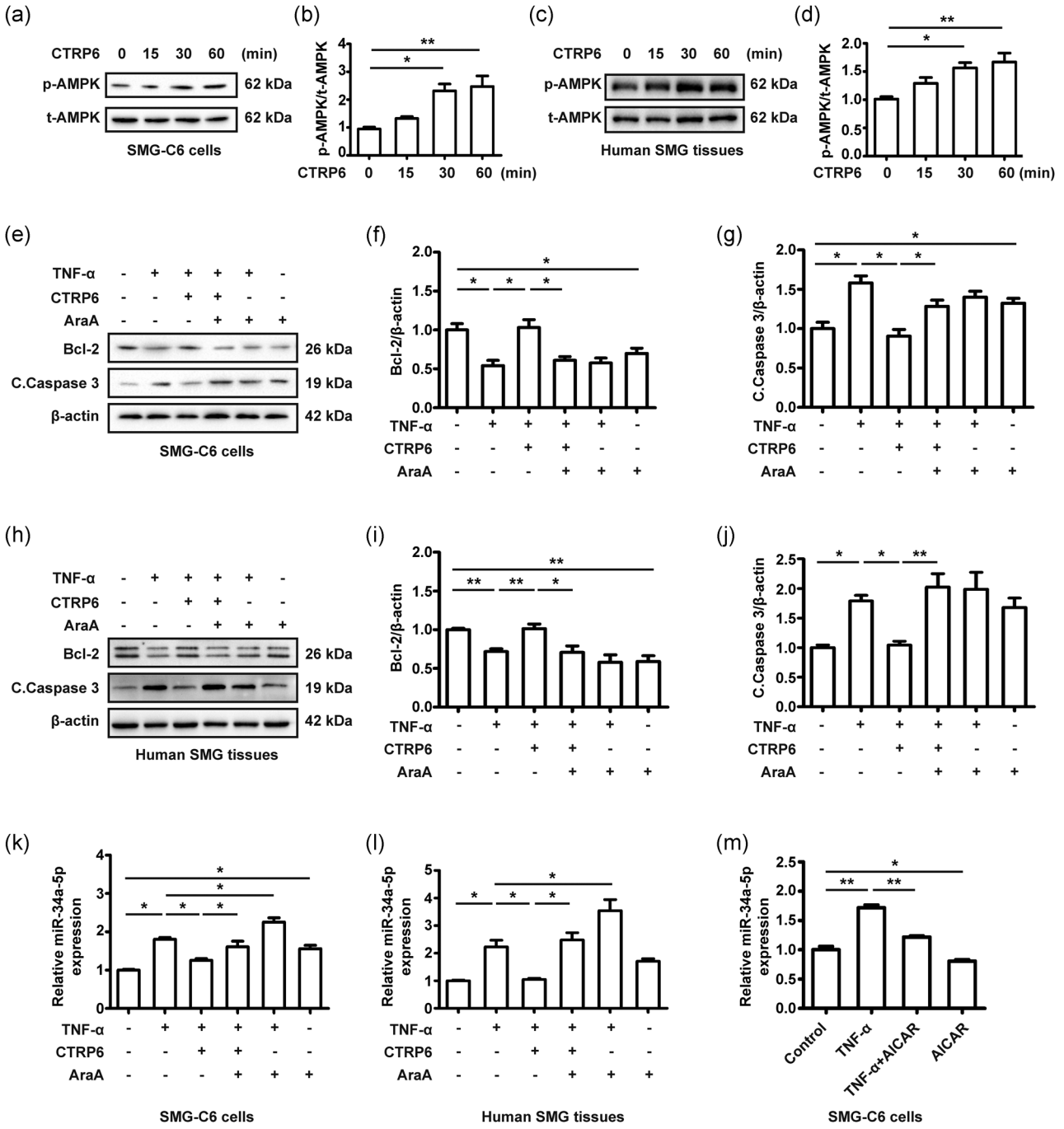

FIGURE 7 Activation of AMPK is required for the protective effects of CTRP6 against TNF-α-induced apoptosis. (a–d) SMG-C6 cells and human SMG tissues were incubated with CTRP6 (2 μg/ml) for the indicated time. AMPK phosphorylation (p-AMPK) was determined using western blot analysis. (e–j) SMG-C6 cells and human SMG tissues were pretreated with AraA (1 mM) for 30 min, incubated with CTRP6 (2 μg/ml) for another 30 min, and further incubated with TNF-α (200 ng/ml) for 36 h and 24 h, respectively. The levels of Bcl-2 and C. Caspase 3 were detected using western blot analysis. (k) and (l) SMG-C6 cells and human SMG tissues were pretreated with AraA (1 mM) for 30 min, incubated with CTRP6 (2 μg/ml) for another 30 min, and then incubated with TNF-α (200 ng/ml) for 24 h. The expression of miR-34a-5p was measured using real-time PCR. (m) SMG-C6 cells were treated with TNF-α (200 ng/ml, 24 h) with or without AICAR treatment (1 mM, 24 h). The expression of miR-34a-5p was measured using real-time PCR. Data are expressed as mean ± *SEM* from six independent experiments. $^{*}p < .05$ and $^{**}p < .01$. CTRP, C1q/tumor necrosis factor-related protein; miR, microRNA; PCR, polymerase chain reaction; SMG, submandibular gland; TNF-α, tumor necrosis factor-α

