Baicalein induces apoptosis and reduces inflammation in LPS-stimulated keratinocytes by blocking the activation of NF-κB: implications for alleviating oral lichen planus

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Abstract: Keratinocytes are the major cellular constituent of oral mucosa epithelium in humans. Inflammation and excessive proliferation of keratinocytes contribute significantly to the progression of oral lichen planus (OLP). In general, the pathogenesis of many chronic inflammatory diseases, including OLP, involves in the activation of the nuclear factor-kappa B (NF-κB) signaling pathway. Baicalein (BAI) is an alcohol soluble flavonoid known for its anti-inflammatory effect. However, its effectiveness on keratinocytes in OLP remains unclear. In the present study, we examined inflammation in oral mucosa tissue from OLP patients. Hematoxylin and eosin staining showed denser subepithelial lymphocytes infiltration compared to the normal oral mucosa epithelium. TNF-α and IL-6 were up-regulated in oral mucosa tissue of OLP patients. We next stimulated humans keratinocytes (HaCaT cells) with lipopolysaccharide (LPS) to create an inflammatory environment like that in the OLP tissue and assessed the effect of BAI on OLP and NF-κB signaling pathways. Our results showed that BAI treatment inhibited the level of TNF-α and IL-6 induced by LPS. However, the cells apoptosis was promoted after BAI treatment. Furthermore, BAI not only inhibited LPS-induced p38 MAPK and ERK1/2 phosphorylation, but also NF-κB activation by reducing IκBα phosphorylation and the nuclear translocation of NFκBp65 and NFκB-p50 from cytoplasm to nucleus in keratinocytes. Our findings suggest that BAI inhibits the production of inflammatory cytokines by negatively regulating the NF-κB signaling pathway under LPS simulation in HaCaT cells.

Key words: Baicalein, oral lichen planus, NF-κB, apoptosis and inflammation.

Introduction

Oral lichen planus (OLP) is clinically characterized by symmetric white thread-like plaques in the oral cavity (1). It affects an estimated 1.0% to 2.0% of the general population and equally affects all racial groups (2). Although there are many presenting treatments, some of them proved its failure or only occur chronically and ineffectively. Therefore, exploitation and development of new agents against OLP is necessary. Previous researches reported that patients with OLP showed more lymphocytes infiltration into the epithelial basal cell layer, which was often accompanied by hyperkeratosis (3). Keratinocytes are implicated as being involved in the perpetuation of exacerbation of OLP lesion sites, and these cells may be a potential target for OLP therapy (4). HaCaT cells are perpetuated human keratinocytes, that are easily cultured and can be passaged infinitely (5). In addition, a local immune response can be induced in HaCaT cells by gram-negative bacteria lipopolysaccharide (LPS), creating a cell environment like that found in the OLP tissue.

Nuclear factor-kappa B (NF-κB) is a primary transcription factor and associated with the pathogenesis of several immune disorders, which can be activated by tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). High concentration of TNF-α promotes the pathological events in OLP (6, 7). IL-6 is a critical cytokine, that can be produced by activated keratinocytes in response to induction by various stimuli (8) and a high level of IL-6 has been observed in the serum of patients with OLP (9, 10). A number of studies on OLP patients highlighted a meaningful correlation between the serum and saliva levels of NF-κB-dependent cytokines (4, 11). Several other studies also demonstrated that mitogen-activated protein kinases (MAPKs) was activated in OLP and MAPK pathway was required for NF-κB p65 transactivation (12). In addition, NF-κB as a typical dimer of p50 and the transactivating subunit p65 (RelA), resides in the cytoplasm in unstimulated cells, and the DNA-binding dimer is bound to the inhibitor of NF-κB (IκB). However, the DNA-binding dimer can be released from IκB and migrated into the nucleus after cell stimulation, which effects the expression of numerous target genes (13, 14).

BAI, a member of the flavone family, can be isolated from the roots of the traditional Chinese medical herb Huangqin (Scutellaria baicalensis). Many reports have shown that BAI possesses antioxidative, antiviral, anti-tumor, neuroprotective and anti-inflammatory activities, and thus it has a wide range of pharmacological effects (15). Previous studies have shown that BAI presented no toxicity to animals and humans (16). In human myeloma cells, BAI induced cell death, but it did not affect normal myeloid cells, indicating a selective cytotoxic effects.
effect (17, 18). Recently, BAI has been shown anti-inflammatory effect by inhibiting COX-2 (cyclooxygenase 2) and blocking CCAAT/enhancer binding protein beta (C/EBPβ) DNA binding activity in Raw 264.7 macrophages with NF-κB activated by LPS (19). Hsieh et al. also found that BAI inhibited the production of inflammatory cytokines by inhibiting NF-κB activation as well as IκBα phosphorylation and degradation in human mast cells (20). However, it still remains unclear whether BAI can affect OLP progression. In this study, our aim was to investigate the role of BAI in OLP disease.

