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RESEARCH PAPER

Fluocinolone acetonide partially restores the mineralization of LPS-stimulated dental pulp cells through inhibition of NF-κB pathway and activation of AP-1 pathway

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BACKGROUND AND PURPOSE

Fluocinolone acetonide (FA) is commonly used as a steroidal anti-inflammatory drug. We recently found that in dental pulp cells (DPCs) FA has osteo-/odonto-inductive as well as anti-inflammatory effects. However, the mechanism by which FA induces these effects in DPCs is poorly understood.

EXPERIMENTAL APPROACH

The effect of FA on the mineralization of DPCs during inflammatory conditions and the underlying mechanism were investigated by real-time PCR, Western blot, EMSA, histochemical staining, immunostaining and pathway blockade assays.

KEY RESULTS

FA significantly inhibited the inflammatory response in LPS-treated DPCs not only by down-regulating the expression of pro–inflammation-related genes, but also by up-regulating the expression of the anti-inflammatory gene PPAR-γ and mineralization-related genes. Moreover, histochemical staining and immunostaining showed that FA could partially restore the expressions of alkaline phosphatase, osteocalcin and dentin sialophosphoprotein (DSPP) and mineralization in LPS-stimulated DPCs. Real-time PCR and Western blot analysis revealed that FA up-regulated DSPP and runt-related transcription factor 2 expression by inhibiting the expression of phosphorylated-NF-κB P65 and activating activator protein-1 (AP-1) (p-c-Jun and Fra-1). These results were further confirmed through EMSA, by detection of NF-κB DNA-binding activity and pathway blockade assays using a NF-κB pathway inhibitor, AP-1 pathway inhibitor and glucocorticoid receptor antagonist.

CONCLUSIONS AND IMPLICATIONS

Inflammation induced by LPS suppresses the mineralization process in DPCs. FA partially restored this osteo-/odonto-genesis process in LPS-treated DPCs and had an anti-inflammatory effect through inhibition of the NF-κB pathway and activation of the AP-1 pathway. Hence, FA is a potential new treatment for inflammation-associated bone/teeth diseases.

Abbreviations

ALP, alkaline phosphatase; AP-1, activator protein-1; DPCs, dental pulp cells; DSPP, dentin sialophosphoprotein; FA, fluocinolone acetonide; OCN, osteocalcin; p-P65, phosphorylated NF-κB P65; RUNX2, runt-related transcription factor 2; t-RA, all-trans-retinoic acid

Introduction

The inflammatory response always follows after an infection, injury or antigenic stimulation (Nathan, 2002). If inflammation occurs in dental pulp, it is hard to resolve due to the specific anatomical structure of the tooth. The healing of inflamed pulp needs at least two requirements: (i) reducing the inflammation and (ii) the formation of a hard tissue barrier to protect the pulp from injurious agents (Tziafas *et al.*, 2000). Previous investigations into the restoration of injured dental pulp have traditionally centred on the mineral role of dental pulp-capping agents. However, controlling dental pulp inflammation is still a big challenge to the success rate of dental pulp capping. A single application of dental pulp-capping agent normally fails to induce reparative dentin formation in inflammatory pulp tissue (Rutherford and Gu, 2000). Furthermore, many studies have shown that inflammation could play an important role in bone loss in diseases such as internal resorption of teeth, periodontal diseases and rheumatoid arthritis (Walsh *et al.*, 2005; Cochran, 2008). Therefore, the relationship between inflammation and mineralization is vitally important to the healing of injured dental pulp. An agent that acts as an anti-inflammatory as well as having an osteo-/odonto-inductive effect may have the potential to promote the restoration of injured dental pulp.

Steroids are the most commonly used anti-inflammatory drugs. Fluocinolone acetonide (FA), a steroidal anti-inflammatory drug, has been used to treat dermal and mucosal disorders (Pauporte *et al.*, 2004; Youngnak-Piboonratanakit *et al.*, 2009). In recent years, steroids have been used systemically to treat endodontic conditions (Chidiac *et al.*, 2009; Fachin *et al.*, 2009; Mahmoud *et al.*, 2010). Muincharern *et al.* showed that FA induces cell proliferation and extracellular matrix formation in human dental pulp cells (DPCs) *in vitro* (Muincharern *et al.*, 2011). However, it is not known whether FA also has an osteo-/odonto-inductive effect in addition to its anti-inflammatory action in inflamed dental pulp tissue.

In this study, we investigated the effect of FA on LPS-stimulated DPCs and the underlying mechanism involved. Our results demonstrated that FA has both an anti-inflammatory and osteo-/odonto-inductive effect in LPS-stimulated DPCs. Notably, FA partially restored the mineralization potential of LPS-stimulated DPCs through blockade of the NF-κB pathway and activation of the activator protein-1 (AP-1) pathway.

Methods

Isolation and culture of human DPCs

All experimental protocols were approved by the Ethics Committee of the Peking University Health Science Center, Beijing, China. DPCs were obtained from healthy permanent premolars extracted during orthodontic treatment at the Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology (from donors 18–28 years old). Briefly, dental pulp tissue was isolated from the teeth and then digested in a mixture of 3 mg·mL^{-1} type I collagenase (Sigma-Aldrich, St Louis, MO, USA) and 4 mg·mL^{-1} dispase (Roche, Indianapolis, IN, USA) for 1 h at 37°C. Next the suspensions of DPCs were passed through a 70 μm strainer to separate the cells (Gronthos *et al.*, 2000). The single-cell suspensions were cultured in DMEM, supplemented with 10% FBS (Hyclone, Logan, UT, USA), 2 mM L-glutamine, 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin, by incubation at 37°C with 5% CO_2. DPCs between the fourth and sixth passage were used for the following experiments.