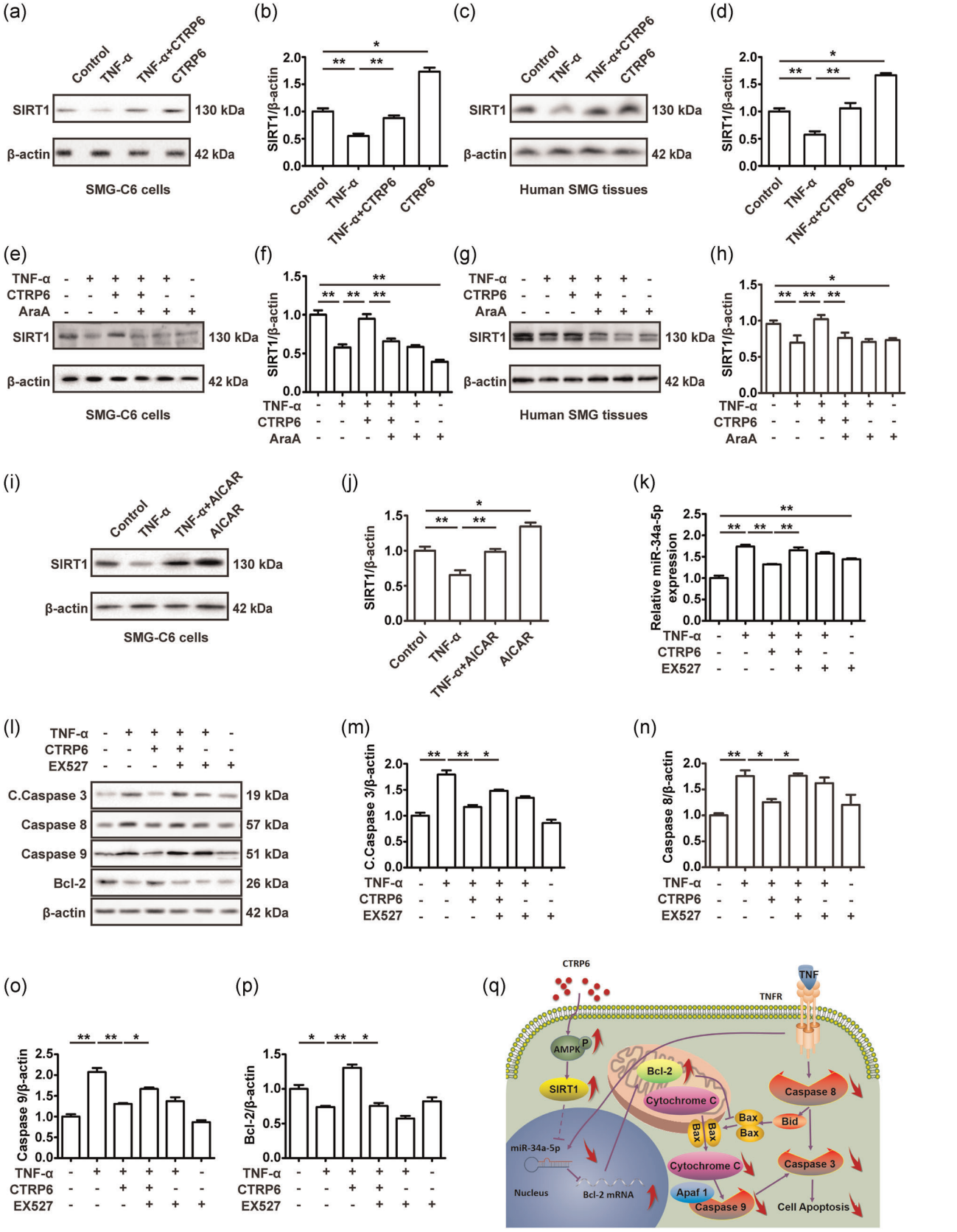

FIGURE 8 (See caption on next page)

