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Original Article

The role of 27-hydroxycholesterol in hypercholesterolemia-associated exacerbation of apical periodontitis and therapeutic potential of felodipine

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Felodipine

Abstract *Background/purpose:* Studies have demonstrated a relation between hypercholesterolemia and development of apical periodontitis (AP), but the underlying mechanism is uncertain. 27-hydroxycholesterol (27HC), produced by cytochrome P450 27A1 (CYP27A1)-catalyzed hydroxylation of cholesterol, is known to possess pro-inflammatory activity. Felodipine is an anti-hypertensive agent able to inhibit CYP27A1. The study aimed to examine the inflammatory response of macrophages to 27HC and the relation between 27HC accumulation and progression of experimental AP. The therapeutic effect of felodipine was also evaluated. *Materials and methods:* J774 murine macrophages were used. Expressions of cyclooxygenase-2 (COX-2) and C–C motif chemokine ligand 20 (CCL20) were examined by Western blot. Concentrations of 27HC were assessed by enzyme-linked immunosorbent assay. Fluorescence assay was used to evaluate cholesterol levels. AP was induced in male rats receiving high fat/high cholesterol diet (HFHCD) or normal diet (ND). Micro-computed tomography and immunohistochemistry were employed to evaluate disease progression and therapeutic effect of felodipine. *Results:* Cholesterol enhanced production of 27HC which in turn stimulated COX-2 and CCL20 synthesis by macrophages. HFHCD consumption significantly augmented serum and lesion

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tissue levels of 27HC in rats. Lesion size and infiltration of COX-2^+ and interleukin (IL)-17^+ cells increased in parallel with 27HC accumulation in AP. Felodipine suppressed cholesterol-induced 27HC production in macrophages. Felodipine treatment reduced serum and tissue levels of 27HC in HFHCD rats and concurrently mitigated AP propagation.
Conclusion: Our results suggest a pivotal role of 27HC in hypercholesterolemia-exacerbated AP. By repressing 27HC production, felodipine may have the potential to help mitigate AP in obese individuals.

Introduction

Apical periodontitis (AP), also known as periapical lesion, is an inflammatory condition predominantly caused by microbial infection in the root canal system.[1] However, host factors, including metabolic status, also play an essential role in modulating treatment outcomes. A study on experimental AP showed more advanced lesions in rats fed with high fat diet than those in hosts taking normal diet.[2] In addition, positive correlation between hypercholesterolemia and periapical bone resorption.[3] The accumulated evidence suggests a connection between AP development and obesity/hypercholesterolemia, however, mechanisms underlying the link remain elusive.

Cholesterol accumulation in macrophages and other immune cells may induce inflammatory responses although many studies have proposed that cholesterol is a poorly reactive molecule.[4,5] In contrast, oxysterols, the oxidized derivatives of cholesterol, possess a much higher biochemical reactivity than that of parent compound.[6] 27-hydroxycholesterol (27HC), the most abundant oxysterol, is produced by cytochrome P450 27A1 (CYP27A1)-catalyzed hydroxylation. In hypercholesterolemia models, macrophage-derived 27HC increases vascular inflammation, which in turn promotes atherosclerosis.[7] Furthermore, 27HC promotes tumor proliferation and facilitates metastasis in human breast cancer.[8,9] Although many cells produce 27HC, its tissue levels are primarily determined by macrophages.[10] Yet its role in the pathogenesis of AP has not been addressed.

Macrophages are a heterogeneous population of myeloid cells that can be polarized to the classically activated type 1 (M1) and the alternatively activated type 2 (M2).[11] M1 macrophages, activated by lipopolysaccharide and interferon-γ, produce pro-inflammatory cytokines. In contrast, M2 macrophages, induced by interleukin-4 (IL-4) and IL-13, are responsible for anti-inflammatory responses and tissue repair.[11] Cyclooxygenase (COX, EC 1.14.99.1) is the rate limiting enzyme in prostaglandin synthesis. COX-2 is induced by endotoxin or cytokines during inflammation.[12] Upregulation of COX-2 is regarded as a biomarker of macrophage polarization to M1 status, indicating the activation of innate immune reaction.[13] Expression of COX-2 was found to be elevated in induced rat AP and inhibition of COX-2 alleviated disease progression.[14] It is interesting to examine the contribution of 27HC to COX-2 induction in the pathogenesis of AP.

Interleukin-17 (IL-17) are secreted by T helper 17 (Th17) cells and its over-production is linked to the onset of many autoimmune diseases.[15,16] The ligand-receptor axis C—C motif chemokine ligand 20 (CCL20)/C—C chemokine receptor 6 (CCR6) is responsible for Th17 cell migration to lesion site.[15,17] Studies have found excessive production of IL-17 and infiltration of Th17 cells in AP.[18,19] Furthermore, high circulating CCL20 induced by low density lipoprotein-cholesterol has been proposed to trigger the pathogenesis of atherosclerosis.[20] Whether 27HC can affect AP development via modulating CCL20 synthesis in obesity/hypercholesterolemic hosts warrants investigation.

Felodipine (Felo) is a calcium channel blocker clinically used as an anti-hypertensive agent. Felo could inhibit 27HC synthesis by disturbing the interaction between cholesterol and CYP27A1.[21] However, its effect on 27HC production in cultured cells and therapeutic action on 27HC-associated diseases remain unconfirmed. In the present study, we aimed to assess the effect of obesity-related hypercholesterolemia on AP progression and the possible involvement of 27HC in the pathogenesis. Specifically, the impact of 27HC on COX-2 and CCL20 production by macrophages and the regulatory function of Felo were examined. AP was established in male rats consuming normal diet (ND) or high fat/high cholesterol diet (HFHCD). Contribution of 27HC in disease development and the therapeutic effect of Felo were evaluated.

