

## Original articles

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## DELAY IN ORAL MUCOSAL ULCER HEALING BY ASPIRIN IS LINKED TO THE DISTURBANCES IN P38 MITOGEN-ACTIVATED PROTEIN KINASE ACTIVATION

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**Background:** Among the early manifestations of oral mucosal impairment by nonsteroidal anti-inflammatory drugs is the delay in soft oral tissue repair brought about by the amplification of apoptotic events. In this study, we investigated the effect of a specific inhibitor of p38 mitogen-activated protein kinase (p38 MAPK), SB 203580, on the rate of buccal mucosal ulcer healing and the apoptotic processes in rats subjected to intragastric administration of aspirin. **Methods:** Groups of rats with experimentally induced buccal mucosal ulcers were administered twice daily for 10 days with SB 203580 (5, 10, and 20 mg/kg) or vehicle followed 30 min later by concomitant administration (twice daily for 10 days) of aspirin at 20 mg/kg. The animals were killed at different periods of treatment and their mucosal tissue subjected to macroscopic assessment of ulcer healing rate, measurement of soluble tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and the assay of epithelial cell apoptosis. **Results:** In the control group the ulcer healed by the tenth day and the rate of healing was not affected by SB 203580 administration, whereas a 54.8% reduction in the ulcer area was attained in the presence of aspirin administration. Moreover, by the tenth day, the delay in ulcer healing caused by aspirin was manifested in a 5.6-fold higher rate of apoptosis and a 5.2-fold higher level of soluble TNF- $\alpha$ . Treatment with SB 203580 produced dose-dependent reduction (59.5–74.8%) in aspirin-induced increase in the mucosal level of soluble TNF- $\alpha$ , evoked 53.2–69.7% decrease in the rate of epithelial cell apoptosis, and led to a marked reversal (51.8–73.9%) in aspirin-induced delay in ulcer healing. **Conclusions:** The results of our findings link the delay in buccal mucosal ulcer healing caused by aspirin ingestion to the disturbances in the p38 MAPK activation.

**Key words:** *Oral mucosa; aspirin; ulcer healing; p38 mitogen-activated protein kinase*

### INTRODUCTION

Over the counter availability of nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, used for the treatment of a wide variety of acute and chronic disorders ranging from headache and toothache to rheumatoid

arthritis and stroke, is a known cause of the impairment in mucosal defenses that are well recognized and clinically emphasized with respect to the gastrointestinal tract (1—3). The interference by NSAIDs with soft oral tissue repair, however, has been demonstrated only recently (4, 5). The available data indicate that untoward oral mucosal manifestation of systemic action of aspirin, reflected in the impairment in healing of buccal mucosal ulceration, is associated with a marked enhancement of epithelial cell apoptosis triggered by up-regulation in the mucosal expression of proinflammatory TNF- $\alpha$  (5). Moreover, the adverse effects of aspirin ingestion on soft oral tissue repair are characterized by a significant increase in the processing of membrane-anchored TNF- $\alpha$  to its mature soluble form (4).

Though both cell surface and released forms of TNF- $\alpha$  are biologically active and capable of signaling through type I (TNFR1) and type II (TNFR2) cellular receptors, the majority of systemic effects of TNF- $\alpha$ , including the initiation of proinflammatory and apoptotic responses, involve the mature 17-kDa soluble form of TNF- $\alpha$  signaling through TNFR1 (6, 7). The engagement of cell surface TNFR1 by soluble TNF- $\alpha$  results in intracellular signal transduction cascade that involves activation of the transcriptional factor NF- $\kappa$ B, a dimeric protein held in the cytoplasm in inactivated form through association with members of a family of inhibitory proteins known as I $\kappa$ Bs (inhibitors of nuclear factor  $\kappa$ B) (6). Upon activation, the I $\kappa$ B proteins undergo rapid phosphorylation at the critical serine residues by the family of activated MAP kinases, which serve as a signal for targeting I $\kappa$ Bs for degradation by the ubiquitin-proteasome pathway and resulting in translocation of NF- $\kappa$ B to the nucleus where it activates genes mediating various aspects of inflammatory responses (6, 8, 9).