the role of CTRP6 in cellular protection and functional preservation in multiple physiological and pathophysiological conditions. Overexpression of CTRP6 in vascular endothelial cells of spontaneously hypertensive rats significantly increases the activation of peroxisome proliferator-activated receptor-γ, and it inhibits vascular endothelial dysfunction and apoptosis (Chi et al., 2017). CTRP6 attenuates doxorubicin-induced cardiac atrophy, inhibits cardiac apoptosis, and improves cardiac functions in vivo and in vitro (Zheng et al., 2019). We observed that CTRP6 reduced the TNF-α-induced apoptosis of submandibular acinar cells, and it inhibited the changes in apoptotic molecules in vitro and ex vivo. These results suggest that CTRP6 is a novel endogenous protective adipokine against TNF-α-induced apoptosis in salivary acinar cells.

miRNAs are widely expressed noncoding RNAs that inhibit the translation or increase the degradation of target genes by binding to their 3′ noncoding regions. They participate in posttranscriptional regulation and play important roles in cellular proliferation, differentiation, and apoptosis (Saliminejad et al., 2019). As NOD mice demonstrated significant autoimmune sialoadenitis with obvious acinar damage at 10–12 weeks and TNF-α is a critical inducer of apoptosis during this process, we compared the miRNA profiles of SMGs of NOD and BALB/c mice. Due to the differences between human diseases and animal models, the reported differentially expressed miRNAs in the salivary glands of Sjögren's syndrome were also considered according to the literature. As Bcl-2 was involved in the inhibitory effects of CTRP6 against TNF-α-induced apoptosis, we focused on the miRNAs targeting Bcl-2. Among the selected miRNAs, miR-34a-5p is a highly conserved miRNA in mammals, and it is widely expressed in multiple tissues and organs. Several studies have revealed its role in promoting the acute lung injuries in murine models, hypoxia-induced cardiomyocyte injury, and apoptosis of HeLa cells and non-small cell lung cancer cells (Jiang et al., 2018; Shah et al., 2019; Shi et al., 2019; Wang, Xie, et al., 2018). It has been reported that the proapoptotic effect of miR-34a-5p is related to its effects on Bcl-2. It reduces Bcl-2 expression in human coronary artery endothelial cells, human cervical cancer cells, NEAT1-overexpressing ovarian cancer cells, rat cardiomyocyte H9c2 cells, and rat insulinoma INS-1 cells (Ding et al., 2017; Lu et al., 2016; Lv et al., 2019; Wang, Xie, et al., 2018; Zhu et al., 2017). In the present study, we demonstrated that miR-34a-5p levels were significantly elevated in the SMGs of NOD mice and TNF-α-treated SMG-C6 cells. The miR-34a-5p inhibitor enhanced whereas the miR-34a-5p mimic suppressed the effect of CTRP6 on TNF-α-induced changes in the apoptotic rate and expression levels of Bcl-2 and cleaved caspase 3, suggesting that miR-34a-5p was involved in the TNF-α-induced apoptosis of submandibular acinar cells. miR-34a-5p expression can be regulated by various molecules or environmental conditions such as p53 and hypoxia (Slabáková et al., 2017), however, few studies have reported the relationship between adipokines and miR-34a. Resistin decreases the ATP levels and impairs the mitochondrial functions of HepG2 cells by upregulating miR-34a (Wen et al., 2015). In this study, we found that CTRP6 inhibited TNF-α-induced miR-34a-5p upregulation whereas the miR-34a-5p mimic reduced the antiapoptotic effects of CTRP6. These results suggest that miR-34a-5p is a critical mediator of the inhibitory effects of CTRP6 against TNF-α-induced apoptosis.

AMPK is a cellular energy sensor that is conserved in all eukaryotic cells, and it regulates the activities of a number of key metabolic enzymes through phosphorylation (Kim & Tian, 2011; Soltani et al., 2019). Recent studies have demonstrated that CTRP6 activates the AMPK signaling pathway in skeletal muscle cells, hepatocellular carcinoma cells, and cardiac fibroblasts (Lee et al., 2010; Lei et al., 2015; Takeuchi et al., 2011). AMPK phosphorylation regulates the expression and functions of certain miRNAs such as miR-451 (Ogawa et al., 2019); however, the regulation of miR-34a-5p via AMPK has not been reported till date. In this study, we demonstrated that CTRP6 promoted the phosphorylation of AMPK in salivary acinar cells. The in vitro and ex vivo inhibition of AMPK activation reversed the effects of CTRP6 on TNF-α-induced miR-34a-5p expression and apoptosis. These results suggest that AMPK is the key signaling molecule that regulates miR-34a-5p expression and mediates the antiapoptotic effects of CTRP6.

