Prunella vulgaris extract and rosmarinic acid suppress lipopolysaccharide-induced alteration in human gingival fibroblasts

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ABSTRACT
Periodontitis is a chronic disease associated with inflammation of the tooth-supporting tissues. The inflammation is initiated by a group of gram-negative anaerobic bacteria. These express a number of irritating factors including a lipopolysaccharide (LPS), which plays a key role in periodontal disease development. Plant extracts with anti-inflammatory and anti-microbial properties have been shown to inhibit bacterial plaque formation and thus prevent chronic gingivitis. In this study we tested effects of Prunella vulgaris L. extract (PVE; 5, 10, 25 µg/ml) and its component rosmarinic acid (RA; 1 µg/ml) on LPS-induced oxidative damage and inflammation in human gingival fibroblasts. PVE and RA reduced reactive oxygen species (ROS) production, intracellular glutathione (GSH) depletion as well as lipid peroxidation in LPS-treated cells. Treatment with PVE and RA also inhibited LPS-induced up-regulation of interleukin 1α (IL-1α), interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α) and suppressed expression of inducible nitric oxide synthase (iNOS). The results indicate that PVE and RA are able to suppress LPS-induced biological changes in gingival fibroblasts. The effects of PVE and RA are presumably linked to their anti-inflammatory and cytoprotective activities and thus use of PVE and RA may be relevant in modulating the inflammation process, including periodontal disease.

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1. Introduction

The relationship between dental plaque and inflammatory periodontal diseases is well known (Teles et al., 2006). Gingivitis begins with dental plaque that is formed by a specific group of bacteria representing more than 300 different species. These produce a number of metabolites which irritate gingival tissue. One of the irritating agents is LPS, the major constituent of outer bacterial membrane, which is a critical determinant in gingivitis initiation and progression. A primary target of LPS are gingival fibroblasts, main constituents of gingival connective tissue which play an important role in the remodelling of periodontal soft tissues (Wang and Ohura, 2002). Several reports have pointed to a possible relationship between periodontitis and expression of pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-α (Prabhu et al., 1996; Yamazaki et al., 1997; Okada and Murakami, 1998). In addition to stimulation of cytokines, LPS production by bacteria is also known to provoke expression of inducible form of NOS (Kim et al., 2007b). Further, ROS such as hydrogen peroxide, singlet oxygen, superoxide radical and hydroxyl radical are frequently detected at regions of tissue inflammation. Imbalance between ROS generation and ROS elimination leads to alteration in cellular metabolism. Exposure of the tissues to increased level of ROS may lead to damage of lipids, proteins and DNA. The adverse effects of ROS can be prevented or stopped via the administration of antioxidants and radical scavengers (Hutter and Greene, 2000; Saccani et al., 2000). Polyphenolic compounds have been reported to assuage both inflammation and oxidative stress (Kim et al., 2007a).

Prunella vulgaris L. (Labiatae) is used in Western and Chinese herbal medicine, especially in the treatment of wounds. It has a wide spectrum of biological effects including anti-microbial, anti-inflammatory and immunomodulatory and thus it has considerable therapeutic potential (Psotová et al., 2003). Its anti-microbial effects include the growth inhibition of Pseudomonas, Bacillus typhi, Escherichium coli, Mycobacterium tuberculi etc. The immune stimulatory and anti-inflammatory effects of P. vulgaris are both receiving increasing interest (Kim et al., 2007c; Fang et al., 2005).

Adámková et al. (2004) performed a 64-day clinical trial to assess the effectiveness of a herbal-based dentifrice containing P. vulgaris and Macleaya cordata extracts in the control of gingivitis. The dentifrice was effective in reducing symptoms of gingivitis, particularly plaque index, community periodontal index of treatment needs and papillary bleeding index (Adámková et al., 2004).