Quantitative real-time PCR

DPCs were exposed to 1 μg·mL^{-1} *Escherchia coli* LPS (Sigma-Aldrich) for 1, 3, 6, 12, 24 h with/without FA (1 and 10 μmol·L^{-1}), the NF-κB inhibitor Bay 11–7082 (Sigma-Aldrich, St Louis, MO, USA), the AP-1 inhibitor all-trans-retinoic acid (t-RA) or the glucocorticoid receptor antagonist mifepristone (Sigma-Aldrich) respectively. At the times indicated, RNA was extracted from the DPCs using TRIzol (Invitrogen, Carlsbad, CA, USA). Then 2 μg total RNA was converted to cDNA with a moloney murine leukaemia virus reverse transcriptase (M-MLV RTase; Promega, Madison, WI, USA) in a total volume of 20 μL. Real-time PCR was carried out in a total volume of 20 μL in SYBR green master mix (Roche, Indianapolis, IN, USA) with 0.5 μL cDNA and 200 nM of the following primers: IL-1β (5′-TGCACGATGCACCTG TACGA-3′ and 3′-AGGCCCAAGGCCACAGGTAT –5′), TNF-α (5′-CAGAGGGAAGAGTTCCCCAG-3′ and 5′-CCTCAGCTTGA GGGTTTGCTAC-3′), IL-6 (5′-GTGAGGAACAAGCCAGAGC-3′ and 5′-TACATTTGCCGAAGAGCC-3′), IL-8 (5′-TTTTGCCAAG GAGTGCTAAAGA-3′ and 5′-AACCCTCTGCACCCAGTTTTC-3′), COX2 (5′-CTGGCGCTCAGCCATACAG-3′ and 5′-ACAC TCATACATACACCTCGGT-3′), alkaline phosphatase (ALP; 5′-ATGGGATGGGTGTCTCCACA-3′ and 5′-CCACGAAGGGG AACTTGTC-3′), runt-related transcription factor 2 (RUNX2; 5′-TCCTATGACCAGTCTTACCCCT-3′ and 5′-GGCTCTTCTT ACTGAGAGTGGAA-3′), DSPP (5′-ATGGGCCATTCCAGTTC CTC-3′ and 5′-ACCATCTTGGGTATTCTCTTGCC-3′), PPAR-γ (5′-TACTGTCGGTTTCAGAAATGCC-3′ and 5′-GTCAGCGG ACTCTGGATTCAG-3′). The housekeeping gene GAPDH was used as an internal control (5′-ATGGGGAAGGTGAAG GTCG-3′ and 5′-GGGGTCATTGATGGCAACAATA-3′). The thermal cycling conditions were as follows: 50°C for 2 min, then 95°C for 10 min, followed by 40 cycles of 94°C for 15 s and 60°C for 1 min. Reactions were performed using ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and FastStart Universal SYBR Green PCR master mix (Rox) from Roche Applied Science (Indianapolis, IN, USA).

ALP staining

DPCs were seeded in 24-well plates at 2×10^4 cells per well for ALP staining. The following day, the DPCs were treated with 1 μg·mL^{-1} LPS with or without either 10 μmol·L^{-1} FA, 5 μmol·L^{-1} Bay 11-7082 (NF-κB inhibitor) or 1 μmol·L^{-1} t-RA (AP-1 pathway inhibitor) for seven days, then they were examined ALP expression using an ALP histochemical staining kit (Cwbiotech, Beijing, China) according to the manufacturer's protocol.

Immunostaining

DPCs were seeded in 24-well plates at 2×10^4 cells per well for immunostaining. The following day, the DPCs were treated with 1 μg·mL^{-1} LPS with/without 10 μmol·L^{-1} FA in osteogenic media [100 nM dexamethasone, 10 mM sodium β-glycerophosphate, 50 μg·mL^{-1} L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma-Aldrich)] in growth medium for 7 days. Then the cells were fixed in 95% alcohol and examined for dentin sialophosphoprotein (DSPP) and osteocalcin (OCN) expression using immunostaining. The procedure was carried out according to the protocol of the SP immunohistochemical kit and DAB coloration kit (ZSGB-BIO Company, Beijing, China). The primary antibodies used were DSPP (sc-73632, Santa Cruz, CA, USA) and OCN (sc-30044, Santa Cruz, CA, USA) respectively. After the samples had been treated with DAB, for substrate colour development, and counterstained with haematoxylin they were examined using an Olympus DP7 microscope (Olympus, Tokyo, Japan).

Alizarin red staining

DPCs were seeded onto 12-well plates at 6×10^4 cells per well. When 60% confluence had been reached, the DPCs were treated with 1 μg·mL^{-1} LPS and cultured in osteogenic media with or without 10 μmol·L^{-1} FA, continued to culture for additional 21 days, then subjected to alizarin red staining. Briefly, the cells were fixed in 4% paraformaldehyde for 20 min, then stained using alizarin red (Sigma-Aldrich).