Interestingly, several recent studies provided well-documented evidence that activation of NF- $\kappa$ B leads to the suppression of apoptotic signals and protects cells from death, while the inhibition of NF- $\kappa$ B promotes the programmed cell death (10—12). Among the agents capable of inhibiting the activation of NF- $\kappa$ B are salicylate and its acetylated derivative, aspirin (9—11). The interference by these agents in the NF- $\kappa$ B pathway is the result of their inhibition of I $\kappa$ B $\alpha$  phosphorylation and degradation as a consequence of p38 MAP kinase activation (10, 12). Moreover, there are strong indications that p38 MAP kinase activation plays a major role in the inhibition of TNF-induced NF- $\kappa$ B activation (10). Hence, pharmacological manipulation of the p38 MAP kinase activation might provide therapeutic benefits in preventing the potentiation of TNF-induced proinflammatory events.

Accordingly, in this study, using the animal model of acetic acid-induced buccal mucosal ulcer model (4), we investigated the mechanism of interference by aspirin with soft oral tissue repair by analyzing the effect of a specific inhibitor of p38 mitogen-activated kinase (p38 MAPK), SB 203580, on the rate of ulcer healing and apoptotic processes in rats subjected to intragastric administration of aspirin.

## MATERIALS AND METHODS

### *Animals*

The study was conducted with 180 to 200 g Sprague-Dawley rats cared for by the professional personnel of the Research Animal Facility. Under ether anesthesia, the buccal surfaces of the animals were exposed for 20 s to contact with glacial acetic acid, using a plastic tube of 4 mm in diameter. This produced an immediate mucosal necrosis within affected area followed 2 days later by the development of chronic ulcer with a well-defined crater, which normally healed within 10 days (4). On the second day after the procedure (designated as ulceration day 0), the animals were divided into groups and subjected twice daily for 10 days to intragastric administration of a specific inhibitor of p38 mitogen-activated protein kinase (p38 MAPK), SB 203580 (Calbiochem, La Jolla, CA) at 5, 10, and 20 mg/kg or vehicle consisting 5% gum arabic in saline, followed 30 min later twice daily for 10 days by intragastric dose of aspirin at 20 mg/kg or vehicle consisting of 5% gum arabic in saline. The applied concentration range of SB 203580 was based on the studies demonstrating the effectiveness of this agent in inhibiting (>90%) endotoxin-induced TNF- $\alpha$  production in rats (13), while the dose of aspirin was chosen based on the data as to the effectiveness of this drug in inhibiting (>75%) mucosal prostaglandin generation without causing gastric mucosal damage (14). The animals were killed at different intervals of ulcer healing for up to 10 days, and the buccal mucosa from the ulcer area together with its margin excised and used for biochemical measurements. The rate of ulcer healing was assessed by measuring the ulcer crater by planimetry (15).

### *Apoptosis Assay*

Quantitative measurements of apoptosis were conducted with epithelial cells prepared from buccal mucosa scraping (15). The cells were incubated in the lysis buffer in accordance with the manufacturer's (Boehringer Mannheim, Indianapolis, IN) instructions, centrifuged, and the diluted supernatant containing the cytoplasmic histone-associated DNA fragments were reacted in the microtiter wells with immobilized anti-histone antibody. After washing, the retained complex was reacted with anti-DNA peroxidase and probed with ABTS reagent for spectrophotometric quantification. The inter- and intra-assay variability range was 5–8%.

### Mucosal Soluble TNF- $\alpha$ Assay

The minced specimens of the excised buccal mucosal tissue were suspended in ice-cold solution, consisting of 0.25 M sucrose in 0.15 M Tris-HCl buffer, pH 7.4, and containing 1 mM PMSF, 20  $\mu$ M pepstatin, 20  $\mu$ M leupeptin and 1 mM EDTA, and homogenized for 1 min in a Polytron tissue mixer. The homogenate was centrifuged at 1000 g for 10 min and the resulting supernatant was centrifuged at 10,000 g to sediment the crude mitochondrial fraction. Centrifugation of the resulting supernatant at 100,000 g for 1 h produced the cytosol-containing supernatant that was used for soluble TNF- $\alpha$  assay (4). The TNF- $\alpha$  was quantitated with an enzyme-linked immunosorbent assay according to the manufacturer's (Genzyme, Cambridge, MA) instructions. The microtiter wells were precoated with monoclonal anti-TNF- $\alpha$  to capture TNF- $\alpha$  from the mucosal cytosol, and the retained complex was probed with horseradish peroxidase-conjugated polyclonal anti-TNF- $\alpha$ . The unbound materials were removed by washing, the wells were incubated with TMB reagent, and TNF- $\alpha$  quantified spectrophotometrically (4). The intra- and inter- assay variability range was 7–9%.