However, no studies have been conducted to evaluate the potential effects of P. vulgaris extract (PVE) and rosmarinic acid (RA), the major phenolic component of the plant, on inflammation and ROS generation in gingival tissues. Therefore, in this work we attempted to assess the effects of PVE and RA on parameters of oxidative stress and inflammation in human gingival fibroblasts.
2. Material and methods

2.1. Materials

Protease inhibitor cocktail tablet (Complete™) was purchased from Roche, Germany. 2,2′-dinitro-5,5′-dithiobenzoic acid was purchased from Serva, Germany. iNOS rabbit polyclonal antibody, actin (1–19) goat polyclonal antibody, horseradish peroxidase conjugated goat anti-rabbit and rabbit anti-goat antibodies, Western Blotting Luminol Reagent were received from Santa Cruz Biotechnology, USA. Dulbecco’s modified Eagle’s medium and heat-inactivated foetal calf serum were purchased from R&D Systems, USA. Rosmarinic acid, penicillin, streptomycin, lipopolysaccharide (from Escherichia coli O55:B5), Immun-Blot™ PVDF (polyvinylidene difluoride) membrane, KO-DAK BioMax light film, phenylmethylsulfonyl fluoride, dihydrodihydroxyamine 123 and all other chemicals were purchased from Sigma-Aldrich, USA.

2.2. Plant material (P. vulgaris extract)

PVE was prepared and kindly provided by IVAX Pharmaceuticals (Opa, Czech Republic). The aerial part of P. vulgaris was collected in bloom in July 2004. The cut dry plant (10.45 kg) was extracted with aqueous ethanol (30% v/v). The primary extract was concentrated on a vacuum rotary evaporator to dryness and further dried in a vacuum dryer (70 °C; 10 mbar) yielding 2.48 kg of the extract. The PVE was characterised by the RA content (9.0% w/w; determined by HPLC).

2.3. Cell culture

Human gingival fibroblasts were obtained from an explant culture of healthy human gingiva. Tissue acquisition protocol was in accordance with the requirements issued by the Ethics Commission of the Czech Republic.

The gingival tissues were taken from the buccal gingiva or interdental papilla. After washing three times in phosphate buffered saline (PBS) with antibiotics (pamycon and colinomycin) the excised gingiva were cut into 1 mm pieces, plated in Petri dishes (10 cm diameter) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum (FCS), penicillin (100 μg/ml) and streptomycin (100 μg/ml). The explant was incubated in humidified atmosphere with 5% CO2 at 37 °C. Cell cultures were fed weekly until the fibroblasts reached confluence. After 4–6 weeks cells were trypsinized and transferred into 75 cm² cultivation flasks. For all experiments the cells were trypsinized and seeded in plates at a density of 105 cells/cm². Cells were used between the 3 and 20 passages for experiments.

2.4. Cell viability

To exclude a possible toxic effect of LPS, PVE and RA on fibroblasts, cell viability was determined by neutral red retention assay.

The cells on 96-well microplates were treated with LPS (0.001–10 μg/ml), PVE (5, 10, 25 μg/ml) or RA (1 μg/ml) in serum-free medium for 4 and 24 h. Control cells were treated with a medium containing dimethylsulfoxide (DMSO; 0.5%, v/v) and sonicated (10 min, 13,000 rpm, 4 °C) and the supernatant was used in the cells viability assay. After incubation period, neutral red (NR; 0.03%, v/v) in PBS was added to the cells (2 h; 37 °C). Then fibroblasts were washed with a mixture of formaldehyde (0.125%, v/v) and CaCl2 (0.25%, w/v) and the retained NR was dissolved using acetic acid (1%, v/v) in methanol (50%, v/v). The plates were read on a microplate reader (Sunrise, Schoeller Instruments, Austria) at 540 nm.

2.5. Treatment of cells

E. coli LPS is the most commonly used LPS in vitro studies and it was therefore employed in the present experiments. LPS stock solutions (0.0002–2 mg/ml) were prepared in sterile water. Stock solutions of PVE (1, 2, 5 mg/ml) and RA (0.2 mg/ml) were prepared in DMSO. The final concentration of DMSO in serum-free medium was 0.5% v/v.

For optimization of LPS-induced oxidative damage and inflammation the cells were treated with LPS (0.1–10 μg/ml; 4 and 24 h) in serum-free medium. After the incubation the media were collected and frozen (−80 °C) for IL-1β, IL-6, TNF-α evaluation. Cells were washed with PBS and harvested for determination of ROS production, lipid peroxidation, level of intracellular GSH and iNOS expression.