EMSA

The DNA-binding activity of NF-κB was assessed by EMSA. Briefly, DPCs were seeded onto 100 mm dishes (1×10^6 cells per dish). When the density of DPCs reached 80% confluency, the cells were treated with 1 μg·mL^{-1} LPS for 3 h with or without either FA (10 μmol·L^{-1}) or Bay 11–7082 (5 μmol·L^{-1}). Nuclear extracts from the DPCs were prepared with a Nuclear-Cytosol Extraction Kit (Applygen Technologies Inc, Beijing, China) according to the manufacturer's protocol. EMSA was applied to determine NF-κB nuclear translocation using the LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL, USA) according to the manufacturer's recommendations. Briefly, nuclear protein (5 μg) was incubated at room temperature for 30 min with 0.2 μg of 3′ biotin-end-labelled double-stranded oligonucleotide containing the NF-κB binding motif (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and 1 μg of poly (dI-dC) as an inhibitor of non-specific binding. In a cold-competition experiment, unlabelled oligonucleotide was used at 100-fold excess and incubated with extracts for 30 min at room temperature before the addition of the biotin-labelled probe. The reaction mixtures were electrophoresed through 6% non-denaturing polyacrylamide Tris-glycine-EDTA gels, and then transferred to a positive charge nylon membrane. After the transfer was completed, the membrane was cross-linked and biotin-labelled DNA was detected using a chemiluminescent detection kit (Pierce, Rockford, IL, USA).

Western blot

DPCs were seeded onto 100 mm dishes (1×10^6 cells per dish). When 80% confluence had been reached, DPCs were treated with LPS with or without either 10 μmol·L^{-1} FA or 5 μmol·L^{-1} Bay 11-7082 for 3 h, lysed in RIPA buffer containing protease inhibitors and quantified by use of the BCA Protein Assay (Pierce). Forty micrograms of protein from each sample was separated on 10% SDS-PAGE gels and transferred to PVDF membranes (Millipore, Bedford, MA, USA) at 100 V for 60 min. The membranes were incubated in blocking buffer (5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween-20, pH 7.4) for 1 h and incubated with the following antibodies in 1:500 dilutions at 4°C overnight: phosphorylated NF-κB P65 (p-P65, Cell Signaling Technology, Beverly, MA, USA), p-c-Jun, Fra-1 (Epitomics, Burlingame, CA, USA), DSPP, RUNX2 and β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). HRP-conjugated anti-rabbit or anti-mouse IgG (Cell Signaling Technology) was added for 1 h at room temperature, based on the source of the corresponding primary antibody. The immunoblots were detected by use of a Western ECL blotting kit (ECL, Applygen Technology Inc. Beijing, China).

Statistical analysis

Data are expressed as means ± SD. Between-group differences were compared using one-way ANOVA. Differences were considered significant if $P < 0.05$.

Results

Effects of FA on mRNA levels of inflammatory mediators in LPS-stimulated DPCs

To investigate whether FA reduces the inflammatory cytokines in DPCs, we examined their expression in DPCs treated with LPS for 1, 3, 6, 12 and 24 h (Figure 1). The inflammatory genes were detected by quantitative PCR. FA at 1 and 10 μmol·L^{-1} significantly decreased the expression of IL-1β at 3, 6 and 12 h; IL-6 from 1 h to 24 h; and IL-8 at 3 and 6 h post-LPS treatment. Meanwhile, at 3, 6, 12 and 24 h after LPS stimulation, 1 and 10 μmol·L^{-1} FA significantly up-regulated the expression of PPAR-γ mRNA, which was clearly down-regulated by 1 μg·mL^{-1} LPS. The results indicate that FA could inhibit inflammation-related genes by up-regulating the expression of the anti-inflammatory gene PPAR-γ.

Effects of FA on the expression of osteo-/odonto-genic genes in DPCs in response to LPS for seven days

It is well known that the ALP and OCN proteins are early and late stage, respectively, biomarkers of osteo-differentiation in the same way that DSPP is considered to represent odonto-differentiation. Therefore, we next investigated the effects of FA on the mineralization of DPCs under inflammatory conditions by measuring these markers. ALP histochemical staining, OCN and DSPP immunostaining were performed in DPCs in response to LPS for 7 days. ALP histochemical staining results showed that under osteogenic conditions, ALP expression was significantly decreased in LPS-treated DPCs, and FA significantly restored the expression of ALP in these

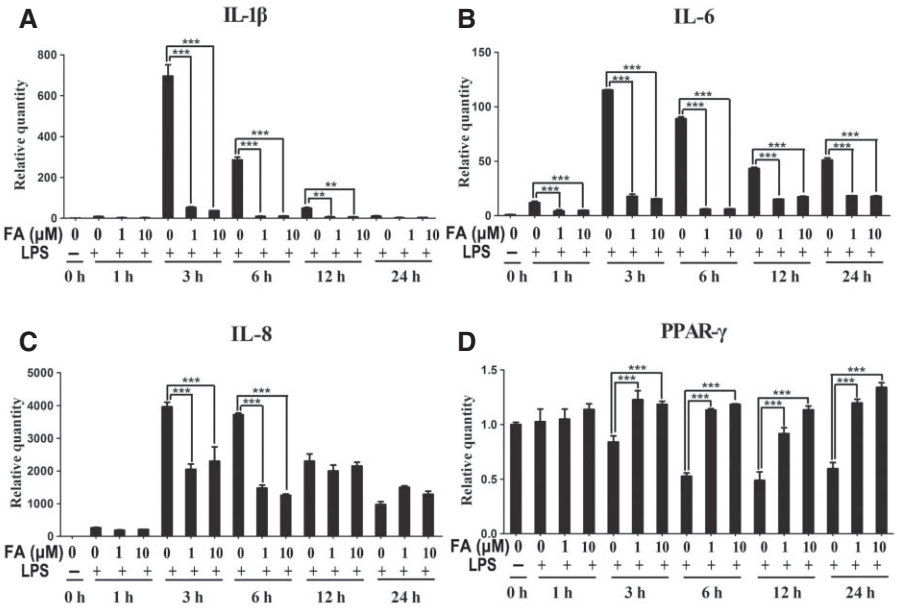

Figure 1

Effects of FA on inflammatory mediators in LPS-treated DPCs. (A–D) LPS-treated DPCs were incubated with 1 and 10 μmol·L^{-1} FA for 1, 3, 6, 12 and 24 h. The mRNA levels of inflammation-related genes, IL-1β, IL-6, IL-8 and PPAR-γ were detected by quantitative PCR. Untreated DPCs at 0 h served as the normal control for comparison of the relative expression of all samples. $^{**}P < 0.01$; $^{***}P < 0.001$.