Materials and methods

Cell culture and treatment

Murine macrophage J774 cells were maintained in RPMI-1640 medium supplemented with 10 % fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C with 5 % CO_2. J774 cells were treated with different concentration of soluble cholesterol (Sigma—Aldrich, St. Louis, MO, USA) or 27HC (Enzo Life Sciences, Farmingdale, NY, USA) for the indicated times.

Western blot analysis

The samples were homogenized in RIPA buffer supplemented with protease inhibitors. Proteins were fractionated by SDS-PAGE and transferred onto PVDF membranes. The membranes were probed with antibodies against COX-2

and CCL20. Antibody against GAPDH was used as a loading control. Primary antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence detection system (Millipore, Burlington, MA, USA).

Measurement of 27-hydroxycholesterol by enzyme-linked immunosorbent assay

The cultured cells, serum and AP tissues, were subjected to 27HC measurement. J774 cells were lysed by RIPA buffer and then the cell lysates were collected. Blood samples were drawn from experimental animals, centrifuged (8,000×*g* for 20 min). Harvested AP tissues were homogenized in tissue lysis buffer and the homogenates were centrifuged to obtain tissue supernatants. 27HC was measured using a two-site-sandwich 27HC ELISA kit (Abbexa Ltd, Cambridge, UK) according to the manufacturer's instructions. Cellular and tissue concentrations of 27HC were normalized to protein content of the samples.

Measurement of serum cholesterol

Rat serum cholesterol level was measured by Amplex Red cholesterol assay kit (Invitrogen, Paisley, UK) according to the manufacturer's instructions. The fluorescence was measured using excitation wave length of 530—560 nm and emission wave length of 590 nm.

Measurement of serum glucose

Glucose levels were assessed using a glucose commercial kit (Thermo Fisher Scientific, Winsford, UK) according to the manufacturer's instructions. The optical density at 560 nm was measured using a Multiskan FC microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Experimental apical periodontitis

AP was induced in rats as described previously.[22] The care and maintenance of animals were in accord with the Guide to Management and Use of Experimental Animals, National Laboratory Animal Center, Taiwan. In the first part of experiments, 6-week-old male SD rats were fed ND and HFHCD (Research Diet, New Brunswick, NJ, USA) for 4 weeks. Mandibular first molars were drilled at the distal fossa with a 0.25 mm round bur to expose the pulps. After 6 weeks, the mandibles were dissected and split into halves. Lesion tissues were carefully excavated and subjected to 27HC level measurement. The right hemimandibles were fixed before image analysis and immunohistochemical examination. In the second part of experiments, 6-week-old male SD rats taking HFHCD were divided into 2 groups. The animals in treatment group received IP injections of Felo (3 mg/kg), starting one day before lesion induction (day 28), then twice a week for 6 weeks.[23] On day 70, the rats were euthanized, and the mandibles were collected and examined as above mentioned.

Microcomputed tomography

Microcomputed tomography (micro-CT) was conducted using SkyScan 1276 (Bruker, Kontich, Belgium) at an X-ray voltage of 70 kV and current of 200 μA. Scanning was performed with a pixel size of 18 μm, rotation step of 0.6° and 180° rotation around the long axis of hemi-mandible, using a 0.5 mm aluminum filter. The acquired images were reconstructed and analyzed by NRecon and CTAn softwares, respectively. DataViewer (v1.5.6.2) software was used to obtain representative images of the specimens.

Immunohistochemistry

The mandibles were decalcified and embedded in paraffin wax. Five 5 μm serial sections of the periapices were deparaffinized, rehydrated, and antigen retrieved. After blocking for nonspecific binding, sections were incubated with antibody against COX-2 and IL-17 overnight and then a biotinylated secondary antibody. Colorization was done with diaminobenzidine, and the sections were counterstained with hematoxylin. For each animal, positive immunostaining signals were counted and three non-overlapping fields exhibiting the strongest inflammatory activity in each section were selected. A 0 to 5 scale was used to quantify the signals as described previously.[24,25]

Statistical analysis

In vitro experiments were performed at least in triplicate. All data sets for comparison between three or more groups were subjected to Levene's test to confirm the equality of error variances across groups and the mean values were compared by one-way ANOVA followed by Tukey's *post hoc* test. Data sets for comparison between two groups were subjected to independent *t*-test. A *P*-value less than 0.05 was considered statistically significant. All analyses were performed with Statistical Package for the Social Sciences (SPSS), version 21.0 (IBM Inc., Chicago, IL, USA). Data are expressed as mean ± standard deviation (SD).

Results

Cholesterol stimulated 27-hydroxycholesterol production in macrophages

Production of 27HC in J774 macrophages following cholesterol treatment was examined by ELISA. Results showed that cholesterol stimulated 27HC formation in a dose- and time-dependent manner. 27HC level escalated steadily after incubation with 1—10 μM cholesterol for 24 h (Fig. 1A). Cellular content of 27HC following treatment with 10 μM cholesterol increased gradually from 8 to 24 h and declined afterward (Fig. 1B).