For the evaluation of PVE and RA effects on LPS-induced oxidative damage and production of pro-inflammatory cytokines, the gingival fibroblasts were pre-treated with LPS (1 and 10 μg/ml; 4/24 h) in serum-free medium, the medium was then removed, cells were washed with PBS and PVE (5, 10, 25 μg/ml) or RA (1 μg/ml) in serum-free medium were applied for 4 h. After the incubation period the medium was collected and frozen (−80 °C) for measurement of IL-1β, IL-6, TNF-α; cells were washed with PBS, harvested and ROS production, lipid peroxidation, level of intracellular GSH and iNOS expression were evaluated.

2.6. Determination of ROS production

To measure the rate of LPS-induced ROS production and the scavenging effect of PVE and RA in living cells, dihydrorhodamine 123 (DHR) assay was performed (Royall and Ischiropoulos, 1993).

Briefly, after the incubation period, the medium was collected for the determination of ROS production, lipid peroxidation, level of intracellular GSH and iNOS expression. The cells on 96-well microplates were treated with LPS (1 and 10 μg/ml; 4/24 h) in serum-free medium were applied for 4 h. After the incubation period the medium was collected and frozen (−80 °C) for measurement of IL-1β, IL-6, TNF-α; cells were washed with PBS, harvested and ROS production, lipid peroxidation, level of intracellular GSH and iNOS expression were evaluated.

The cells were washed with cooled PBS, scraped into the cooled perchloric acid (1%, v/v) and sonicated. The aliquots were used for protein determination by Bradford assay. The suspension was centrifuged (10 min, 13,000 rpm, 4 °C) and the supernatant was used for estimation of GSH, based on the reaction with 2,2′-dinitro-5,5′-dithiobenzoic acid (Sedlak and Lindsay, 1968). The absorbance was read on a microplate reader at 412 nm.

2.8. Lipid peroxidation

The cells rinsed with cooled PBS were scraped into trichloroacetic acid (2.8%, w/v), sonicated and aliquots were used for protein determination by Bradford assay. The suspension was centrifuged (10 min, 13,000 rpm, 4 °C) and the supernatant was used for estimation of GSH, based on the reaction with 2,2′-dinitro-5,5′-dithiobenzoic acid (Sedlak and Lindsay, 1968).

2.9. Cytokine determination

The effect of LPS, PVE and RA on IL-1β, IL-6 and TNF-α were determined using specific immunoassays (Quantikine®). The assays were performed according to the manufacturer’s protocols. Briefly, after the incubation period, the medium was collected for the evaluation of LPS-induced oxidative damage and inflammation.
3. Results

3.1. Cell viability

To exclude the possible toxic effect of PVE (5, 10, 25 μg/ml) and RA (1 μg/ml) on human gingival fibroblasts, microscopic changes and NR retention was monitored after 4 and 24 h treatment. Using an inversion microscope (Olympus, Japan) we found no visible alteration to fibroblasts monolayer. Neither PVE nor RA caused any changes in NR retention at concentrations tested after 4 and 24 h treatment. The concentration (5, 10, 25 μg/ml) of PVE and RA did not cause any augmentation of fibroblasts viability. We observed no microscopic changes and NR incorporation was not affected after 4 and 24 h compared to untreated cells.

3.2. Effect of PVE and RA on LPS-induced oxidative damage

In order to evaluate the ability of PVE and RA to reduce ROS generation in LPS-stimulated cells, total ROS levels were measured using DHR as a probe. We found that PVE (5, 10, 25 μg/ml) and RA (1 μg/ml) themselves did not increase basal ROS production (Fig. 1A) in gingival fibroblasts; on the contrary PVE at the concentration of 25 μg/ml non-significantly reduced the ROS level in gingival fibroblasts. In fibroblasts treated with LPS (0.001–10 μg/ml; 4 h) we observed a dose-dependent increase in ROS generation (Fig. 1B). The concentration of 1 μg/ml significantly increased ROS production and thus was chosen for monitoring of the PVE/RA protective effect. Application of both PVE and RA to LPS-treated cells (1 μg/ml) resulted in reduction of the ROS level after 4 h (Fig. 1C). The effect of PVE was dose-dependent.

