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ORIGINAL ARTICLE

E prostanoid receptor-3 promotes oxidized low-density lipoprotein-induced human aortic smooth muscle cells inflammation

Chuang-Jia Hu[1,2,3], Yan-Wei Wang[1], Wei-Xing Huang[4] and Yu-Bin Xia[5]*

[1]*Department of Cardiology, First Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong Province, China;* [2]*Laboratory of Molecular Cardiology, First Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong Province, China;* [3]*Laboratory of Medical Molecular Imaging, First Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong Province, China;* [4]*Department of Cardiac Surgery, First Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong Province, China; and* [5]*Department of Nephrology, First Affiliated Hospital of Shantou University Medical College, No. 57, Changping Rd, Shantou, 515000, Guangdong Province, China*

Abstract

Aim The progression of atherosclerosis can lead to the occurrence of multiple cardiovascular diseases (coronary heart disease, etc.). E prostanoid receptor-3 (EP3) is known to participate in the progression of atherosclerosis. This study aimed to investigate the mechanism by which EP3 modulates the development of atherosclerosis.

Methods and results ApoE$^{-/-}$ mice were used to construct *in vivo* model of atherosclerosis. Human aortic smooth muscle cells (HASMCs) were stimulated with oxidized low-density lipoprotein (ox-LDL) to construct *in vitro* model of atherosclerosis. mRNA expressions were assessed by qRT-PCR, and western blot was applied to assess the protein levels. CCK-8 assay was applied to assess the cell viability. The inflammatory cytokines levels were assessed by enzyme-linked immunosorbent assay, and flow cytometry was applied to assess cell apoptosis. *In vivo* experiment was constructed to investigate the impact of EP3 in atherosclerosis development. L-798106 (EP3 inhibitor) significantly inhibited the levels of pro-inflammatory cytokines in atherosclerosis *in vivo*. EP3 inhibitor (L-798106) significantly reversed ox-LDL-caused HASMCs injury via inhibiting the apoptosis and inflammatory responses ($P < 0.05$). The levels of interleukin-17 (IL-17) and intercellular adhesion molecule-1 (ICAM-1) in HASMCs were elevated by ox-LDL, whereas L-798106 or knockdown of cyclic AMP (cAMP) response element-binding protein (CREB) notably restored this phenomenon ($P < 0.05$). EP3 overexpression further aggravated ox-LDL-induced inflammation in HASMCs, and EP3 up-regulated the levels of IL-17 and ICAM-1 in ox-LDL-treated HASMCs ($P < 0.05$). EP3 up-regulation promoted the inflammatory responses in ox-LDL-treated HASMCs through mediation of cAMP/protein kinase A (PKA)/CREB/IL-17/ICAM-1 axis ($P < 0.05$).

Conclusions EP3 inhibitor alleviates ox-LDL-induced HASMC inflammation via mediation of cAMP/PKA/CREB/IL-17/ICAM-1 axis. Our study might shed new lights on discovering novel strategies against atherosclerosis.

Keywords Atherosclerosis; EP3; cAMP/PKA; CREB; IL-17; ICAM-1

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**Correspondence to: Dr Yu-Bin Xia, Department of Nephrology, First Affiliated Hospital of Shantou University Medical College, No. 57, Changping Rd, Shantou 515000, Guangdong Province, China. Email: xiayubin1979@163.com*

Introduction

Atherosclerosis is known to be a subtype of inflammatory diseases, and its progression can lead to the occurrence of vascular diseases (myocardial infarction, cardiovascular disease, etc.).[1] In addition, dysfunction of human umbilical vein endothelial cells (HUVECs) is one of the major causes of atherosclerosis progression.[2] It has been reported that migration, inhibited proliferation, and apoptosis of HUVECs are key cellular events causing the development and progression of atherosclerosis.[3] However, the function of human aortic smooth muscle cells (HASMCs) has been poorly studied; it is important to explore oxidized low-density lipoprotein (ox-LDL)-induced HASMCs changes in atherosclerosis treatment.

Prostaglandin E2 (PGE2) is implicated in pathological and physiological angiogenesis. PGE2 could exhibit its functions via four G protein-coupled receptors. Among these four members, E prostanoid receptor-3 (EP3) can suppress protein kinase A (PKA) activity through coupling to Gi.[4,5] In addition, the level of EP3 was down-regulated in sponge granulation tissues, and a EP3 receptor agonist restored the inhibited angiogenesis.[6] This function could contribute to the progression of cardiovascular disease.[7,8] Also, it has been confirmed that EP3 could be involved in the progression of atherosclerosis. For instance, EP3 down-regulation aggravated the effect of thromboxane-prostanoid receptor (TP) knockdown in alleviating endothelial dysfunction in atherosclerosis[9]; Yu *et al*. found that EP3 activation promoted endothelial cell apoptosis.[10] Nevertheless, the detailed mechanism by which EP3 modulates atherosclerosis development is unknown.

Cyclic AMP (cAMP)/PKA pathway is known to be involved in multiple cellular processes (cell proliferation, apoptosis, etc.).[11,12] In addition, cAMP/PKA signalling can play a vital role in multiple diseases (inflammation, cancer, etc.).[13,14] Meanwhile, activation of cAMP/PKA can lead to the development of atherosclerosis. For example, Fan *et al*. found that glucagon-like peptide 1 (GLP-1) rescued the myocardial hypertrophy in atherosclerosis by activation of cAMP/PKA signalling[15]; T-cell death-associated gene 8 could accelerate atherosclerosis by up-regulation of cAMP/PKA signalling.[16] Thus, we sought to investigate the relation between EP3 and cAMP/PKA in atherosclerosis.

The activated PKA catalytic subunit can enter the nucleus and regulate cell metabolism and gene expression by catalysing the phosphorylation of serine or threonine residues of proteins in the cell, the most famous of which is cAMP response element-binding protein (CREB).[17] In addition, CREB is known to be involved in multiple diseases including atherosclerosis.[18,19] However, the relation between PKA and CREB in atherosclerosis remains largely unknown.

Based on the above backgrounds, we hypothesize that EP3 receptor activation promoted the progression of atherosclerosis by mediation of cAMP/PKA/CREB signalling. Then, this work decided to investigate the function of EP3 in atherosclerosis. We hope this study would supply a new theoretical basis for exploring new strategies against atherosclerosis.

Materials and methods

Cell culture and treatment

HASMCs (ATCC) were maintained in RPMI-1640 medium with foetal bovine serum (FBS) (10%) and glutamine (2 mM, Sigma) in the condition of 37°C. To construct *in vitro* model of atherosclerosis, ox-LDL (10, 25, 50, 100, 150, or 200 μg/mL, Sigma) was applied to treat HASMCs for 48 h.[2,20]

Reagents

EP3 inhibitor (L-798106), PGE2 (EP3 activator), and H-89 (PKA inhibitor) were bought from Sigma. Cells were treated with H-89 (15 μM) for 4 h. In addition, HASMCs were treated with L-798106 (1 μM) for 6 h.[21] Besides, cells were treated with PGE2 (0.1 μg/mL) for 4 h.

Cell transfection

SiRNAs against CREB (si-CREB, 10 nM; Riobo) were applied to transfect HASMCs by Lipofectamine® 2000. For CREB overexpression, HASMCs were transfected with pcDNA3.1 or pcDNA3.1-CREB (CREB overexpression, Genepharma) by Lipofectamine 2000 for 48 h.

qRT-PCR

TRIZOL (Invitrogen) was applied to extract total RNAs from cells. PrimeScript RT (Takara) and SYBR (Takara) were applied in reverse transcription and real-time PCR assays. β-Actin was performed as a loading control. The primer sequences were listed in *Table 1*. $2^{-\Delta\Delta CT}$ method was applied for quantification.