27-hydroxycholesterol induced inflammatory reaction in macrophages

Expressions of pro-inflammatory mediators following 27HC treatment in J774 were assessed. Western blot showed that

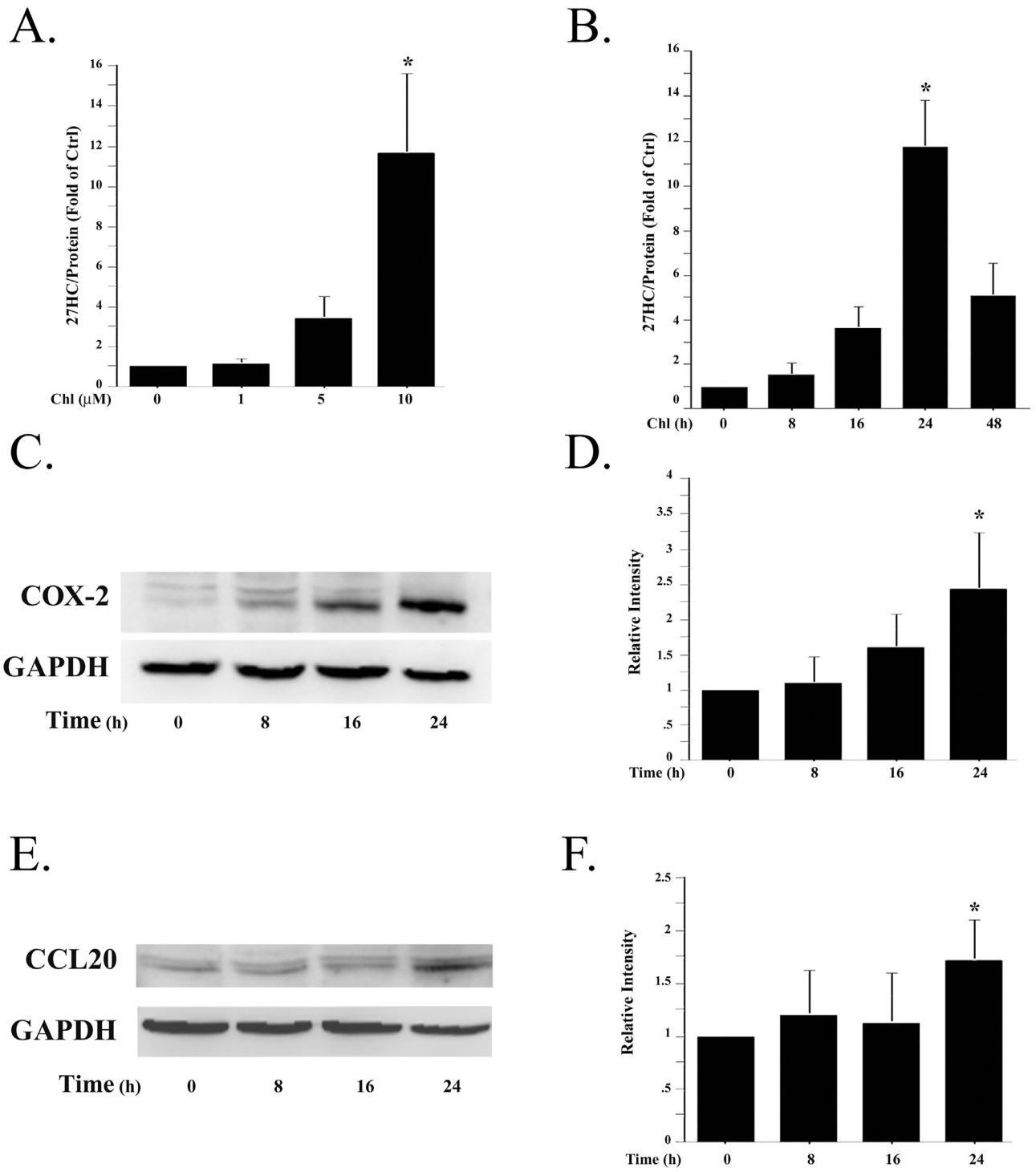

Figure 1 Cholesterol enhanced 27HC production and 27HC induced COX-2 and CCL20 expressions in macrophages. (A) Murine macrophages J774 were treated with 10 μM cholesterol (Chl) for the indicated time points and 27-hydrocyxholesterol (27HC) was measured by ELISA. (B) J774 cells were treated with various concentrations of cholesterol for 24 h and 27HC was measured. (C) J774 cells were incubated with 27HC (10 μM) for the indicated time points and expression of cyclooxygenase-2 (COX-2) was analyzed by Western blot. (D) Densitometric analysis of COX-2 expression. (E) Expression of C—C motif chemokine ligand 20 (CCL20) after 27HC treatment. (F) Densitometric analysis of CCL20 expression. Densitometric data are expressed as mean ± standard deviation (SD) of 3 independent experiments. *$P < 0.05$ vs. control.

27HC significantly upregulated COX-2 (Fig. 1C and D) and CCL20 (Fig. 1E and F) at 24h.

Elevated 27-hydroxycholesterol was associated with apical periodontitis exacerbation in high fat/high cholesterol diet rats

Experimental AP was established in male rats fed with two types of diet (ND and HFHCD). After euthanasia, serum levels of cholesterol, 27HC and glucose were assessed. Tissue content of 27HC as well as lesion volume of AP were evaluated. While serum concentrations of cholesterol (Fig. 2A) and 27HC (Fig. 2B) in HFHCD rats were significantly higher than those in ND group, similar glucose levels (Fig. 2C) between these animals were noted. In parallel with elevated serum 27HC, 27HC concentrations in AP lesions were significantly higher in HFHCD rats (Fig. 2D). Concomitantly, micro-CT disclosed that HFHCD rats had more prominent periapical bone resorption than the ND animals (Fig. 2E and F).