### Data Analysis

All experiments were carried out in duplicate, and the results are expressed as the means  $\pm$  SD. Analysis of variance (ANOVA) was used to determine significance, and the significance level was set at  $p < 0.05$ . The protein content of samples was assayed with the BCA protein assay kit (Pierce, Rockford, IL).

## RESULTS

The acetic acid-induced buccal mucosal ulcer model was used to investigate the mechanism of interference by aspirin with soft oral tissue repair as reflected in the delay in healing and upregulation of apoptotic events. The delay in ulcer healing, and the changes in the mucosal levels of soluble TNF- $\alpha$  and epithelial cell apoptosis, caused by aspirin were assessed using rats subjected to intragastric pretreatment with SB 203580, a specific inhibitor of p38 MAPK. As depicted in *Fig. 1*, the ulcer crater at the onset of the experiments (day, 0) averaged 12.4 mm<sup>2</sup>, and in the absence of drugs administration (control group) healed by the tenth day. The rate of ulcer healing was not affected by SB 203580 administration, while in the group subjected to aspirin by the tenth day of healing the mean ulcer crater area still measured 5.6 mm<sup>2</sup>. Pretreatment with SB 203580 prior to aspirin administration produced dose-dependent reduction in the delay in ulcer healing caused by aspirin. A 51.8% reduction in the aspirin-induced delay in healing was attained at the end of ten days with SB 203580 at 5 mg/kg, while a 73.9% increase in ulcer healing occurred with the agent at 10 mg/kg. Increasing the dose of SB 203580 to 20 mg/kg, however, produced no discernible additional increase in the rate of ulcer healing (*Fig. 1*).

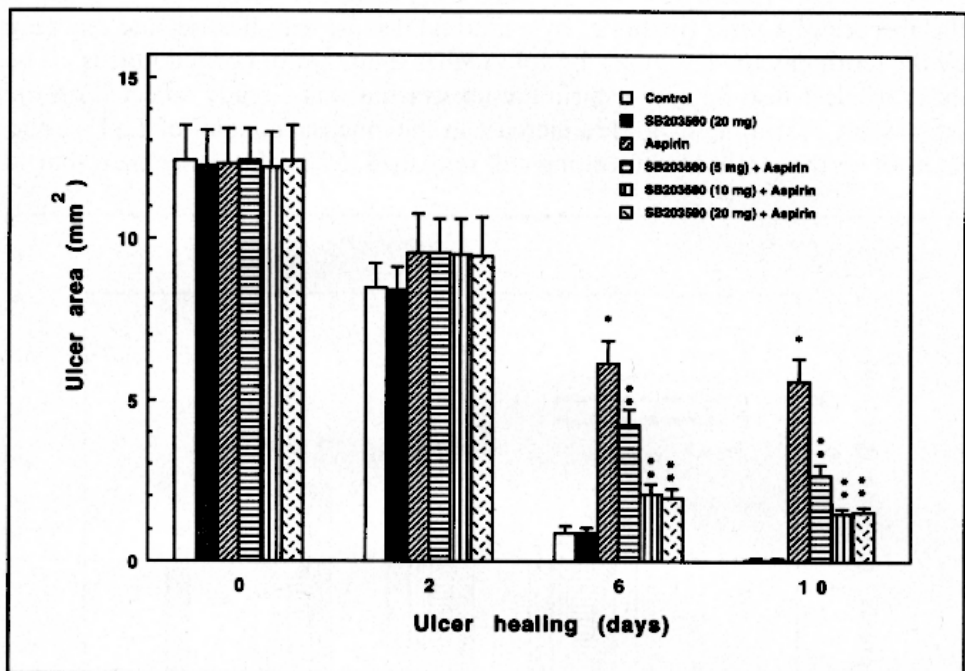


Fig. 1. Effect of pretreatment with p38 MAPK inhibitor, SB 203580 on the rate of buccal mucosal ulcer healing in the presence of intragastric administration of aspirin. Administration (twice daily for 10 days) of SB 203580 (at 5, 10, and 20 mg/kg) followed 30 min later by aspirin (twice daily for 10 days at 20 mg/kg) was commenced on the day of ulcer development (day, 0). Values represent the means  $\pm$  SD obtained with 10 animals in each group. \* $P < 0.05$  compared with that of the control. \*\* $P < 0.05$  compared with that of aspirin.