CCK-8 assay

HASMCs (5×10^3 per well) were seeded overnight. Cells were stimulated with 100 μg/mL ox-LDL or ox-LDL + L-798106 for 48 h. CCK-8 (10 μL, Beyotime) was applied to treat cells for 2 h. Finally, the absorbance (450 nm) was assessed by a microplate reader.

Wound healing assay

HASMCs were plated into a 24-well Cell Culture Cluster and allowed to grow to 80–90% confluence. Then, cells were underlined perpendicular to the cell culture plate with a small pipette head. After washing with phosphate-buffered saline (PBS) three times, serum-free medium was used for further culture, and the scratch widths at 0 and 24 h were recorded under an optical microscope.

Table 1 The sequences for primers

Gene	Sequence of primer
CREB	Forward: 5′-GGTAAACCGGGCTCGTGTAC-3′ Reverse: 5′-ATTCAGCCAGAGTTCTCCGC-3′
β-Actin	Forward: 5′-GTCCACCGCAAATGCTTCTA-3′ Reverse: 5′-TGCTGTCACCTTCACCGTTC-3′

CREB, cyclic AMP response element-binding protein.

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Enzyme-linked immunosorbent assay (ELISA)

Interleukin-17 (IL-17) (ab100556/ab100702), tumour necrosis factor-α (TNF-α) (ab181421/ab208348), IL-8 (ab214030/ab256609), IL-1β (ab178013/ab197742), and IL-6 (ab178013/ab222503) levels in serum of mice or HASMC supernatants were assessed by ELISA kit (Abcam).

Western blotting

Radioimmunoprecipitation assay (RIPA) was applied to extract total protein from tissues or cell lysates, and bicinchoninic acid (BCA) (Beyotime) was applied to quantify the protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%) was applied to resolve proteins, and then proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. Primary antibodies were applied to incubate membranes after membranes were blocked with skim milk (5%) for 1 h. Afterwards, secondary anti-rabbit antibody (1:5000) was applied to incubate the membranes for 1 h. Odyssey Imaging System was applied to scan the membranes, and membranes were then analysed with Odyssey v2.0 software. The primary antibodies used in this study were as follows: anti-IL-1β (1:1000), anti-TNF-α (1:1000), anti-IL-6 (1:1000), anti-IL-8 (1:1000), anti-IL-17 (1:1000), anti-intercellular adhesion molecule-1 (ICAM-1, 1:1000), anti-Bax (1:1000), anti-cleaved caspase 3 (1:1000), anti-total caspase 3 (1:1000), anti-Bcl-2 (1:1000), anti-Bim (1:1000), anti-Bad (1:1000), anti-CREB (1:1000), anti-p-CREB (1:1000), anti-PKA (1:1000), anti-p-PKA (1:1000), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000). GAPDH was applied as a loading control. All antibodies originated from Abcam.

cAMP detection

cAMP level in tissues or HASMCs was assessed by cAMP kit (Beyotime). The procedure was in line with the instruction of manufacturer.

Cell apoptosis analysis

HASMCs were resuspended after trypsinized and washed. Then, Annexin V (5 μL, BD bioscience) and propidium (PI; 5 μL, BD bioscience) were applied to stain cells for 15 min. Flow cytometry (BD) was applied to analyse the cells.

In vivo experiment

ApoE$^{-/-}$ mice (n = 40, aged 7 weeks old, male, 20 ± 2 g, Vital River) were placed in a room with dedicated specific pathogen free (SPF) facility. Meanwhile, mice were classified into control, high-fat diet (HFD), and HFD + L-798106, HFD + H-89. Normal diet (21% kcal from protein, 68.5% kcal from carbohydrate, and 10.5% kcal) was applied to fed control mice. HFD (59% basic mice feed, 20% sugar, 18% lard, and 3% egg yolk) was applied to fed mice in other groups for 10 weeks. In addition, mice in HFD + L-798106 and HFD + H-89 group were injected with L-798106 (10 μg/day per mouse) and H-89 (2 mg/kg, every 2 days) via the tail vein for 10 days, respectively.[22] Meanwhile, mice in other two groups were administrated with the same dosages of saline for 10 days. Finally, mice were sacrificed for collection of serum and arterial tissues. All the experiments were performed in line with the National Institutes of Health (NIH) guide. Moreover, the Ethics Committees of The Medical Animal Care and Welfare of Shantou University Medical College approved the protocol of this study (No. SUMC2021-200).

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining

Tissues were washed and permeabilized, and then TUNEL reaction mixture (50 μL) was used to incubate the samples in the dark for 60 min. Then, peroxidase (POD; 50 μL) was applied to incubate the slides for 30 min, and then the slides were incubated with diaminobenzidine (DAB; 50 μL) for 10 min. Finally, the tissues were captured.

Statistical analysis

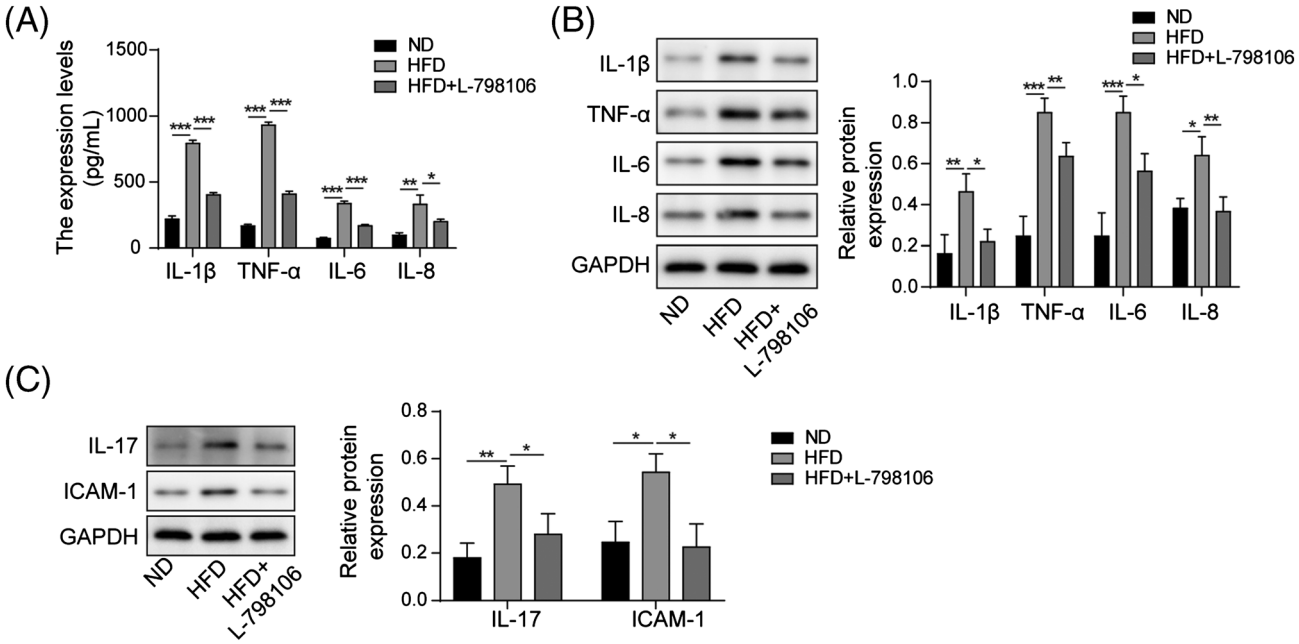
Mean ± SD was applied to express data. Student's t-test (only two groups) or one-way analysis of variance (ANOVA) followed by Tukey's test (more than two groups, GraphPad Prism 7) was used to analyse the differences. In addition, one-way ANOVA followed by Tukey's test (more than two groups, GraphPad Prism 7) was used in animal study. Student's t-test (only two groups) or one-way ANOVA followed by Tukey's test (more than two groups, GraphPad Prism 7) was used in *in vitro* analysis. The data were in accordance with the normal distribution. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