LPS-stimulated cells (Figure 2A). In addition, immunostaining of OCN and DSPP showed the same trend as ALP. OCN and DSPP expression were inhibited by LPS, and were partially restored by FA at day 7 post-treatment (Figure 2B, C).

Effects of FA on the formation of mineralized nodules in LPS-treated DPCs

To determine the 'long-term' effects of FA on osteo-/odontogenesis in LPS-stimulated DPCs, the stimulated cells were subjected to alizarin red staining on day 21 post-treatment. Compared with untreated control DPCs, the number of mineralized nodules was significantly increased in osteo-/odonto-inductive medium and this effect was completely inhibited by LPS. However, when the cells were treated with LPS plus FA, a few mineralized nodules were found on the surface of DPCs (Figure 2D). This indicates that FA can partially restore the mineralization of LPS-stimulated DPCs.

Effects of FA and the NF-κB inhibitor Bay 11–7082 on the expression of osteo-/odonto-genic genes in DPCs in response to LPS for 3 h

To investigate whether FA induces the expression of osteogenic genes in LPS-stimulated DPCs, the expressions of ALP, RUNX2 and DSPP mRNA were determined after DPCs had been treated with LPS in the absence or presence of FA or the NF-κB inhibitor Bay 11–7082 for 3 h. Quantitative PCR results showed that compared with untreated control, LPS significantly suppressed the expression of the osteogenesis-related genes ALP, RUNX2 and DSPP in DPCs after 3 h of treatment. Both FA and Bay 11-7082 restored the expression of ALP, RUNX2 and DSPP in LPS-treated DPCs (Figure 3A-C). Western blot results were further confirmed with real-time PCR results via densitometry using Image J software (National Institutes of Health, Bethesda, MD, USA; Figure 3D-F).

FA inhibited inflammation in LPS-stimulated DPCs via blockade of the NF-κB pathway

To identify the possible involvement of NF-κB in the effect of FA, we compared the anti-inflammatory effect of FA on LPS-stimulated DPCs with that of Bay 11–7082. DPCs were exposed to LPS for 3 h with either 10 μmol·L^{-1} FA or 5 μmol·L^{-1} NF-κB pathway inhibitor Bay 11-7082. Quantitative PCR results showed that treatment with FA or Bay 11–7082 could effectively inhibit the expression of pro-inflammatory genes such as IL-1β, TNF-α, IL-6, IL-8, COX-2 mRNA in LPS-treated DPCs (Figure 4A-E). Relationship analysis was carried out using the parameters mentioned earlier, and a positive correlation ($r = 0.911$) was found between the FA and the NF-κB inhibitor group (Figure 4F). This result indicates that FA acts as a NF-κB inhibitor. We also did a Western blot analysis and EMSA to further confirm the inhibitory effect of FA on NF-κB. Using EMSA, it was found that the expression of p-P65 in DPCs was increased by LPS, and either FA or Bay11-7082 significantly inhibited this effect (Figure 5A). This EMSA result was further confirmed by Western blot analysis (Figure 5B).