High 27-hydroxycholesterol linked to increased expression of cyclooxygenase-2 and interleukin-17 in apical periodontitis

Elevated 27HC also related to enhanced expressions of inflammatory mediators in AP. Comparing with ND group (Fig. 3A), HFHCD group (Fig. 3B) showed more extensive bone destruction and heavier inflammatory cell infiltration in AP upon histopathological examination. Immunohistochemistry revealed more numerous COX-2-expressing cells scattering around Howship's lacunae in lesions of HFHCD rats (Fig. 3D) as compared with the ND animals (Fig. 3C). Abundant IL-17 signal was detected in small round cells and larger polygonal cells infiltrating lesions of the HFHCD group (Fig. 3G). Scarce IL-17 signal was found in the ND lesions (Fig. 3F). Staining scores for COX-2 (Fig. 3E) and IL-17 (Fig. 3H) in HFHCD group were significantly higher than those of the ND group.

Felodipine suppressed 27-hydroxycholesterol production in macrophages

J774 cells with or without Felo pre-incubation were treated by cholesterol. ELISA demonstrated that cholesterol-induced production of 27HC was markedly repressed by Felo (Fig. 4A).

Felodipine alleviated apical periodontitis progression in high fat/high cholesterol diet rats

AP was induced in HFHCD rats receiving IP injections of Felo or vehicle. Administration of Felo had no obvious effect on serum level of cholesterol (Fig. 4B). In contrast, concentrations of 27HC in serum (Fig. 4C) and AP tissues (Fig. 4D) decreased significantly in Felo-treated animals. In parallel with 27HC reduction, micro-CT revealed that Felo therapy markedly alleviated bone resorption associated with AP in HFHCD rats (Fig. 4E and F).

Felodipine attenuated cyclooxygenase-2 and interleukin-17 expressions in apical periodontitis

Histopathological study showed excessive infiltration of inflammatory cells in AP lesions of rats receiving vehicle injection (Fig. 5A). Treatment with Felo attenuated inflammatory infiltration (Fig. 5B). Immunohistochemistry demonstrated plentiful COX-2-positive cells in AP of vehicle-treated animals (Fig. 5C) and the number of COX-2-positive cells was significantly lower in Felo-treated lesions (Fig. 5D and E). Similarly, many IL-17-expressing cells were found in control lesions (Fig. 5F) and Felo diminished infiltration of IL-17-positive cells in AP (Fig. 5G and H).

Discussion

The present study demonstrated that cholesterol induced macrophages to produce 27HC, which in turn stimulated the synthesis of COX-2 and CCL20. In our AP models, rats receiving HFHCD showed significantly higher concentrations of 27HC in serum and lesion tissues. In parallel, more advanced AP progression and heavier infiltration of COX-2 and IL-17-expressing cells were noted in HFHCD rats. In addition, we confirmed that Felo prohibited 27HC production in macrophages. In vivo experiments revealed that Felo effectively decreased serum and tissue concentrations of 27HC without changing cholesterol level in HFHCD rats. Concomitant with 27HC suppression, Felo therapy reduced periapical bone destruction and accumulation of cells expressing COX-2 and IL-17 in HFHCD rats. Taken together, our findings imply that 27HC is a pivotal player in hypercholesterolemia-exacerbated AP progression and Felo mitigates disease development via repressing 27HC formation (Fig. 6).

COX-2 signaling participates in multiple inflammatory reactions, including production of IL-1β and TNF-α and secretion of CCL2 to promote macrophage recruitment.[26] The involvement of COX-2 pathway in AP pathogenesis has been validated in previous studies.[27,28] Lipopolysaccharide (LPS) has been regarded as the most common triggering factor of this pathway in AP.[29] Interestingly, a recent investigation demonstrated that 27HC accumulated in atherosclerotic plaques could activate COX-2 to trigger the development of advanced atherosclerosis.[30] Hypercholesterolemia-derived 27HC may stimulate COX-2 expression to enhance AP progression has never been reported before. Our findings support that dysregulation of cholesterol metabolism can affect prostaglandin homeostasis in bacteria-induced inflammation.

In the study, 27HC promoted prominent CCL20 synthesis from macrophages. Corresponding to the notion that CCL20 can trigger IL-17 production through binding to the cell surface receptor CCR6,[16,17] abundant infiltration of IL-17-expressing cells was noted in AP from rats fed with HFHCD diet. Although we cannot verify precisely the presence of Th17 cells in the AP lesions because specific biomarker of Th17 cells is still unavailable. Excessive differentiation of Th17 cells secondary to 27HC accumulation has also been shown contributing to cognitive impairment in humans or animals.[31,32] It is interesting to find out whether 27HC participates in the pathogenesis of other Th17-associated diseases as well.