EP3 inhibition alleviated the secretion of IL-6, TNF-α, IL-8, IL-1β, ICAM-1, and IL-17

To investigate the function of EP3 in atherosclerosis, we constructed *in vivo* model of atherosclerosis. As shown in *Figure 1A*, IL-6, TNF-α, IL-8, and IL-1β levels in serum of mice were elevated by HFD, which were abolished by EP3 inhibitor. Fur-

Figure 1 E prostanoid receptor-3 (EP3) inhibition alleviated the progression of atherosclerosis. *In vivo* model of atherosclerosis was established. Mice were divided in three groups [normal diet (ND), high-fat diet (HFD), and HFD + L-798106]. (A) Interleukin-8 (IL-8), tumour necrosis factor-α (TNF-α), IL-1β, and IL-6 levels in serum of mice were assessed by enzyme-linked immunosorbent assay (ELISA). (B) IL-6, TNF-α, IL-8, and IL-1β levels in tissues of mice were assessed by western blot. (C) Intercellular adhesion molecule-1 (ICAM-1) and IL-17 levels in tissues of mice were assessed by western blot. $N = 10$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.



thermore, HFD-induced increase of IL-6, TNF-α, IL-8, IL-1β, ICAM-1, and IL-17 levels in mice was abolished by L-798106 (*Figure 1B,C*). Altogether, EP3 inhibition alleviated the secretion of IL-6, TNF-α, IL-8, IL-1β, ICAM-1, and IL-17.

EP3 down-regulation alleviated ox-LDL-induced cell injury

In order to mimic atherosclerosis *in vitro*, HASMCs were treated with ox-LDL. Based on the above result, 100 μg/mL ox-LDL was selected of use in subsequent experiments. Next, to investigate the inflammatory responses in HASMCs, ELISA was performed. As illustrated in *Figure 2A*, ox-LDL significantly up-regulated the levels of IL-6, TNF-α, IL-8, and IL-1β in HASMC supernatants, whereas L-798106 markedly reversed this phenomenon. Consistently, IL-1β, TNF-α, IL-8, and IL-6 levels in HASMCs were obviously increased by ox-LDL, which were rescued by EP3 inhibitor (*Figure 2B*). ox-LDL-caused inhibition of cell viability was significantly restored by L-798106 (*Figure 2C*), and L-798106 markedly abolished ox-LDL-caused HASMC apoptosis (*Figure 2D*). Meanwhile, ox-LDL significantly elevated cleaved-caspase 3, Bax, IL-17, ICAM-1, Bad, and Bim levels and decreased Bcl-2 expression in HASMCs, whereas the impact of ox-LDL was greatly rescued by L-798106 (*Figure 2E,F*). Taken together, EP3 down-regulation alleviated ox-LDL-caused HASMCs injury.

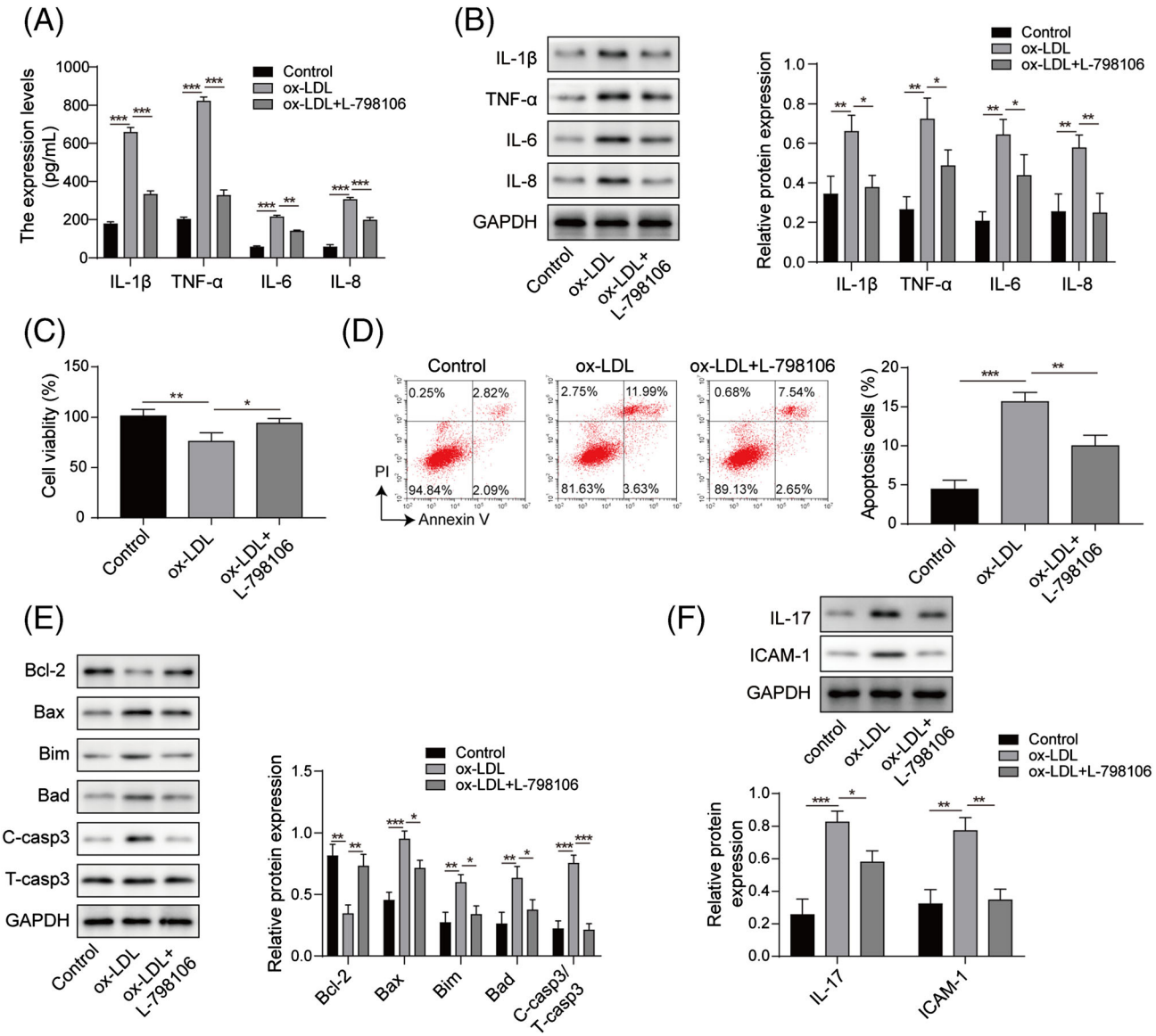
EP3 activation aggravated ox-LDL-induced up-regulation of IL-17 and ICAM-1

To further assess the impact of EP3 in ox-LDL-induced cell injury, ELISA and western blot were used. In *Figure 3A,B*, ox-LDL-induced up-regulation of TNF-α, IL-1β, IL-17, IL-8, and IL-6 levels was further aggravated by EP3 activator (PGE2). In addition, PGE2 further aggravated ox-LDL-caused decrease of HASMC viability by inducing the apoptosis (*Figure 3C,D*). Meanwhile, cleaved caspase 3, Bax, IL-17, ICAM-1, Bad, and Bim levels in HASMCs were significantly increased by ox-LDL, which were further increased in the presence of PGE2 (*Figure 3E,F*). In contrast, ox-LDL markedly suppressed Bcl-2 level in HASMCs, and PGE2 further enhanced the effect of ox-LDL (*Figure 3E*). In summary, EP3 activation aggravated ox-LDL-caused activation of IL-17 and ICAM-1.