As elevated ROS production is associated with oxidation of cellular molecules, we further evaluated effect of PVE and RA on GSH, one of the oxidative stress markers. GSH is the major intracellular antioxidant and its decline is linked to oxidative damage (Rousar et al., 2005). As shown in Fig. 2. PVE (5, 10, 25 μg/ml) and RA (1 μg/ml) themselves did not affect the GSH level (Fig. 2A) in cells after 4 h application. In cells treated with LPS (0.001–10 μg/ml; 4 h), a dose-dependent decrease in intracellular GSH was evident, particularly at the concentration of 0.1 μg/ml and higher (Fig. 2B). LPS-induced (1 μg/ml) depletion of intracellular GSH level was reversed by PVE (in a dose-dependent manner) and RA treatment (Fig. 2C). These results are in agreement with the ROS generation data.

ROS overproduction is also connected with increased levels of lipid peroxidation in cell biomembranes. PVE (5, 10, 25 μg/ml) and RA (1 μg/ml) themselves did not cause any augmentation of

2.10. iNOS expression (Western blot analysis)

The cells were properly washed with cold PBS and scraped into the ice-cold lysis buffer (20 mM Tris, 5 mM EGTA, 150 mM NaCl, 20 mM glycerol phosphate, 1 mM NaF, 1% Triton X-100, 1 mM Na3VO4, 0.1% Tween 20, protease inhibitor cocktail tablet). After incubation (15 min, 4°C) the lysate was cleared by centrifugation (14,000 rpm, 10 min, 4°C). The supernatant protein concentration was determined by Lowry assay. Proteins were separated by 8% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. Residual binding sites on the membrane were blocked using blocking buffer (5% non-fat dry milk (w/v) in 100 mM Tris-buffered saline (pH 7.5) with Tween 2% (v/v); 1 h; room temperature). The membrane was then incubated with a primary antibody (iNOS rabbit polyclonal antibody or actin (1–19) goat polyclonal antibody, overnight, 4°C) and then with a secondary horseradish peroxidase conjugated antibody or actin (1–19) goat polyclonal antibody, 2 h, room temperature). iNOS and actin expression was detected by chemiluminescence using Western Blotting Luminol Reagent and autoradiography using a KODAK BioMax light film.

2.11. Statistical analysis

Data are expressed as means ± SD of three independent experiments performed in triplicate for each sample. The Student’s t-test was used for statistical analysis. Statistical significance was determined at p < 0.05.
TBARS after 4 h application (Fig. 3A). Moreover, in cells treated with the highest concentration of PVE (25 µg/ml) the TBARS level was lower than that of control cells (91%). This finding is in agreement with the results of DHR assay. LPS (0.001–10 µg/ml; 4 h) caused a dose-dependent increase in TBARS level. The maximal damage was observed at concentrations of 1 and 10 µg/ml (Fig. 3B). As shown in Fig. 3C, application of PVE and RA markedly reduced LPS-induced (1 µg/ml) TBARS level in fibroblasts. The maximal diminution was found in cells treated with PVE at the concentration of 10 µg/ml.

3.3. Effect of PVE and RA on LPS-stimulated pro-inflammatory cytokines

IL-1β, IL-6 and TNF-α are known pro-inflammatory mediators that possess a multitude of biological activities linked to chronic inflammatory diseases (Okada and Murakami, 1998). Therefore, we examined whether PVE and RA (4 h) are capable of modulating cytokine production in gingival fibroblasts pre-treated with LPS (10 µg/ml; 24 h), using specific ELISA kits. As shown in Fig. 4A, PVE (25 µg/ml) and RA (1 µg/ml) themselves did not increase expression of IL-1β. PVE (5 and 10 µg/ml) reduced the level of IL-1β compared to the control. Neither PVE (5, 10, 25 µg/ml) nor RA (1 µg/ml) influenced the level of IL-6 (Fig. 4A). TNF-α was not affected by PVE (5, 10, 25 µg/ml) treatment. However, application of RA (1 µg/ml) decreased the amount of TNF-α (Fig. 6A). LPS (0.001–10 µg/ml) itself induced production of the cytokines in gingival fibroblasts (Fig. 4B, 5B, 6B) in a dose dependent manner. LPS-stimulated (10 µg/ml) expression of IL-1β (20-fold increase in comparison to control cells, Fig. 4C), IL-6 (25-fold increase in comparison to control cells, Fig. 5C) and TNF-α (15-fold increase in comparison to control cells, Fig. 6C) was significantly blocked in fibroblasts treated with PVE (5, 10, 25 µg/ml; concentration dependently) and RA (1 µg/ml). Effect of RA on IL-6 expression was comparable with PVE (25 µg/ml). However its effect on IL-1β and TNF-α decrease was 2–3 times stronger than PVE at the highest concentration tested.