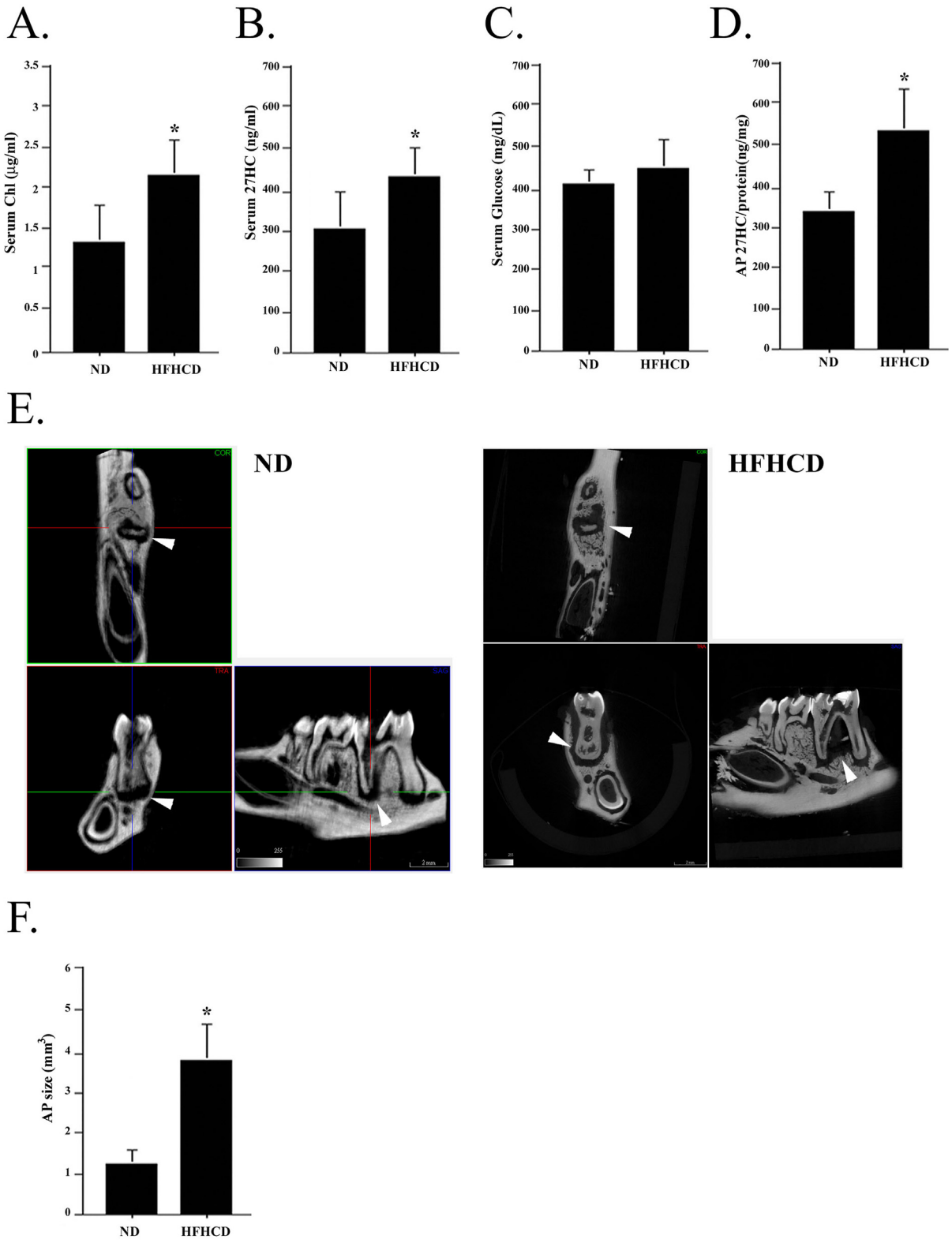

Figure 2 HFHCD caused 27HC accumulation and aggravated apical periodontitis propagation. Experimental apical periodontitis (AP) was established in male rats consuming normal diet (ND) or high fat/high cholesterol diet (HFHCD). Rats were sacrificed 6 weeks after lesion induction. (A) Serum concentrations of cholesterol (Chl)were measured by fluorescence assay. (B) Serum concentrations of 27-hydroxycholesterol (27HC) were measured by enzyme-linked immunosorbent assay (ELISA). (C) Serum glucose levels were assessed by colorimetric assay. (D) 27HC contents in AP lesions were measured by ELISA. (E) Representative microcomputed tomography (micro-CT) pictures of AP (arrowheads). (F) Quantitative assessment of AP lesion size in ND and HFHCD groups. Data are expressed as mean ± standard deviation (SD). *$P < 0.05$ vs. ND group.