Materials and Methods

Antibodies and reagents

The BAI was purchased from Sigma (St. Louis, MO, USA). The anti-TNF-α, anti-IL-6, anti-NF-κB-p65, anti-NF-κB-p50, anti-Bax and anti-Bcl-2 antibodies were from Abcam (Cambridge, UK). The anti-β-actin antibody and anti-mouse secondary antibodies were from Millipore (Boston, MA, USA). Enzyme-linked immunosorbent assay (ELISA) kits of IL-6 and TNF-α were obtained from R&D (Minneapolis, MN). The phospho-specific antibodies were from Cell Signaling Technology (Boston, MA, USA). Fetal bovine serum was from Atlanta Biologicals (Atlanta, GA). The NE-PER nuclear and cytoplasmic extraction kit was from Pierce Biotechnology (Rockford, IL, USA).

Population investigated and ethics statement

The study material was collected from 26 subjects where the first affiliated hospital of Zhengzhou university. The participants’ ages ranged from 15 to 50 years. A total of 19 cases (5 women and 14 men) with OLP occurring on the buccal mucosa were included in the study, and the control group comprised specimens from 11 subjects (5 women and 6 men) with normal buccal mucosa who were recruited for the study. OLP patients had symmetric white thread-like plaques in the oral cavity. None of the subjects had lupus or graft versus host disease. The diagnosis was made on the basis of the modified World Health Organization diagnostic criteria for OLP (21). This study was approved by the ethical committee of The First Affiliated Hospital of Zhengzhou University and had been carried out in accordance with The Code of Ethics of the World Medical Association, and written informed consent was obtained from each subject.

Tissue specimen

After local anesthesia with articaine HCl, a 4-mm punch biopsy of lesional tissue extending into the submucosa was collected from each patient, while normal oral mucosa tissue was collected from the control group participants. The mucosa tissue samples were immediately frozen at -70°C until the beginning of analysis.

Cell culture

Cell line HaCaT was purchased from Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China) and cultured in T-75 flasks with high glucose DMEM media with 10% FBS, 20 μM L-glutamine and Ampcillin/Streptomycin in a 37°C incubator with 5% CO₂. To develop a local inflammatory environment like that in OLP tissue, HaCaT cells were starved in serum-free medium for 24 h and then treated with 10 μg/mL of LPS purified from Escherichia coli for up to 24 h under serum-free conditions. To investigate the anti-inflammatory roles of BAI in OLP, HaCaT cells were treated with BAI (30 μM) for 24 h after addition of LPS.

HE staining and evaluation

The mucosa tissue samples were fixed in 10% buffered formalin for 48 h and then dewaxed in xylene, rehydrated with a series of graded alcohols, and then embedded in paraffin. These samples were cut into 4 μm thick sections and stained with hematoxylin and eosin (HE) for histopathological analysis under light microscope using a Zeiss microscope.

Western blot

Radioimmunoprecipitation lysis buffer and cytoplasmic extraction kit were used to isolate the nuclear and cytoplasmic fraction from the HaCaT cells according to the manufacturer’s instructions. Cytoplasmic and nuclear proteins were separated by 10% SDS–PAGE electrophoresis and transferred on to PVDF membranes. After the transfer, the membranes were blocked in 5% free-fat dry milk containing 0.1% Tween-20 for 2 h. The PVDF membranes were immunoprobed with the interest antibody at 4 °C overnight, and then incubated in goat anti-rabbit Ig G for 1 h at room temperature. The conjugated peroxidase was visualized by enhanced chemiluminescence according to the manufacturer's instructions. The lanes were calculated by the Quantity One software, and the values were expressed as an optical density ratio with respect to β-actin.

Enzyme-linked immunosorbent assay

After BAI treatment for 24 h, medium from the cultured HaCaT cells was collected and centrifuged at 500 g for 5 min. The IL-6 and TNF-α levels in the supernatant were determined using IL-6 and TNF-α ELISA kits respectively. The commercially available NF-κB-p65 and NF-κB-p50 transcription factor ELISA assay kits were used according to the manufacturer’s protocol.