Up-regulation of CREB promoted IL-17 and ICAM-1 levels

For the purpose of investigating the function of CREB in ox-LDL-caused cell injury, CREB siRNA was applied to

Figure 2 E prostanoid receptor-3 (EP3) down-regulation alleviated oxidized low-density lipoprotein (ox-LDL)-caused cell injury. Human aortic smooth muscle cells (HASMCs) were stimulated with ox-LDL or ox-LDL + L-798106. (A) Interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), IL-8, and IL-1β levels in HASMC supernatants were assessed by enzyme-linked immunosorbent assay (ELISA). (B) IL-6, TNF-α, IL-8, and IL-1β levels in HASMCs were assessed by western blot. (C) The viability of HASMCs was assessed by CCK-8 assay. (D) Cell apoptosis was assessed by flow cytometry. (E) Bcl-2, Bim, Bax, Bad, total-caspase 3, and cleaved-caspase 3 levels in HASMCs were tested by western blot. (F) Intercellular adhesion molecule-1 (ICAM-1) and IL-17 levels in HASMCs were tested by western blot. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.



transfect HASMCs. As shown in *Figure 4A,B*, CREB mRNA level in HASMCs was elevated by ox-LDL, which was reversed by CREB siRNA. In addition, ox-LDL-induced increase of pro-inflammatory agents (TNF-α, IL-1β, IL-6, and IL-8) was partially abolished in the presence of CREB knockdown (*Figure 4C,D*). Moreover, knockdown of CREB reversed ox-LDL-caused HASMC apoptosis via mediation of Bax, Bcl-2, Bad, Bim, and cleaved-caspase 3 (*Figure 4E,F*). Meanwhile, the impact of ox-LDL on IL-17 and ICAM-1 levels was reduced by CREB silencing (*Figure 4G*). To sum up, up-regulation of CREB promoted IL-17 and ICAM-1 levels.

EP3 activation aggravated the inflammation and migration in ox-LDL-caused HASMCs via regulation of cAMP/PKA/CREB axis

To investigate the correlation between EP3 and PKA in ox-LDL-induced HASMC injury, H-89 (PKA inhibitor) was applied.

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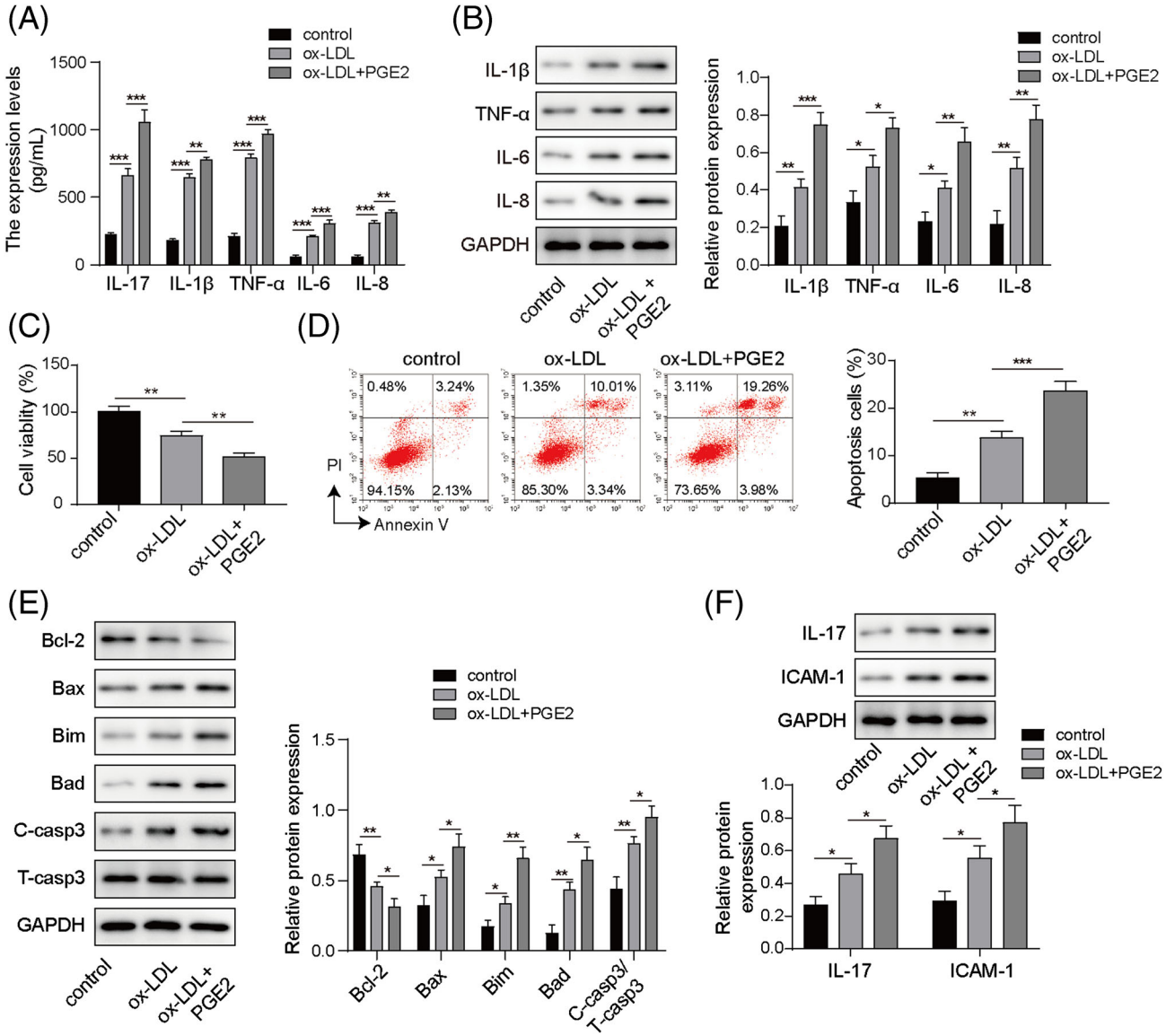
Figure 3 E prostanoid receptor-3 (EP3) activation aggravated oxidized low-density lipoprotein (ox-LDL)-caused elevation of intercellular adhesion molecule-1 (ICAM-1) and interleukin-17 (IL-17). Human aortic smooth muscle cells (HASMCs) were stimulated with ox-LDL + prostaglandin E2 (PGE2) or ox-LDL. (A) IL-17, IL-6, tumour necrosis factor-α (TNF-α), IL-8, and IL-1β levels in HASMC supernatants were investigated by enzyme-linked immunosorbent assay (ELISA). (B) TNF-α, IL-6, IL-1β, and IL-8 levels in HASMCs were assessed by western blot. (C) HASMC viability was assessed by CCK-8 assay. (D) The apoptosis of HASMCs was assessed by flow cytometry. (E) The protein levels of Bcl-2, Bim, Bax, Bad, total caspase 3, and cleaved caspase 3 were tested by western blot. (F) ICAM-1 and IL-17 levels were tested by western blot. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