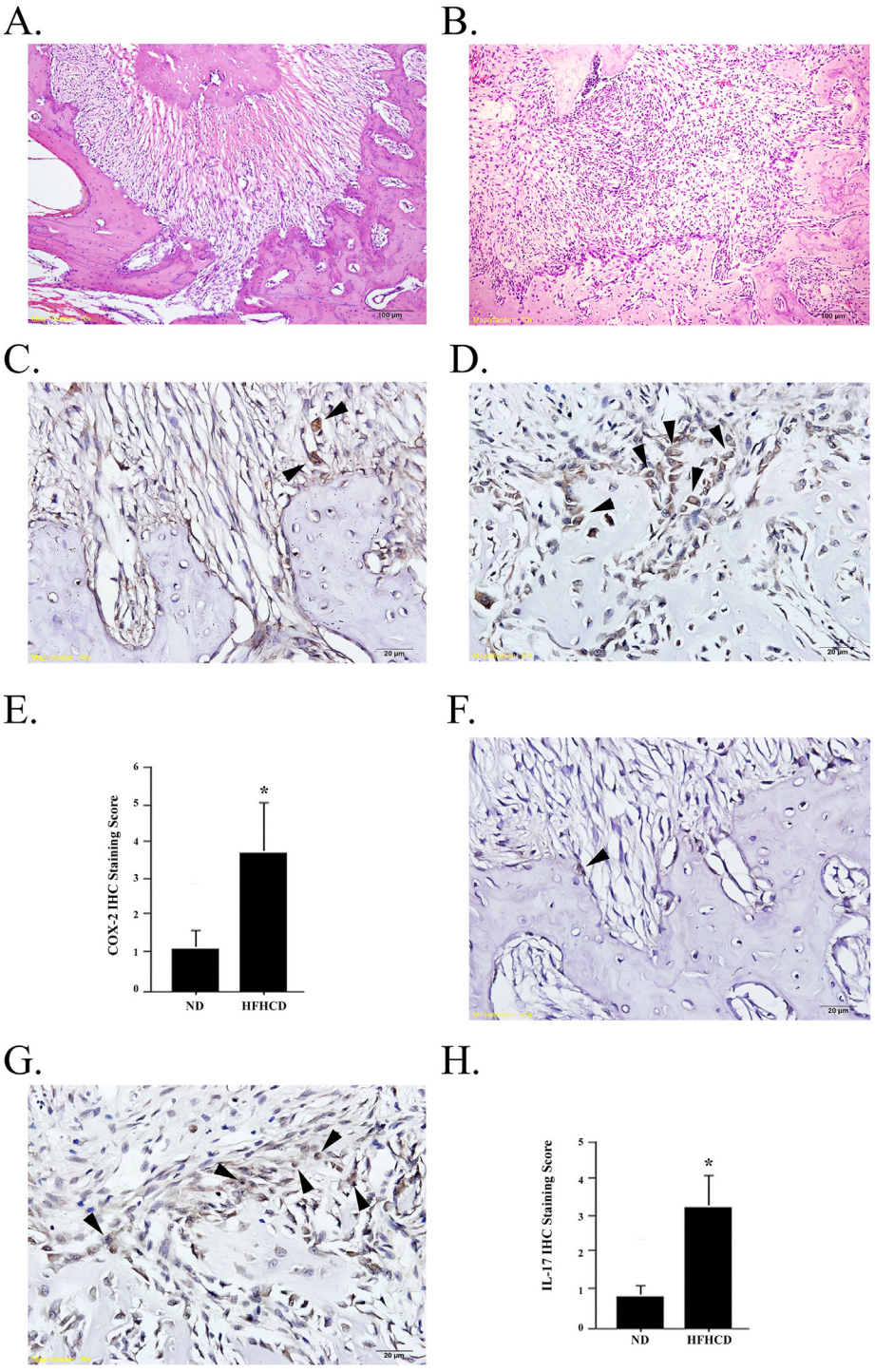

Figure 3 Immunohistochemical analysis of COX-2 and IL-17 expressions in experimental apical periodontitis. (A) Hematoxylin-eosin staining of apical periodontitis (AP) lesion in normal diet (ND) group. (B) A representative AP lesion in high fat/high cholesterol diet (HFHCD) group shows heavier infiltration of inflammatory cells and more numerous resorption lacunae along the bone surface comparing to ND group. (C) Expression of cyclooxygenase-2 (COX-2) was examined by immunohistochemical analysis (IHC). Only a few cells were stained positive for COX-2 in lesions of ND group. (D) More prominent COX-2 expression was found in HFHCD lesions. (E) Quantitative comparison of COX-2 expression (by a 0 to 5 scale) between the 2 groups. (F) Immunohistochemistry showed scanty interlukin-17^{+} (IL-17^{+}) cell infiltration in ND lesions. (G) More abundant IL-17^{+} cells were found in HFHCD lesions. Note that many small round cells and larger polygonal cells were stained positive. (H) Quantitative comparison of IL-17 expression (by a 0 to 5 scale) between the 2 groups. Data are expressed as mean ± standard deviation (SD). *$P < 0.05$ vs. ND group.