3.4. Effect of PVE and RA on LPS-induced iNOS expression in human gingival fibroblasts

iNOS expression is strongly associated with cytotoxicity and tissue damage, and is involved in a number of processes such as inflammation and immunoregulation (Kim et al., 2007b). Thus we also investigated the PVE and RA effect on LPS-induced iNOS expression in human gingival fibroblasts (Fig. 7). The results showed that treatment with PVE and RA did not affect iNOS expression while LPS (10 µg/ml) induced its expression. We further
found that PVE at the concentration of 10 μg/ml and RA (1 μg/ml) suppressed LPS-induced iNOS expression.

4. Discussion

Human gingival fibroblasts play a key role in periodontal disease initiation and development. These are the main cell type in gingival tissue and thus represent the major target for oral bacteria endotoxins such as LPS, which irritate the cells and provoke several reactions. Regulation of gingival fibroblasts responses therefore may be one way of preventing and/or control periodontal disease (Bodet et al., 2007b). Polyphenols exhibit a variety of biological and pharmacological activities, including antioxidant, anti-inflammatory, anti-bacterial, or immunomodulatory. Moreover, polyphenols have been found to inhibit chronic inflammation in several experimental models (Kim et al., 2007a). They also prevent tissue damage and stimulate wound healing (Phan et al., 2001). In this regard, polyphenols (e.g. PVE and RA) would be suitable candidates for a gingival tissue inflammation treatment.

Periodontal disease is also connected with increased ROS production (Kim do et al., 2007). Reactive oxygen intermediates are released from phagocytic cells that are important for regulating microbial activity. However, a high level of ROS may be harmful to surrounding cells and tissue matrix components (Skaleric et al., 2000). The imbalance between ROS production and their elimination may cause damage to cellular macromolecules in gingival fibroblasts. ROS are capable of oxidizing lipids, proteins and DNA that lead to the formation of oxidized products such as lipid hydroperoxides, protein carbonyls or 8-hydroxyguanosine (Hutter and Greene, 2000; Saccani et al., 2000). Oxidative damage of lipids affects mainly polyunsaturated fatty acids in biological membranes and induces further pro-oxidants generation. Membrane destruction is linked to a loss of fluidity, inactivation of membrane enzymes, increase in cell membrane permeability to ions and eventually to rupture of membrane and release of organelles (Girotti and Kriska, 2004). Both ROS and oxidized molecules also affect various cellular pathways and gene expression. Fibroblasts are equipped with an enzymatic and non-enzymatic defence system consisting of several endogenously produced scavenging molecules that suppress ROS-caused injury (Yang et al., 2003). Among these GSH is the most important. It is necessary for maintaining the intracellular redox state (Rousar et al., 2005) and intracellular levels of GSH have been identified as a critical regulator of the induction of stress-activated signal transduction pathways (Wilhelm et al., 1997). Further, GSH is necessary for DNA and protein synthesis, cell proliferation and apoptosis, cytokine production and immune response (Bagchi et al., 2006). In an earlier report,
an aqueous extract of \textit{P. vulgaris} was demonstrated to have strong superoxide- and hydroxyl radical-scavenging activity (Liu and Ng, 2000). Psotová et al. also showed that the organic fraction of \textit{P. vulgaris} (25.7\% RA, w/w) scavenged the DPPH radical and inhibited human LDL Cu(II)-mediated oxidation in vitro (Psotová et al., 2003). In our experiments we showed that both PVE and RA have a strong ability to eliminate LPS-induced ROS generation as well as lipid peroxidation. PVE and RA also efficiently suppressed LPS-caused depletion of intracellular GSH. Treatment of LPS-activated cells with PVE at the concentration of 25 \(\mu\)g/ml and RA (1 \(\mu\)g/ml) resulted in a recovery of ROS and GSH levels to that of the control cells.