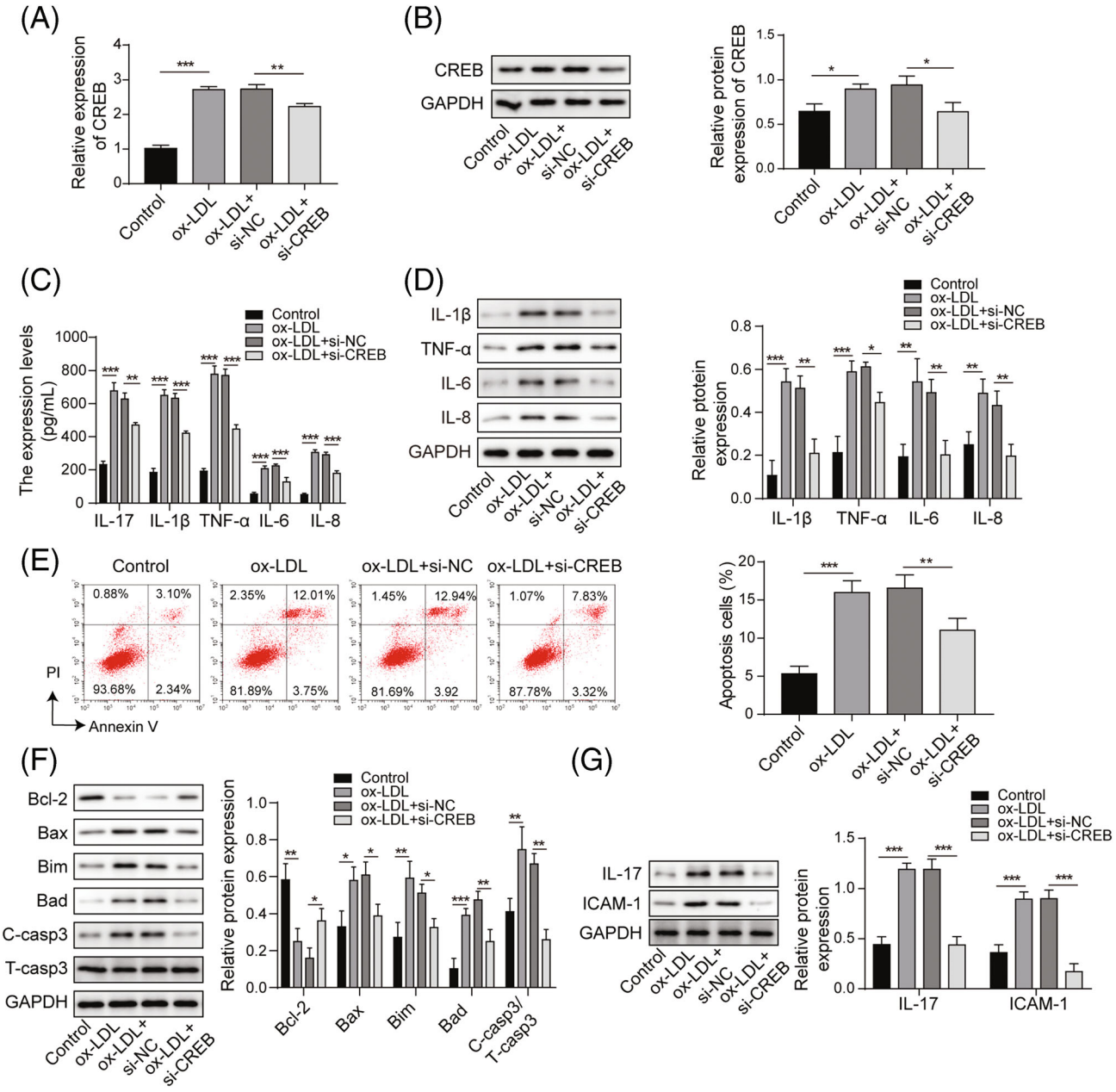


As demonstrated in *Figure 5A*, the protein levels of p-PKA and p-CREB in HASMCs were elevated by ox-LDL, whereas the impact was abolished by L-798106 or H-89. Meanwhile, the level of cAMP in HASMCs was obviously up-regulated by ox-LDL, whereas it was abolished by L-798106 (*Figure 5B*). However, H-89 did not affect the impact of ox-LDL on cAMP level (*Figure 5B*). Moreover, ox-LDL-induced up-regulation of IL-8, TNF-α, IL-1β, IL-17, IL-6, and ICAM-1 proteins was markedly restored in the presence of L-798106 or H-89 (*Figure 5C–E*). In summary, EP3 activation induced the inflammation in ox-LDL-treated HASMCs via regulation of cAMP/PKA/CREB axis.

EP3 activation up-regulated ICAM-1 and IL-17 levels via mediation of cAMP/PKA/CREB axis in HASMCs after ox-LDL treatment

To further assess the relation between EP3 and cAMP/PKA/CREB axis in ox-LDL-stimulated HASMCs, the following exper-

Figure 4 Up-regulation of cyclic AMP (cAMP) response element-binding protein (CREB) promoted interleukin-17 (IL-17) and intercellular adhesion molecule-1 (ICAM-1) levels. Human aortic smooth muscle cells (HASMCs) were stimulated with oxidized low-density lipoprotein (ox-LDL) + NC siRNA, ox-LDL, or ox-LDL + CREB siRNA. (A) The expression of CREB mRNA in HASMCs was assessed by qRT-PCR. (B) CREB level in HASMCs was detected by western blot. (C) The levels of IL-17, tumour necrosis factor-α (TNF-α), IL-6, IL-1β, and IL-8 in HASMC supernatants were investigated by enzyme-linked immunosorbent assay (ELISA). (D) The protein levels of TNF-α, IL-6, IL-1β, and IL-8 in HASMCs were assessed by western blot. (E) HASMC apoptosis was assessed by flow cytometry. (F) Bcl-2, Bim, Bax, total-caspase 3, Bad, and cleaved-caspase 3 levels in HASMCs were tested by western blot. (G) IL-17 and ICAM-1 levels in HASMCs were tested by western blot. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.



iments were performed. The data indicated that PGE2 further promoted ox-LDL-induced up-regulation of cAMP, whereas H-89 or H-89 plus CREB did not affect this phenomenon (*Figure 6A*). In addition, PGE2 further enhanced ox-LDL-induced up-regulation of CREB level, whereas H-89 markedly reversed the effect of PGE2 (*Figure 6B*). Meanwhile, CREB overexpression partially restored the effect of H-89 on CREB expression in HASMCs co-treated with ox-LDL, PGE2, and H-89 (*Figure 6B*). On the other hand, the expressions of p-CREB, p-PKA, and CREB in HASMCs co-treated with ox-LDL and PGE2 were significantly decreased by H-89 (*Figure 6C*). Nevertheless, the impact of H-89 on p-CREB and CREB levels was restored