NF-κB p65 DNA-binding activity

DNA-binding activity of the p65 NF-κB and p50 NF-κB subunits were determined in protein fraction using the ELISA kits (Active Motif, Rixensart, Belgium). All procedures were performed according to the manufacturer's instructions. Activity at baseline is expressed as absorbance at 450 nm.

Real-time PCR

The total RNA was extracted from HaCaT cells using Trizol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed using RevertAid First Strand cDNA Synthesis Kit. Maxima SYBR Green Master Mix (2X) was obtained from Thermo scientific, and the manufacturer’s instructions were followed. The quantitative primers for NF-κB-p65 was designed and synthesized by Ribobio Inc. (Guangzhou, China). Data were analyzed using the comparative 2−ΔΔCt method.

Flow cytometry analysis

Analysis of apoptosis was performed by using An-
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Statistical analysis

All values are expressed as mean ± SD. Statistical comparisons were made between groups with one-way ANOVA using the software SPSS 18.0. A \( P < 0.05 \) was regarded statistically significant.

Results

Inflammation in oral mucosa tissue of OLP patients

To explore the inflammatory status in oral mucosa tissue of OLP patients, we performed HE staining to observe the subepithelial lymphocytes. In the OLP group (Fig. 1B), a band of infiltrating lymphocytes were observed close to the subepithelium, and the basal cells had liquefied and disappeared. In addition, tissue from OLP patients showed denser subepithelial lymphocytes infiltration \( (P < 0.05) \) compared with the control group (Fig. 1A). We also investigated the expression of TNF-α, IL-6, and NF-κB-p65 in oral mucosa tissue (Fig. 1C) by Western blotting. The expression of TNF-α, IL-6, and NF-κB-p65 in the OLP group were higher than in the control group \( (P < 0.05, \text{respectively}) \). Our present results not only confirmed the presence of inflammation but also suggested that NF-κB pathway might involve in the pathogenesis of OLP.

BAI induced apoptosis and attenuated LPS-induced inflammation in HaCaT cells

To determine whether BAI treatment could influence the apoptosis in keratinocytes, the percentage of apoptotic cells in BAI-treated HaCaT cells was evaluated by FCM assay. The percentage of early apoptotic cells was 8.19% in the control group and 0.74% for the LPS group, the percentage increased to 8.52% in the BAI-treated group (Fig. 2A). In addition, we tested the expression of Bcl-2 and Bax in HaCaT cells (Fig. 2B). Bax and Bcl-2 proteins level among groups were detected by Western blot after treating with BAI. C. IL-6 and TNF-α protein secretion among groups were analyzed with the ELISA assay. D. NF-κB-p65 mRNA level among groups were detected with RT-PCR. Values are the means ± SD (n = 8). * \( P < 0.05 \) and ** \( P < 0.01 \) vs. CON group; # \( P < 0.05 \) vs. LPS group.

BAI inhibited phosphorylation of p38MAPK, ERK1/2, and IκBα

Based on the above results, to determine whether the inhibition of BAI to NF-κB-p65 was correlated with NF-κB pathway, we subsequently investigated the key enzymes in activation of the NF-κB (Fig. 3A-3C). Our results showed that LPS increased the p38MAPK \( (P < 0.05) \), but not ERK1/2. In the presence of BAI, the p38MAPK protein was decreased \( (P < 0.05) \) compared to the LPS alone group. At the same time, decreased IκBα was found in the LPS group \( (P < 0.05) \) and BAI treatment rescued the down-regulated IκBα induced by
LPS ($P < 0.05$).

We next examined the effect of BAI on IκBα, p38MAPK, and ERK1/2 phosphorylation in HaCaT cells (Fig. 3A-3C). There were higher level of IκBα, p38MAPK, and ERK1/2 phosphorylation in the LPS group compared to the control group ($P < 0.05$, respectively). BAI treatment significantly prevented phosphorylation of IκBα, p38MAPK and ERK1/2 ($P < 0.05$, respectively) induced by LPS. The consistent results of p-p38MAPK/p38 MAPK, p-ERK/ERK and p-IκBα/IκBα ratio (Fig. 3A, B and C) showed densitometric analysis for relative expression of p-p38MAPK/p38 MAPK, p-ERK/ERK and p-IκBα/IκBα. Values are expressed as means ± SD (n = 8). *$P < 0.05$ and **$P < 0.01$ vs. CON group; # $P < 0.05$ vs. LPS group.