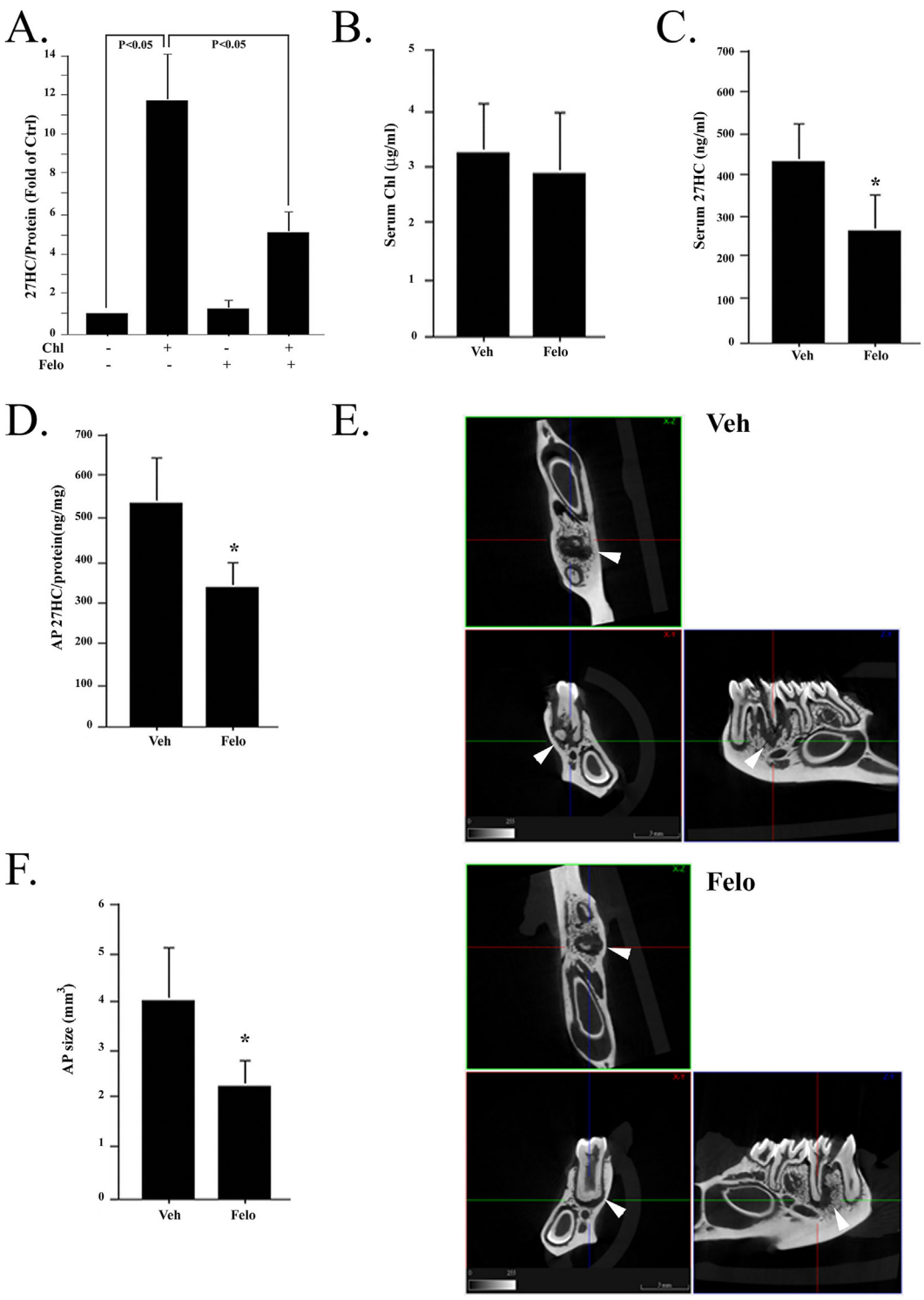

Figure 4 Felodipine inhibited 27HC synthesis and suppressed apical periodontitis development. (A) J774 cells were stimulated by 10 μM cholesterol (Chl) for 24 h, with or without the addition of felodipine (Felo, 2.5 nM, 3 h before cholesterol treatment). Cellular 27-hydroxycholesterol (27HC) was examined. (B) to (C) Experimental apical periodontitis (AP) was induced in high fat/high cholesterol diet (HFHCD) rats which received IP injections of either Felo (3 mg/kg, twice a week) (n = 6) or vehicle (Veh) (n = 6) for 6 weeks. After euthanasia, serum cholesterol (B) serum 27HC (C) and AP lesion 27HC (D) were assessed. (E) Representative microcomputed tomography (micro-CT) pictures of AP lesions (arrowheads). (F) Quantitative comparison of AP lesion size between Veh and Felo-treated rats. Data are expressed as mean ± standard deviation (SD). *$P < 0.05$ vs. control.