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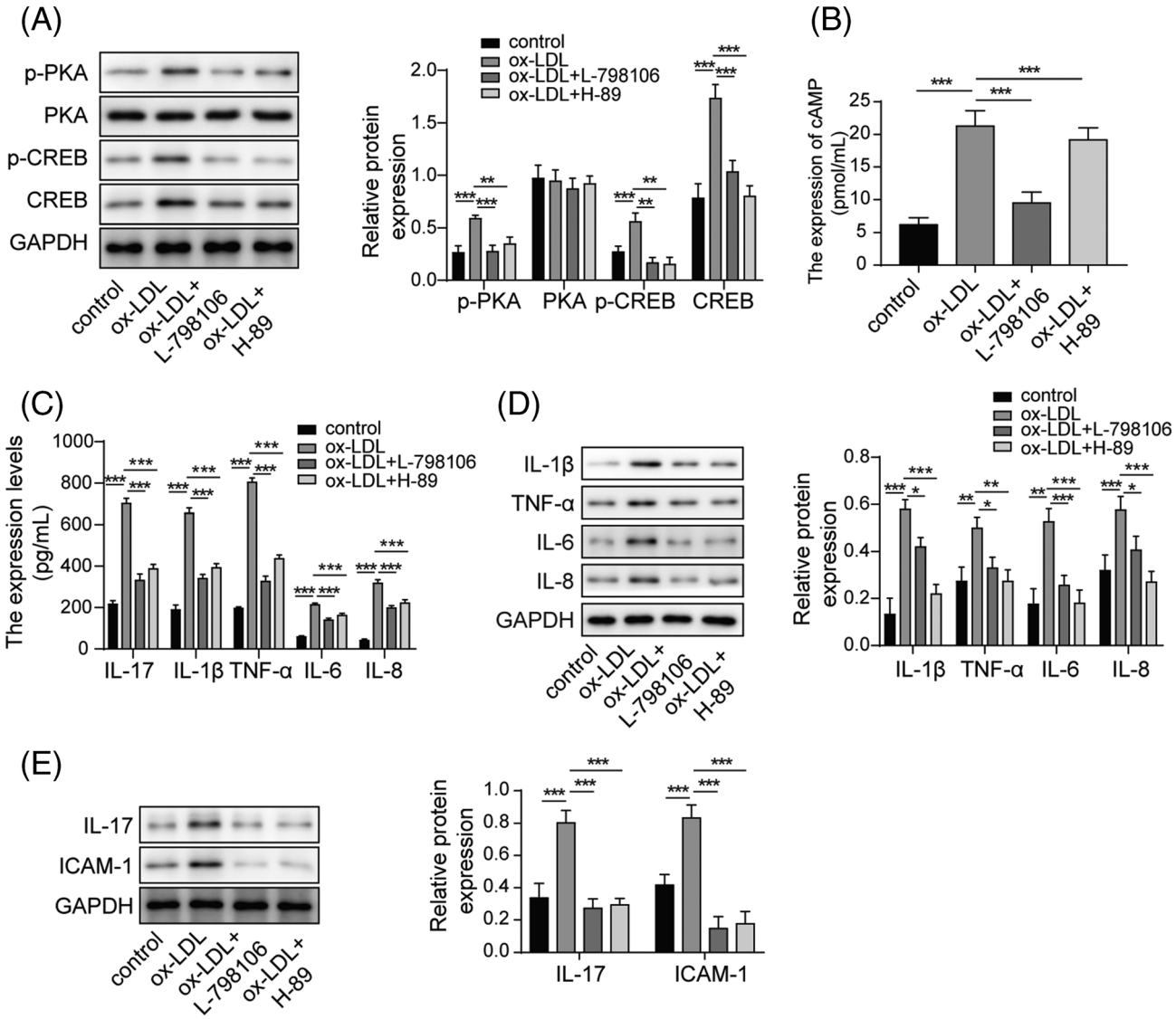
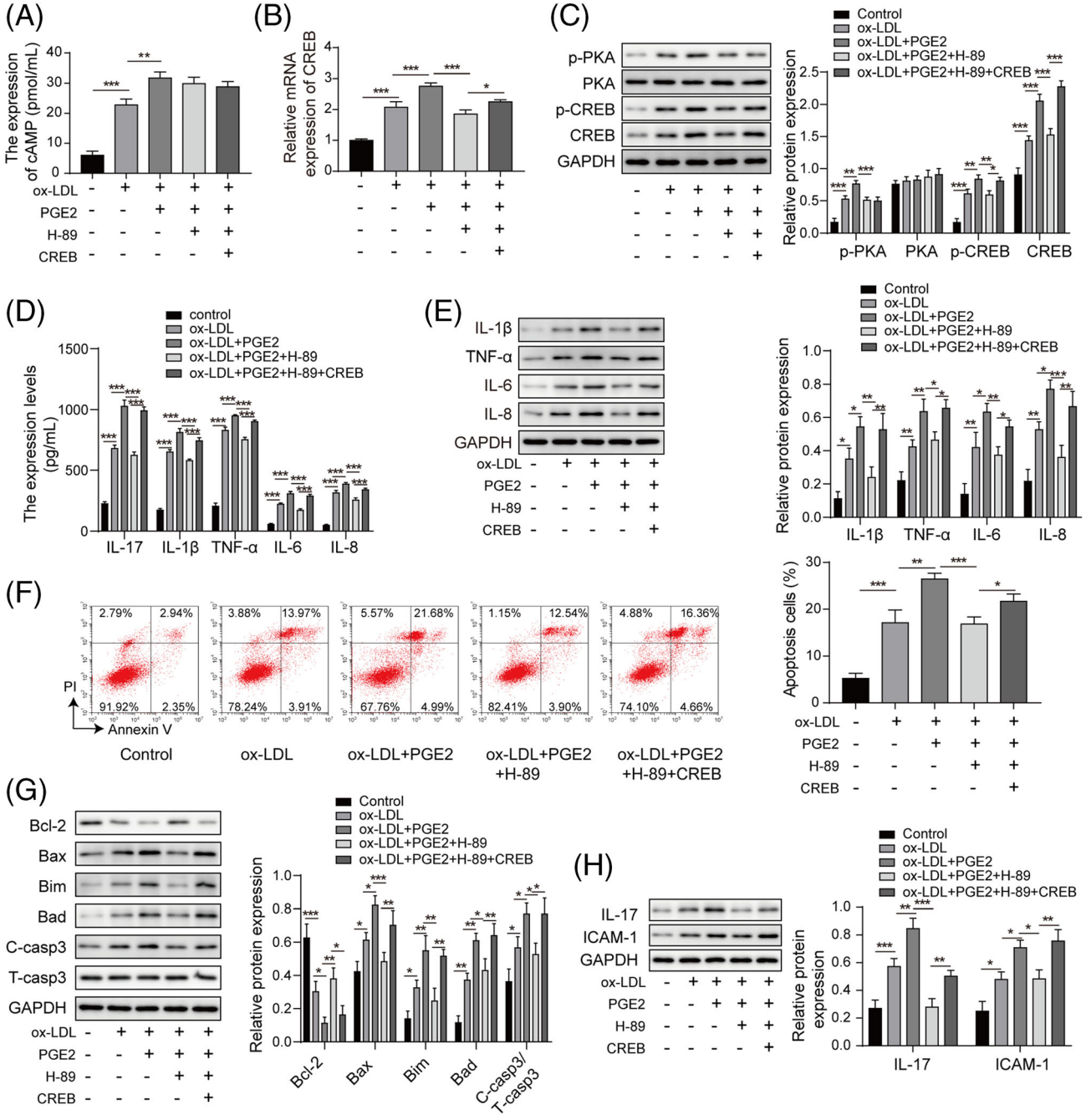
Figure 5 E prostanoid receptor-3 (EP3) activation induced the inflammation in oxidized low-density lipoprotein (ox-LDL)-treated human aortic smooth muscle cells (HASMCs) via regulation of cyclic AMP (cAMP)/protein kinase A (PKA)/cAMP response element-binding protein (CREB) axis. HASMCs were stimulated with ox-LDL, ox-LDL + L-798106, or ox-LDL + H-89. (A) p-PKA, PKA, CREB, and p-CREB levels in HASMCs were assessed by western blot. (B) cAMP level in HASMCs was tested by cAMP detection kit. (C) Tumour necrosis factor-α (TNF-α), interleukin-17 (IL-17), IL-1β, IL-8, and IL-6 levels in HASMC supernatants were investigated by enzyme-linked immunosorbent assay (ELISA). (D) The protein levels of TNF-α, IL-6, IL-1β, and IL-8 in HASMCs were assessed by western blot. (E) The protein levels of IL-17 and intercellular adhesion molecule-1 (ICAM-1) in HASMCs were tested by western blot. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.