**Discussion**

Here, our results from HE staining and evaluation of inflammatory cytokine expression show that patients with OLP experience inflammation in the oral mucosa tissue. To investigate the effects and mechanisms of BAI on OLP, we simulated an inflammatory environment similar to that in the OLP tissue via stimulation of HaCaT cells with LPS. After treatment with BAI, the inflammation was alleviated while the apoptosis of keratinocytes was promoted. Furthermore, BAI inhibited LPS-induced NF-κB activation by suppressing IκBα phosphorylation.

Up-regulation of TNF-α and IL-6 is known to usually accompany with activation of NF-κB (22). NF-κB activation involves IκBα degradation, IκBα phosphorylation, and translocation of NF-κB from the cytoplasm to the nucleus (13, 14). Meanwhile, NF-κB activation has been shown to have an anti-apoptotic role. Ge et al. reported that caspase-8 was up-regulated in LPS-induced keratinocytes, which was positively correlated with NF-κB-p65(4). It is worth noting that the expression of p38 MAPK in response to TNF-α further induces the activation of NF-κB (23). Our present results further supported that TNF-α, IL6 and their upstream NF-κB were activated in the oral mucosa of patients with OLP and in vitro model with HaCaT cells. We also observed that apoptosis of HaCaT cells was reduced when NF-κB was activated through p38 MAPK-regulated IκBα phosphorylation.
rylation. All of these findings suggest that NF-κB signaling pathway may be associated with the perpetuation processes of OLP.

In recent reports, BAI has been proved to exert a strong inhibitory effect on inflammation in various diseases. Mabalirajan et al. found that BAI treatment for allergic and airway inflammation induced by IL-13 in mice reduces airway epithelial injury and various features of airway inflammation, which could have important implications in developing therapeutic strategies for airway injury in asthma (24). Orally administered BAI could inhibit inflammation induced by intraperitoneal injection of LPS in mice (25), and BAI may be a therapeutic candidate for acute liver injury by accelerating liver regeneration (26). In the present study, treatment with BAI significantly decreased the LPS-induced inflammatory cytokine expression in keratinocytes. Therefore, we confirmed that BAI could strongly inhibit inflammation in immune circumstance in vitro OLP model as well, which suggests BAI may be clinically useful for OLP.

BAI alleviate inflammation through inhibiting the activation of NF-κB or/and MAPKs signaling pathways in many inflammatory diseases. In mice, BAI attenuates inflammatory responses by suppressing NF-κB and MAPK signaling pathways in LPS-induced mastitis (27), which suggest that BAI may be a potential agent for prophylaxis of LPS-induced mastitis. BAI may inhibit IL-1β and TNF-α induced inflammation in human mast cells by suppressing IkBα phosphorylation (28), which would hence restrain activation of NF-κB. Combination of baicalin and baicalein (BAI) enhanced. Combination of baicalin and BAI enhanced the human breast cancer cells apoptosis, by activating caspase-3 and caspase-9 and down-regulating the level of bcl-2, and up-regulating the level of Bax via the ERK/p38 MAPK pathway (29). Bidya Dhar Sahu et al. found that BAI inhibited the cisplatin-induced expression of nitric oxide synthase and concealed redox-sensitive transcription factor NF-κB activation via reduced DNA-binding activity and p38 MAPK, ERK1/2, c-Jun N-terminal kinase (JNK), and IkBα phosphorylation in kidneys. Furthermore, BAI preserved cisplatin-induced apoptosis by suppressing Bax expression and Bax/Bcl-2 imbalance during this process (30). In the present study, we noted that treating HaCaT cells with BAI blocked LPS-induced apoptosis and suppressed the release of TNF-α and IL-6. Meanwhile, BAI attenuated NF-κB activation by inhibiting phosphorylation and degradation of IkBα and subsequently inhibited LPS-induced inflammation in keratinocytes. Our experiments provide evidence that BAI restrains the inflammation development by inhibiting NF-κB signaling in OLP. Since IkBα phosphorylation is the key point for NF-κB activation, inhibition of this action by BAI is essential to its mechanism.

Taken together, our present results indicate that BAI may be a novel potential candidate for OLP therapy. The protective effects of BAI seem to be due to up-regulation of apoptosis and down-regulation inflammation on inhibition of the NF-κB signaling pathways. The physiological inhibitory effect of BAI on the NFκB-p65 activation pathway and its pro-apoptotic effect have major implications for therapeutic strategies targeting NF-κB and apoptosis under the immune circumstance of the OLP patients.

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