Recently, several studies have reported the cross-talk between miR-34a and SIRT1. Silencing miR-34a leads to an increased expression of SIRT1, and it activates AMPK in steatosis liver cells and HFD C57BL/6 liver tissues (Ding et al., 2015). The inhibition of SIRT1 mediates the effect of miR-34a-5p on the apoptosis of doxorubicin-induced cardiomyocytes and Caco-2 cells, which are incubated under

FIGURE 8 SIRT1 mediates CTRP6-modulated cellular protection in submandibular cells. (a–d) SMG-C6 cells and human SMG tissues were treated with TNF-α (200 ng/ml) for 36 h and 24 h, respectively with or without CTRP6 (2 μg/ml) pretreatment for 30 min. The expression of SIRT1 was determined using western blot analysis, and it was quantified. (e–h) SMG-C6 cells and human SMG tissues were pretreated with AraA (1 mM) for 30 min, incubated with CTRP6 (2 μg/ml) for another 30 min, and then incubated with TNF-α (200 ng/ml) for 36 h and 24 h, respectively. The expression of SIRT1 was detected using western blot analysis. (i) and (j) SMG-C6 cells were treated with TNF-α (200 ng/ml, 36 h) in the presence or absence of AICAR (1 mM, 24 h). The SIRT1 levels were measured using western blot analysis. (k) SMG-C6 cells were pretreated with CTRP6 (2 μg/ml) for 30 min, and this was followed by TNF-α (200 ng/ml, 24 h) treatment with or without EX527 (10 μM, 24 h). The expression of miR-34a-5p was measured using real-time PCR. (l–p) SMG-C6 cells were pretreated with CTRP6 (2 μg/ml) for 30 min, and this was followed by TNF-α (200 ng/ml, 36 h) treatment with or without EX527 (10 μM, 24 h). The levels of apoptosis-related proteins were determined using western blot analysis. Data are expressed as mean ± *SEM* for 4–8 independent experiments. $^{*}p < .05$ and $^{**}p < .01$. (q) Schematic illustrations showing the possible mechanism of CTRP6 that protects salivary acinar cells against TNF-α-induced apoptosis via activating AMPK, upregulating SIRT1 level, inhibiting miR-34a-5p expression, and increasing Bcl-2 protein level. CTRP, C1q/tumor necrosis factor-related protein; miR, microRNA; PCR, polymerase chain reaction; SMG, submandibular gland; TNF-α, tumor necrosis factor-α

hypoxia/reoxygenation conditions (Wang et al., 2016; Zhu et al., 2017). Moreover, the activation of AMPK almost completely prevented miR-34a-induced cellular stress in C2C12 cells (Simão et al., 2019). In this study, we demonstrated that CTRP6 increased the expression of SIRT1 and reversed the inhibitory effect of TNF-α on SIRT1 expression in both rat SMG cells and human SMG tissues. Inhibition of AMPK and SIRT1 abolished the effects of CTRP6 on TNF-α-induced changes in miR-34a-5p and the expression of apoptosis-related molecules. These results indicate that SIRT1 is the downstream mediator of the AMPK pathway, which is activated by CTRP6, and it could negatively regulate the expression of miR-34a-5p. However, it is still unclear whether the regulation is due to a direct interaction between SIRT1 and miR-34a-5p or via some other mediators, such as the SIRT1/p53 negative feedback loop. These findings indicate a novel role of the AMPK/SIRT1/miR-34a-5p axis in the protective effects of CTRP6 against TNF-α-induced apoptosis.

In summary, we reported for the first time that CTRP6 is a novel salivary adipokine that ameliorates TNF-α-induced apoptosis. AMPK/SIRT1-modulated miR-34a-5p expression mediates the anti-apoptotic effects of CTRP6 against TNF-α (Figure 8q). Our findings provide new insights into the important roles of CTRP6 in salivary glands, reveal a novel mechanism of antiapoptotic effects mediated by CTRP6, and provide the experimental basis for new therapeutic strategies for salivary gland diseases.

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CONFLICT OF INTERESTS

The authors declare that there are no competing financial interests.

AUTHOR CONTRIBUTIONS

L.H Qu performed the experiments, analyzed the data, and drafted the manuscript. X. Hong designed and performed the experiments, interpreted the data, and drafted the manuscript. Y. Zhang designed and performed the experiments and revised the manuscript. X. Cong, R.L. Xiang, M. Mei, and J.Z. Su contributed to data analysis and revised the manuscript. L.L. Wu and G.Y. Yu designed the experiments, interpreted the data, and critically revised the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the study.

DATA AVAILABILITY STATEMENT

The data supporting the findings of our study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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