by pcDNA3.1-CREB, and CREB overexpression had very limited effect on p-PKA expression (*Figure 6C*). Moreover, the pro-inflammatory impact of PGE2 on ox-LDL-treated HASMCs was significantly inhibited by H-89, whereas CREB overexpression partially restored this phenomenon (*Figure 6D,E*). Consistently, the anti-apoptotic effect of H-89 on HASMCs co-treated with ox-LDL and PGE2 was abolished by pcDNA3.1-CREB (*Figure 6F*). Mechanistically, pcDNA3.1-CREB reversed this phenomenon via regulation of apoptosis-related proteins (Bax, Bad, Bim, cleaved-caspase 3, and Bcl-2) (*Figure 6G*). In consistent, H-89 greatly decreased ICAM-1 and IL-17 levels in HASMCs co-treated with ox-LDL and PGE2, whereas it was abolished by CREB overexpression (*Figure 6H*). Thus, EP3 activation up-regulated ICAM-1 and IL-17 levels via mediation of cAMP/PKA/CREB.

Figure 6 E prostanoid receptor-3 (EP3) activation up-regulated interleukin-17 (IL-17) and intercellular adhesion molecule-1 (ICAM-1) levels via mediation of cyclic AMP (cAMP)/protein kinase A (PKA)/cAMP response element-binding protein (CREB) axis in oxidized low-density lipoprotein (ox-LDL)-stimulated human aortic smooth muscle cells (HASMCs). HASMCs were stimulated with ox-LDL, ox-LDL + prostaglandin E2 (PGE2), ox-LDL + PGE2 + H-89, or ox-LDL + PGE2 + H-89 + pcDNA3.1-CREB. (A) The level of cAMP in HASMCs was tested by cAMP detection kit. (B) CREB level in HASMCs was detected by qRT-PCR. (C) p-PKA, PKA, p-CREB, and CREB levels in HASMCs were assessed by western blot. (D) IL-17, tumour necrosis factor-α (TNF-α), IL-6, IL-1β, and IL-8 levels in HASMC supernatants were assessed by enzyme-linked immunosorbent assay (ELISA). (E) IL-6, TNF-α, IL-8, and IL-1β levels in HASMCs were assessed by western blot. (F) HASMC apoptosis was assessed by flow cytometry. (G) Bcl-2, Bim, Bax, total-caspase 3, Bad, and cleaved-caspase 3 levels in HASMCs were assessed by western blot. (H) IL-17 and ICAM-1 levels in HASMCs were assessed by western blot. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.



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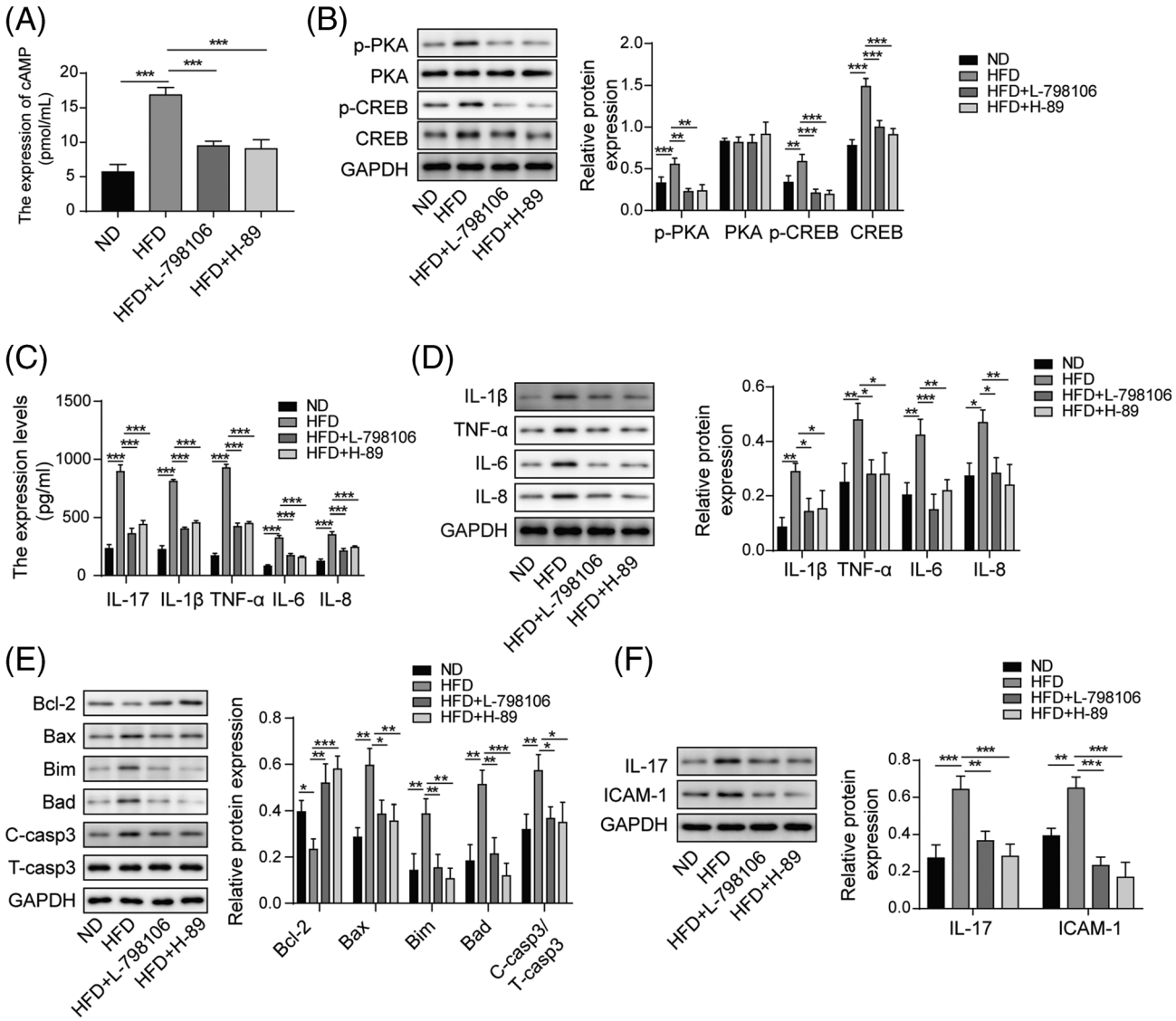
EP3 inactivation attenuated atherosclerosis development via modulation of cAMP/PKA/CREB axis