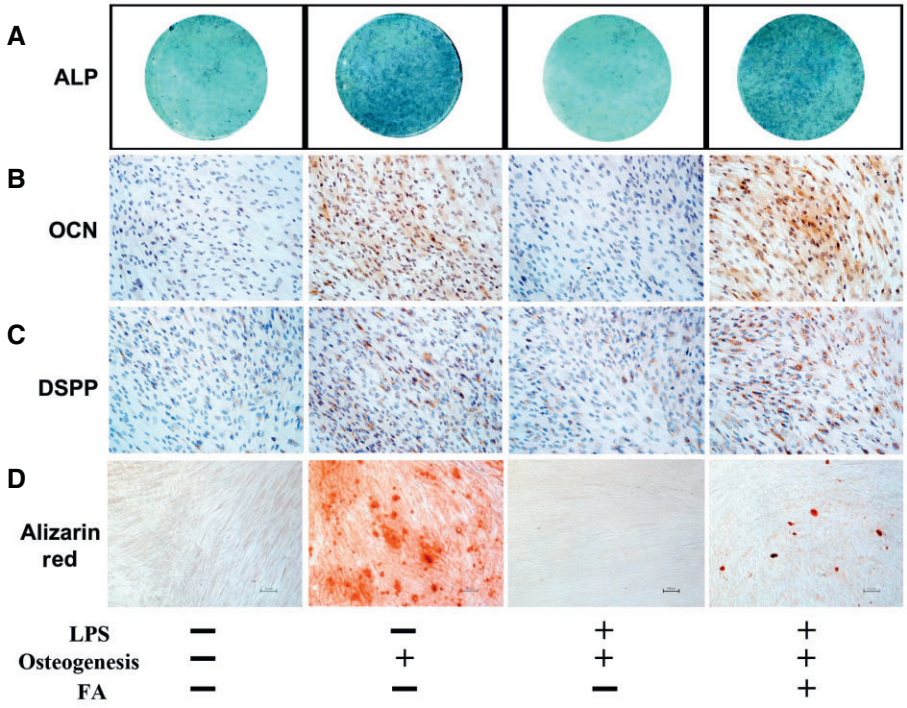

Figure 2

Effects of FA on the mineralization of LPS-stimulated DPCs; 2×10^4 DPCs in 24-well plates were exposed to 1 μg·mL^{-1} LPS with or without 10 μmol·L^{-1} FA in osteogenic media for 7 days or 21 days. (A) The ALP activity of DPCs was tested by ALP histochemical staining on day 7. (B–C) Immunostaining of OCN and DSPP was performed on day 7. (D) The formation of mineralized nodules was detected by alizarin red staining on day 21.

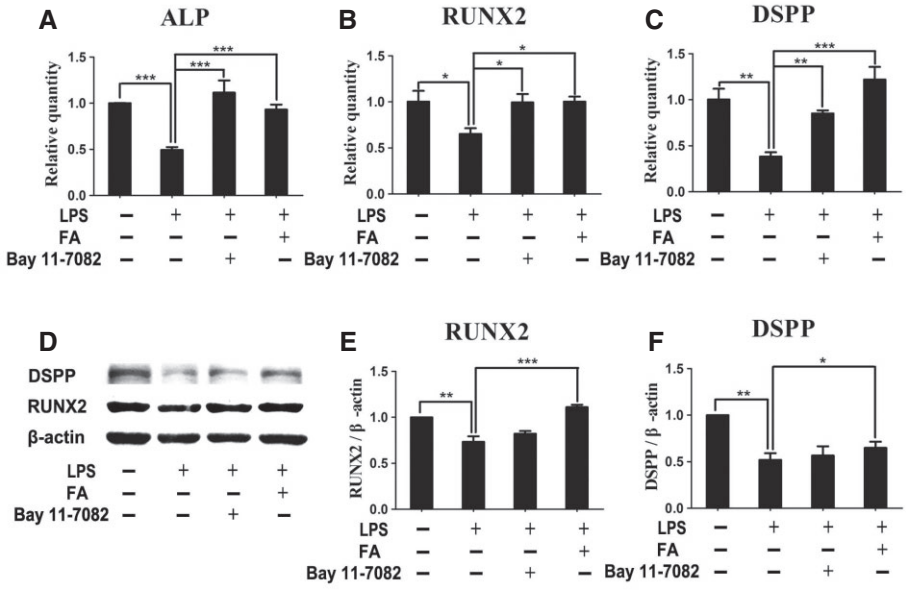

Figure 3

Effects of FA on the expression of osteo-/odonto-genic genes after 3 h in LPS-stimulated DPCs. (A–C) The mRNA expressions of ALP, RUNX2 and DSPP were detected by quantitative PCR. (D) The expressions of RUNX2 and DSPP were examined by Western blot, and (E & F) the quantification of the Western blots was determined by densitometry using Image J software. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

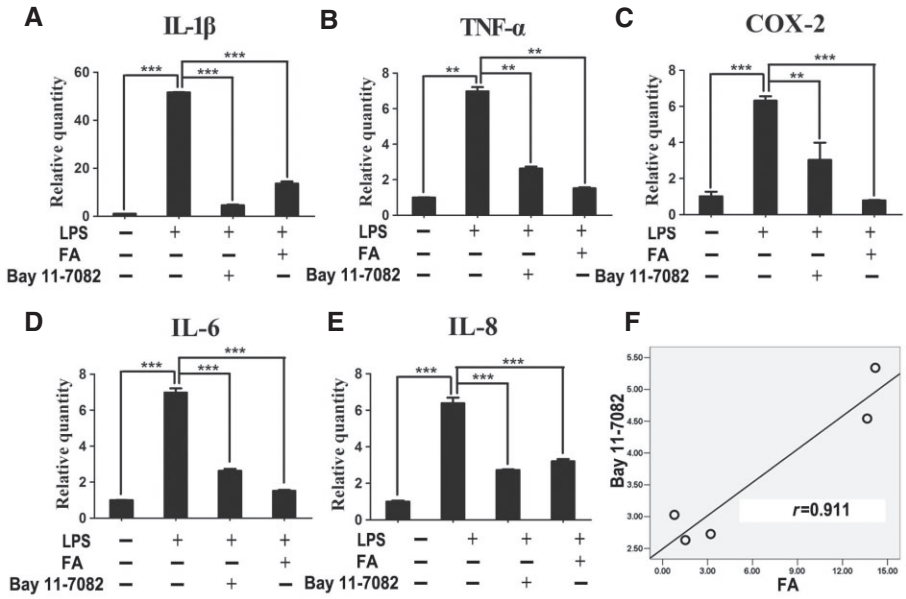

Figure 4

Effects of FA on the expression of inflammatory genes in DPCs in response to LPS for 3 h. DPCs were exposed to LPS for 3 h with either 10 μmol·L^{-1} FA or 5 μmol·L^{-1} of the NF-κB inhibitor Bay11-7082. (A–E) The expression of inflammation-related genes IL-1β, TNF-α, IL-6, IL-8 and COX-2 was detected by quantitative PCR. (F) Relationship analysis was carried out using the parameters mentioned above and a positive correlation ($r = 0.911$) was found between the effects of FA and those of the NF-κB inhibitor. $^{**}P < 0.01$; $^{***}P < 0.001$.