A large number of reports point to a connection between the expression of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-\(\alpha\) in gingival tissue, and progression of periodontitis. Cytokines production is stimulated by oral bacteria metabolites. These are capable of regulating several fibroblast functions such as migration, proliferation, differentiation, regeneration and homeostasis. Synthesis of cytokines is inducible although some are known to be produced constitutively. IL-1 is synthesized in various cell types including fibroblasts. It has a large number of diverse activities and has the critical role in the tissue homeostasis during periodontal disease and unrestricted production of IL-1 may lead to tissue damage (Okada and Murakami, 1998). IL-6 is a multifunctional cytokine that plays an important role in regulating the immune response and inflammatory reactions during disease. It has been reported that gingival fibroblasts isolated from a diseased tissue produce a larger amount of IL-6, both constitutively and after stimulation, in comparison to gingival fibroblasts isolated from healthy tissue (Kent et al., 1999; Bodet et al., 2007a). IL-6 production is up-regulated by IL-1\(\beta\), IL-1\(\beta\) and TNF-\(\alpha\) in a dose- and time-dependent manner. Their action is synergistic and hundred times more potent than that of LPS (Kent et al., 1998). TNF-\(\alpha\) is generated in the early stage of inflammation and is known to provoke superoxide generation from neutrophils and is also the most active trigger of superoxide production in gingival fibroblasts (Kim et al., 2007a). Thus it may contribute to oxidative damage of gingival tissue. Here we demonstrated that PVE and RA were able to reduce significantly IL-1\(\beta\), IL-6 and TNF-\(\alpha\) production. The effectiveness of RA (1 \(\mu\)g/ml) on IL-1\(\beta\) and TNF-\(\alpha\) level was 3 times stronger than PVE (25 \(\mu\)g/ml). Suppression of IL-6 was comparable in both PVE (25 \(\mu\)g/ml) and RA. The obtained results are in agreement with a previous study where an aqueous extract of \textit{P. vulgaris} attenuated phorbol 12-myristate 13-acetate and calcium ionophore A23187-stimulated TNF-\(\alpha\), IL-6, and IL-8 secretion in human mast cells. The inhibitory effect of the extract on pro-inflammatory cytokines was nuclear factor-kappaB-dependent (Kim et al., 2007c).

The other pathway contributing to periodontal disease pathogenesis involves NO production by NO synthase (NOS). At least three types of NOS isoforms exist. Neuronal and endothelial are expressed constitutively, whereas iNOS is produced in a variety of cell types in response to normal and mainly pathological conditions. iNOS has the critical function in inflammation-related diseases (Murakami and Ohigashi, 2007). A number of phytochemicals, such as curcumin, kaempferol or resveratrol, have been shown to suppress a LPS-induced iNOS production (Kim et al., 2007a; Murakami and Ohigashi, 2007). Harput et al. showed that aqueous extract of \textit{P. vulgaris} suppressed NO production in LPS-stimulated macrophages dose-dependently without any cytotoxicity. However, they found no changes in iNOS protein synthesis detected by Western blot analysis (Harput et al., 2006). We found that PVE (10 \(\mu\)g/ml) and RA (1 \(\mu\)g/ml) down-regulates the production of iNOS in LPS-pre-treated gingival fibroblasts.

In conclusion, modulation of pro-inflammatory cytokines and ROS generation is a promising approach to managing periodontitis, particularly for those with a predisposition for the disease. Both \textit{P. vulgaris} and rosmarinic acid have been reported to be effective in conditions of oxidative stress and inflammation. In this study we confirmed the ability of PVE and RA to suppress ROS generation, TBARS production and GSH depletion as well as expression of IL-1\(\beta\), IL-6, TNF-\(\alpha\) and iNOS. This suggests that PVE and RA, by attenuating of inflammatory response and oxidative mediators production in gingival fibroblasts, may be beneficial in the treatment of periodontitis to slow down the disease development.
Conflict of interest statement

None declared.

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