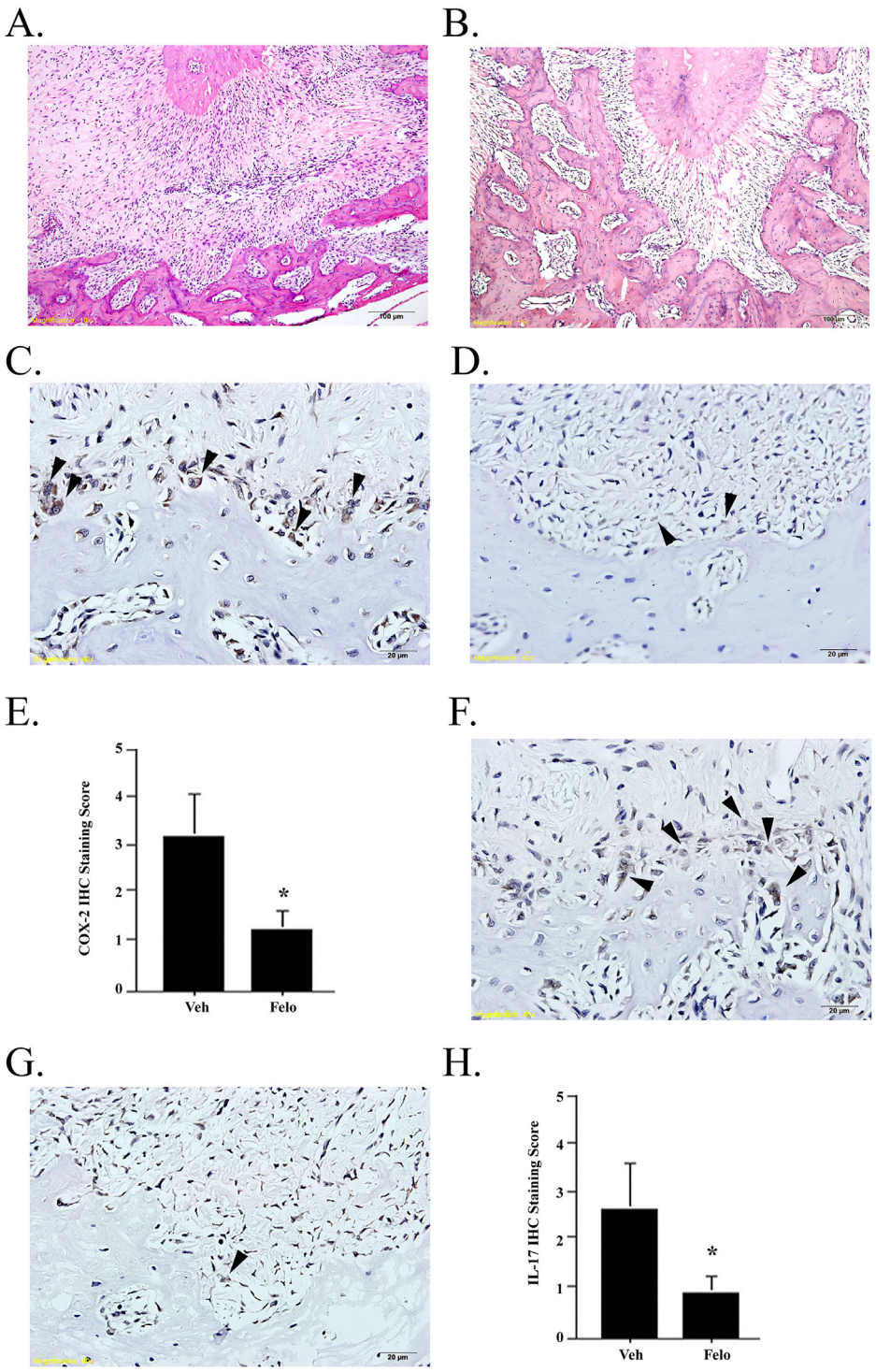

Figure 5 Felodipine attenuated inflammation and infiltration of COX-2 and IL-17-synthesizing cells in apical periodontitis. (A) A representative Hematoxylin-eosin section of apical periodontitis (AP) in vehicle (Veh) group. (B) Felodipine (Felo) treatment attenuated inflammation. (C) Immunohistochemistry (IHC) revealed numerous cyclooxygenase-2^{+} (COX-2^{+}) cells (arrowheads) in Veh group. (D) Felo treatment diminished COX-2^{+} cell infiltration. (E) Quantitative comparison of COX-2 expression (by a 0 to 5 scale) between the 2 groups. (F) Infiltration of interlukin-17 (IL-17)-producing cells (arrowheads) was marked in Veh group. (G) AP lesions in Felo group had fewer IL-17^{+} cells. (H) Quantitative comparison of IL-17 expression (by a 0 to 5 scale) between the 2 groups. Data are expressed as mean ± standard deviation (SD). *$P < 0.05$ vs. control.

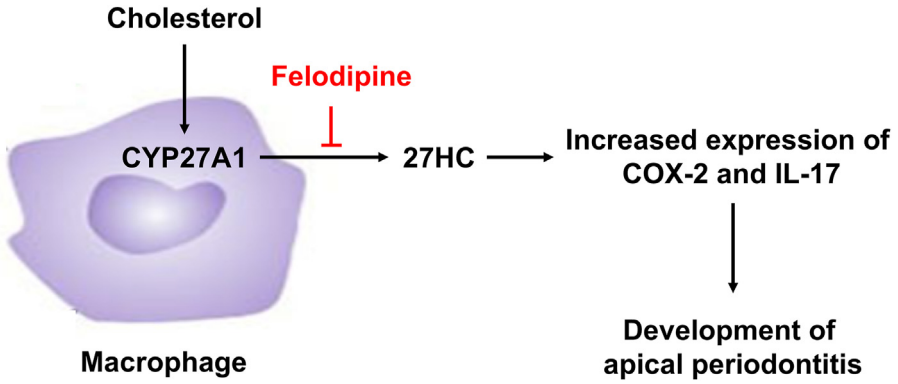

Figure 6 Working model of felodipine action on 27HC signaling pathway in apical periodontitis.

We showed that some IL-17 signals were located in macrophage-like polygonal cells, especially in the HFHCD group. Indeed, IL-17 is also upregulated in infections of bacteria, fungi and mycobacteria, in addition to taking part in autoimmune reactions.[33] Previous investigations found that LPS alone could not stimulate IL-17 production from macrophages.[34] However, with the presence of complement-activated product C5a, LPS could induce IL-17 production from macrophages via toll-like receptor (TLR)-4-mediated pathway.[35,36] Along with this well-known pathway for induction of IL-17 in macrophages to promote AP development,[37] results of our study suggest that in hosts with hypercholesterolemia, 27HC may collaborate in the generation of IL-17-differentiated macrophages in AP.

A previous ex vivo assay by Lam et al. disclosed that Felo could suppress CYP27A1 and inhibit 27HC synthesis.[21] In the present study, Felo prohibited 27HC production in macrophages. Administration of Felo reduced serum 27HC and 27HC level in AP lesion, implying that the therapeutic effect of Felo is secondary to its 27HC-lowering activity. It is noteworthy that Lam et al. also found Felo treatment did not alter cholesterol levels in serum, liver and brain in mice.[21] Mechanistically, our data suggest that Felo diminishes 27HC production by macrophages in AP of hosts with hypercholesterolemia. The subsequent inflammatory reactions elicited by 27HC are then alleviated.

In conclusion, the study suggests a pivotal role of 27HC in potentiating AP development associated with hypercholesterolemia status. Our data also imply that through alleviating 27HC synthesis in macrophages, Felo may have the potential to help mitigate AP in obese individuals. Local delivery methods that allow direct administration of Felo to AP lesion warrant further investigations.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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