FA up-regulated the expression of osteo-/odonto-genic genes in DPCs through activation of the AP-1 pathway

To investigate whether FA induced mineralization through the AP-1 pathway, we performed a pathway blockade assay. DPCs were exposed to 10 μmol·L^{-1} FA for 3 h. The levels of expression of osteo-/odonto-genic proteins such as DSPP and RUNX2, and AP-1 family members such as Fra-1 and p-c-Jun were assessed by Western blot analysis. It was found that FA up-regulated the expressions of DSPP, RUNX2, Fra-1 and p-c-Jun, and down-regulated that of p-P65 compared with untreated controls (Figure 6A).

Blockade of the AP-1 pathway inhibited FA-triggered mineralization and up-regulated the expression of inflammatory mediators in DPCs

To confirm the role of the AP-1 pathway in FA-induced mineralization and anti-inflammatory effects, DPCs were exposed to 10 μmol·L^{-1} FA and/or 1 μmol·L^{-1} t-RA, an AP-1 pathway inhibitor, for 3 h. Quantitative PCR results showed that FA significantly inhibited the expression of pro-inflammatory genes such as IL-1β and IL-6 and up-regulated the expression of the osteogenesis-related gene ALP in DPCs. When the AP-1 pathway inhibitor was added, these anti-inflammatory and osteo-/odonto-inductive effects of FA were inhibited. The expression of IL-1β and IL-6 were increased and ALP expression was decreased at mRNA level (Figure 6B–D). The ALP mRNA result was further confirmed by ALP histochemical staining, which also showed the same trend for an increased expression of ALP mRNA at 7 days post-treatment in FA-treated DPCs (Figure 6E).

The anti-inflammatory and osteo-/odonto-inductive effects of FA were inhibited by the glucocorticoid receptor antagonist mifepristone

To further verify the anti-inflammatory and osteo-/odonto-inductive effects of FA, DPCs were exposed to 1 μg·mL^{-1} LPS with 10 μmol·L^{-1} FA and/or 1 μmol·L^{-1} mifepristone, a glucocorticoid receptor antagonist, for 3 h. Quantitative PCR results showed that mifepristone suppressed both the anti-inflammatory and osteo-/odonto-inductive effects of FA in DPCs. FA significantly suppressed the expression of the inflammatory genes IL-6 and IL-8 and also markedly increased the expression of the osteogenesis-related genes ALP and RUNX2 in LPS-stimulated DPCs. When mifepristone was added, these anti-inflammatory and osteo-/odonto-inductive effects of FA were clearly reversed; IL-6 and IL-8 expressions were significantly up-regulated, whereas ALP and RUNX2 were significantly down-regulated in DPCs treated with a combination of mifepristone, FA and LPS (Figure 7).

Discussion

LPS is the major element of the outer membrane in gram-negative bacteria and it can induce the expression of inflammatory cytokines and cell apoptosis in DPCs (Massey *et al.*, 1993; Rupf *et al.*, 2000; Tokuda *et al.*, 2001; Yang *et al.*, 2010). In the present study, we showed that LPS could create an inflammatory environment in DPCs by up-regulating the expression of the pro-inflammatory cytokines IL-1β, TNF-α, IL-6, IL-8 and the inflammation-related gene COX-2 and down-regulating that of the transcription factor PPAR-γ. Our

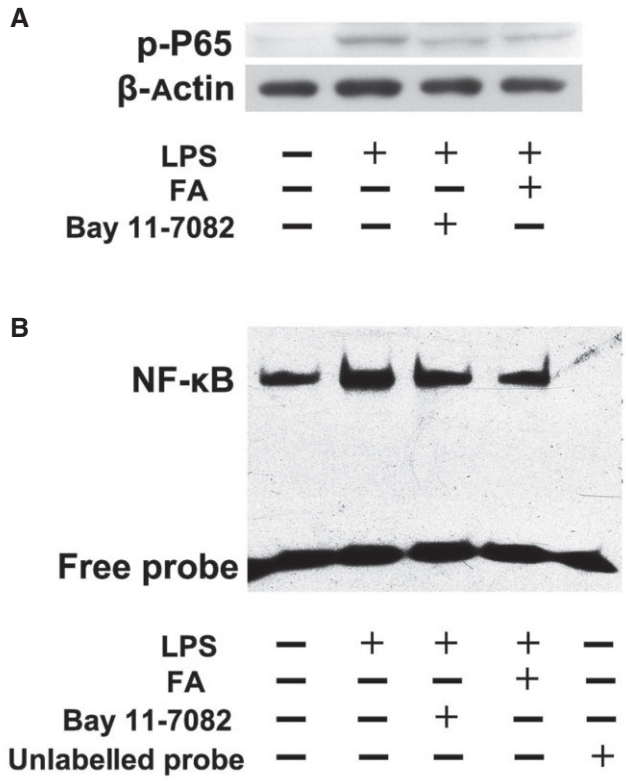

Figure 5

Effects of FA on NF-κB activity in DPCs in response to LPS for 3 h. DPCs were exposed to LPS for 3 h with either 10 μmol·L^{-1} FA or 5 μmol·L^{-1} NF-κB inhibitor Bay11-7082. (A) p-P65 expression was examined by Western blot. (B) DNA-binding activity of NF-κB was assessed by EMSA.