To further confirm the mechanism by which EP3 modulates atherosclerosis progression, the following analysis was performed. L-798106 or H-89 notably reversed HFD-induced up-regulation of cAMP level (*Figure 7A*), and p-PKA, CREB, and p-CREB levels in HFD-treated mice were also decreased by L-798106 or H-89 (*Figure 7B*). Meanwhile, the pro-inflammatory effect of HFD in mice was significantly alleviated by L-798106 or H-89 (*Figure 7C,D*). In addition, the proteins expressions of Bad, Bim, cleaved-caspase 3, Bax, IL-17, and ICAM-1 in mice were markedly increased by HFD, which were reversed in the presence of L-798106 or H-89 (*Figure 7E,F*). In contrast, HFD-induced inhibition of Bcl-2 level was

Figure 7 E prostanoid receptor-3 (EP3) inactivation attenuated the progression of atherosclerosis *in vivo* via mediation of cyclic AMP (cAMP)/protein kinase A (PKA)/cAMP response element-binding protein (CREB) axis. (A) cAMP level in mice was tested by cAMP detection kit. (B) p-PKA, PKA, CREB, and p-CREB levels in mice were assessed by western blot. (C) Interleukin-17 (IL-17), tumour necrosis factor-α (TNF-α), IL-6, IL-1β, and IL-8 levels in serum of mice were investigated by enzyme-linked immunosorbent assay (ELISA). (D) The protein levels of TNF-α, IL-6, IL-1β, and IL-8 in tissues of mice were assessed by western blot. (E) Bcl-2, Bim, Bax, total-caspase 3, Bad, and cleaved-caspase 3 levels in mice were assessed by western blot. (F) IL-17 and intercellular adhesion molecule-1 (ICAM-1) levels in mice were assessed by western blot. $N = 10$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.



restored by L-798106 or H-89 (*Figure 7E*). In summary, EP3 inactivation attenuated atherosclerosis development via cAMP/PKA/CREB axis.

Discussion

Atherosclerosis is a major vascular disease formed by lipid and fibre accumulation within the artery wall.[23] Atherosclerotic cardiovascular diseases, including stroke and ischaemic heart attack, continue to have a high incidence and affect the health of a large number of patients worldwide.[24] The oxidation of low-density lipoproteins (LDLs) has been shown to promote the formation of atherosclerotic lesions.[25,26] Although the therapeutic effect of antioxidants is still under debate,[27] removal of ox-LDL has been shown to prevent the progression of atherosclerosis. On the other hand, HFD could induce the production of ox-LDL, thereafter promoting the accumulation of arterial plaque, leading to the progression of atherosclerosis.[28] In this study, we found that EP3 inhibitor alleviated ox-LDL-induced HASMC inflammation and HFD-induced lipid accumulation via mediation of cAMP/PKA/CREB/IL-17/ICAM-1 axis. In addition, it could attenuate the progression of atherosclerosis *in vivo*. It has been demonstrated that EP3 promoted the severity of atherosclerosis. For instance, EP3 inhibition enhanced the impact of TP deficiency in alleviation of endothelial dysfunction in atherosclerosis[9]; EP3 activated ACE/TLR4/PTGS2 signalling in patients with atherosclerosis.[29] Our research firstly explored the correlation between EP3 and cAMP/PKA/CREB/IL-17/ICAM-1 axis in ox-LDL-treated HASMCs, further suggesting EP3 as a promoter in atherosclerosis.

cAMP/PKA signalling is known to be involved in cellular process. For example, activation of cAMP/PKA signalling promoted luteinizing hormone (LH)-induced luteolysis in pregnant rats[30]; Xie *et al*. found that GLP-1 improved the neuronal ability in astrocytes by cAMP/PKA.[31] Meanwhile, cAMP/PKA signalling was able to be involved in the progression of atherosclerosis.[32,33] However, the relation between EP3 and cAMP/PKA signalling in atherosclerosis development remains largely unknown. Based on the above backgrounds, we aimed to confirm this relation. Our finding revealed that EP3 activation up-regulated cAMP/PKA signalling in HASMCs. Thus, our study was in line with the previous data, indicating that EP3 acted as a promoter in cAMP/PKA signalling.

Vascular inflammatory responses contribute to the pathogenesis of atherosclerosis,[34] and endothelial cells play an important role in vascular inflammation.[35] Pro-inflammatory stimuli such as ox-LDL activated the endothelial inflammatory response, resulting in the secretion of pro-inflammatory cytokines that contribute to the pathogenesis of cardiovascular disease.[36] Endothelial inflammatory responses are characterized by the overproduction of inflammatory mediators, including cytokines (e.g. IL-17), and adhesion molecules (e.g. ICAM-1).[37] In this study, ox-LDL significantly up-regulated the expression of IL-17 and ICAM-1; however, this pro-inflammatory effect of ox-LDL was significantly inhibited by EP3 down-regulation.

CREB is known to be a mediator in multiple diseases (cancer, inflammation, etc.).[38,39] In addition, its up-regulation promoted the inflammatory responses.[40,41] Meanwhile, it has been reported that activation of CREB lead to the development of atherosclerosis.[18,42] However, the effect of EP3 on CREB expression during the development of atherosclerosis remains unclear. Consistent to these backgrounds, our study further suggested that EP3 played a crucial role in atherosclerosis via up-regulation of CREB. On the other hand, some pro-inflammatory agents (IL-8, TNF-α, IL-6, IL-17, IL-1β, and ICAM-1) were found to be modulated by EP3 in this research. As we know, CREB could mediate the pro-inflammatory cytokines.[43,44] Thus, it can be concluded that EP3 promoted the inflammatory responses through up-regulation of CREB.

Meanwhile, lectin-like oxidized lipoprotein receptor-1 (LOX-1) was one of the major receptors of ox-LDL. Moreover, it has been reported that LOX-1 inhibition could be involved in the progression of atherosclerosis, and it could mediate Nox2, Nox4, and p47phox.[45,46] Thus, LOX-1-mediated downstream signalling is also of great significance in progression of atherosclerosis, and this point will be further explored in coming future.

Of course, some limitations are needed to be addressed as follows: (i) More mechanisms by which EP3 regulates atherosclerosis progression need to be further explored; (ii) we still need to further explore animal experiment; (iii) the relation between LOX-1-mediated downstream signalling and atherosclerosis needs to be further investigated; (iv) in this research, PGE2 has well-known receptors, namely, EP1, EP2, and EP3, and L-798106 also has micromolar activities at the EP4, EP1, and EP2 receptors. Thus, the possible involvement of these receptors should be excluded in the future.

Conclusions

In conclusion, EP3 inhibitor attenuated atherosclerosis development via cAMP/PKA/CREB/IL-17/ICAM-1 axis. Thereby, EP3 could be used as a target for atherosclerosis treatment.

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Conflict of interest

The authors declare that there is no conflict of interest.

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