results also indicate that LPS-induced inflammation is the main reason that the ability of injured dental pulp tissues to recover is limited. Our findings corroborate those from many previous studies in other kinds of osteogenic cells. Lacey *et al.* showed that the key pro-inflammatory cytokines IL-1β and TNF-α inhibit the mesenchymal stem cells to differentiate into osteoblasts and suppress the mineralization process (Lacey *et al.*, 2009). Nomiyama *et al.* showed that LPS inhibits the expression of ALP, DSPP and RUNX2 and the formation of mineralized nodules in odontoblasts (Nomiyama *et al.*, 2007). Shanbhag *et al.* verified that local inflammation has a negative effect on new bone formation (Shanbhag *et al.*, 1995). In addition, PPAR-γ has been shown to have an anti-inflammatory effect in inflamed adipose tissue (Foryst-Ludwig *et al.*, 2010). Therefore, both mineralization and an anti-inflammatory effect are needed for restoration of injured dental pulp. Reducing the release of inflammatory mediators may have a positive effect on the restoration of inflamed pulp tissues. However, only a few studies have investigated the effects of inhibiting the release of inflammatory mediators on pulpal inflammation previously (Lee *et al.*, 2008). Based on these results, it could be concluded that the anti-inflammatory effect of FA is induced through up-regulation of PPAR-γ expression.

In the present study, we found that the mineralization-related genes, ALP, RUNX2 and DSPP were suppressed in DPCs treated with LPS for 3 h. Furthermore, the expressions of ALP, OCN and DSPP were down-regulated on day 7 and the formation of mineralized nodules inhibited in LPS-treated DPCs on day 21. These results indicate that inflammation induced by LPS reduces the ability of DPCs to initiate mineralization. We further investigated the effect of FA on osteogenesis by inhibiting the inflammation in DPCs. Our results clearly show that osteogenesis-related proteins and the formation of mineralized nodules could be partially restored

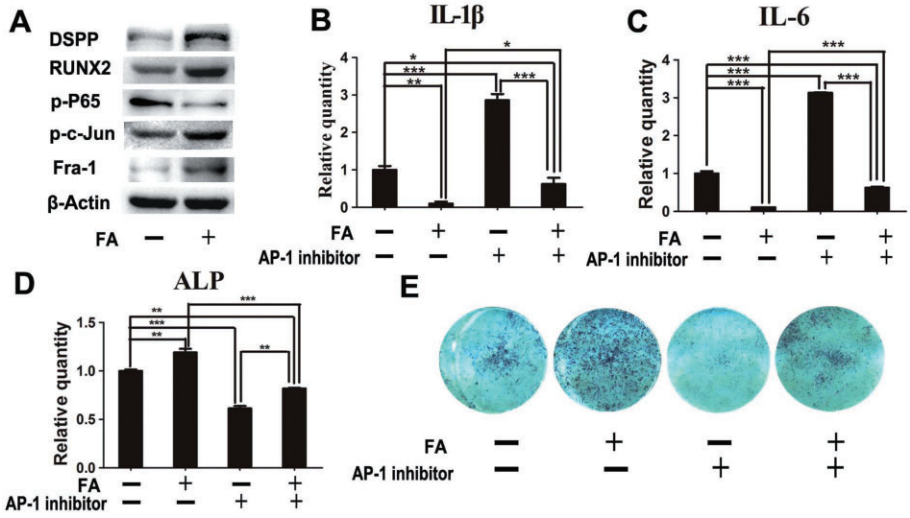

Figure 6

FA affects the expression of the inflammatory genes and osteo-/odonto-genic genes in DPCs partially through activation of the AP-1 pathway. (A) DSPP, RUNX2, p-P65 and AP-1 family members, Fra-1 and p-c-Jun, were determined by Western blot. (B–D) Inflammatory genes including IL-1β and IL-6 expression and the osteogenesis-related gene ALP were detected by quantitative PCR (E) ALP histochemical staining results showing the levels of expression of ALP 7 days post-treatment. *P < 0.05; **P < 0.01; ***P < 0.001.

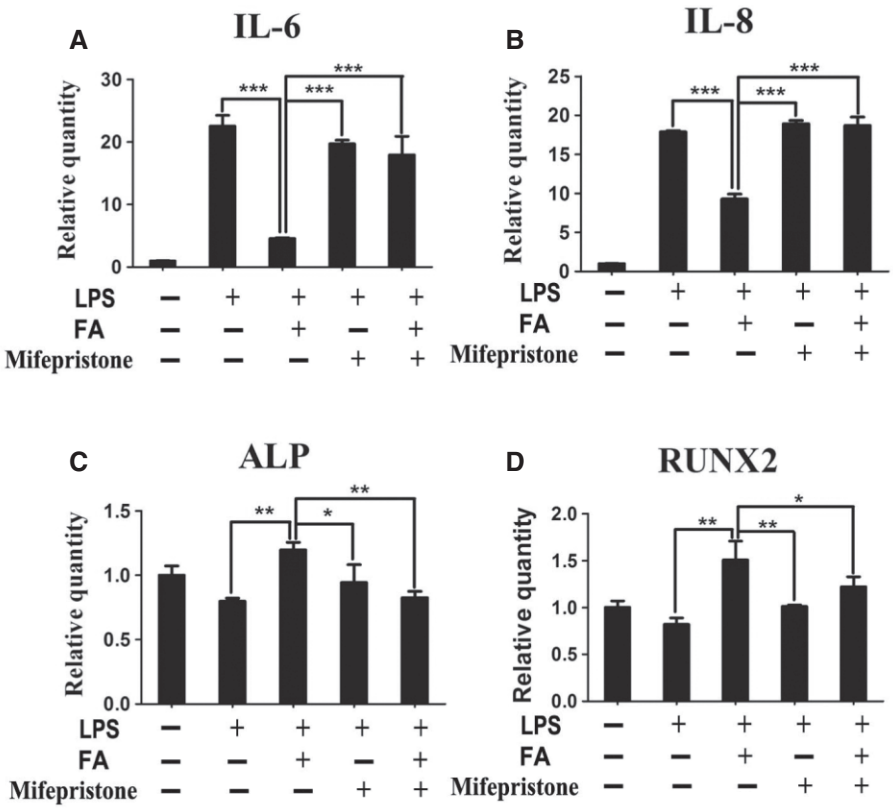

Figure 7

The glucocorticoid receptor antagonist mifepristone suppressed the effects of FA in LPS-stimulated DPCs. (A, B) The expressions of the inflammatory genes IL-6 and IL-8 were up-regulated by mifepristone in LPS-treated DPCs with/without FA. (C, D) Mifepristone was added with or without FA, the expression of osteogenesis-related genes ALP and RUNX2 were down-regulated by mifepristone in LPS-stimulated DPCs. *P < 0.05; **P < 0.01; ***P < 0.001.

by FA on days 7 and 21 in LPS-stimulated DPCs. This indicates that anti-inflammatory drugs could have an osteo-/odonto-genesis effect via inhibition of inflammation in injured osteo-/odonto-genic cells. Our opinion was further confirmed by results from a recent study by Liu *et al.*, who showed that aspirin, a typical non-steroidal anti-inflammatory drug improved the repair of calvarial-based bone marrow mesenchymal stem cells by reducing the release of inflammatory mediators such as IFN-γ or TNF-α (Liu *et al.*, 2011). Hence, we speculate that a steroidal drug such as FA has the ability to promote the remineralizing process in an inflammatory environment and could be used for the regeneration of dental pulp and bone tissue under inflammatory conditions.

Next, we investigated the underlying mechanism of FA-induced odontogenesis of DPCs. Our Western blot and EMSA results revealed that FA similar to the NF-κB pathway inhibitor, Bay 11–7082, down-regulated the expression of pro-inflammatory cytokines in LPS-treated DPCs. This result was consistent with Renard's study, which showed that NF-κB controls the level of many genes in pro-inflammatory, immune responses (Renard and Raes, 1999). In the present study, we confirmed that FA not only induces the expression of mineralization-related biomarkers ALP, RUNX2 and DSPP, but also inhibits the LPS-evoked inflammatory response in DPCs by inhibiting the NF-κB pathway. This indicates that FA has both anti-inflammatory and osteo-/odonto-inductive effects in inflamed DPCs.

AP-1 is a transcription factor mainly consisting of Jun(c-Jun, JunB, JunD) and Fos (Fra-1, Fra-2, c-Fos, FosB) subunits. In a previous study it was reported that inducing the expression of Fra-1 promotes bone formation through inhibition of the NF-κB pathway (Chang *et al.*, 2009). We speculate that FA promotes mineralization through up-regulation of AP-1 activity. Retinoic acid (RA) is a negative regulator of AP-1 and potently inhibits the activity of AP-1 (Schule *et al.*, 1991; Salbert *et al.*, 1993). Therefore, in the present study we used t-RA to inhibit AP-1 activity. In the absence of t-RA, FA up-regulated the expression of AP-1 family members p-c-Jun and Fra-1, significantly suppressed the expression of inflammatory genes IL-1β and IL-6 and up-regulated the expression of osteogenesis-related gene ALP in DPCs. Whereas the presence of t-RA partially inhibited these anti-inflammatory and osteo-/odonto-inductive effects of FA. This indicates that FA mediates its effects on inflammatory and mineral genes in DPCs partially via activation of the AP-1 pathway; and there is probably crosstalk between the NF-κB and AP-1 pathways.

From our results we hypothesize that blockade of the NF-κB pathway inhibits inflammation as well as promoting mineralization and, in turn, blockade of the AP-1 pathway inhibits osteogenesis as well as up-regulating the expression of inflammatory mediators. Hence, FA has not only an anti-inflammatory effect through inhibition of the NF-κB pathway, but also an osteo-/odonto-inductive effect via activation of the AP-1 pathway. To verify this hypothesis, we performed a pathway blockade assay using the glucocorticoid receptor antagonist mifepristone to block the effect of FA. The results clearly showed that mifepristone reduced the anti-inflammatory effects as well as the mineralization potential of FA, and are consistent with those from previous studies (Ayalasomayajula *et al*., 2009; Strehl *et al*., 2011). Ayalasomayajula *et al*. showed that FA inhibited the expression of VEGF via stimulation of glucocorticoid receptors in human retinal pigment epithelial cells. In addition, Strehl *et al*. showed that the steroidal drugs induce their effects through glucocorticoid receptors. Our results indicate that FA acts on both the NF-κB and AP-1 pathways through stimulation of glucocorticoid receptors. Crosstalk between the NF-κB pathway and AP-1 pathway could influence the balance between inflammation and osteogenesis, although the exact mechanism needs to be elucidated in further studies.

In conclusion, LPS decreased the expression of the mineralization-related biomarkers, ALP, OCN, RUNX2 and DSPP, and suppressed the formation of mineralized nodules in DPCs. FA partially restored the osteo-/odonto-genesis process in LPS-treated DPCs, as well as having an anti-inflammatory effect through inhibition of the NF-κB pathway and activation of the AP-1 pathway.

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Conflict of interest

The authors declare that there are no potential conflicts of interest.

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