The delay in buccal mucosal ulcer healing in the presence of aspirin administration was reflected in a significantly higher rate of epithelial cell apoptosis. Compared with the controls, the animals subjected to aspirin exhibited already a 2.4-fold higher rate of apoptosis by the second day of healing and a 5.6-fold higher rate of apoptosis was observed on the tenth day of healing (Fig. 2). The effect of aspirin was countered by the pretreatment with SB 203580, which, when administered at 5 mg/kg, by the tenth day of healing caused a 53.2% reduction in the extent of epithelial cell apoptosis elicited by aspirin, a 69.4% reduction in apoptosis occurred with the agent at 10 mg/kg, and a 69.7% reduction at 20 mg/kg (Fig. 2).

The data on buccal mucosal level of soluble TNF- $\alpha$  during ulcer healing in the presence of aspirin administration in the absence and the presence of pretreatment with SB 203580 are presented in Fig. 3. The ulcer onset (day, 0) was characterized by a 9-fold increase in the level of soluble TNF- $\alpha$ , followed in

the absence of aspirin treatment by a gradual decline with healing and reaching by the tenth day the level only 1.5-fold higher than that of normal mucosa. The delay in ulcer healing with aspirin administration was already reflected on the second day of healing by a 31% increase in the mucosal content of TNF- $\alpha$ , and its level by the tenth day of healing still remained a 5.2-fold higher than that of

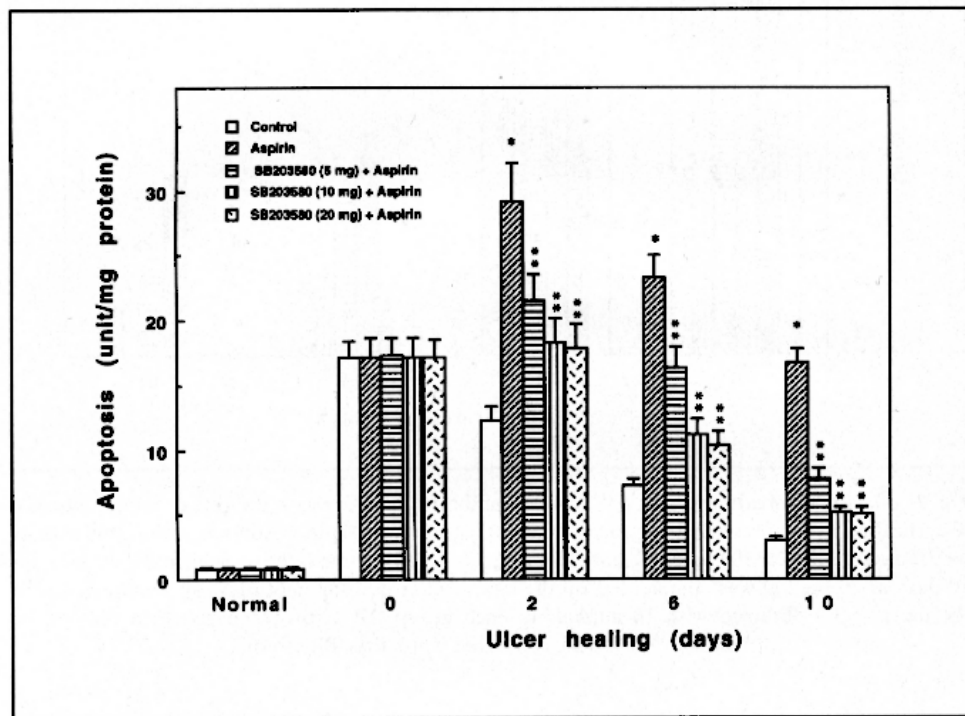


Fig. 2. Effect of pretreatment with SB 203580, twice daily for 10 days, on the extent of epithelial cell apoptosis during buccal mucosal ulcer healing in the presence of intragastric administration of aspirin (twice daily at 20 mg/kg). The bars presented under "Normal" represent the extent of apoptosis prior to ulcer induction. Values represent the means  $\pm$  SD of duplicate analyses performed with 10 animals in each group. \* $P < 0.05$  compared with that of the control. \*\* $P < 0.05$  compared with that of aspirin.

the control group. Intra-gastric administration of SB 203580 preceding aspirin treatment produced dose-dependent reduction in aspirin-induced elevation in the mucosal level of soluble TNF- $\alpha$ . Indeed, by the tenth day of healing, a 59.4% reduction in the mucosal level of soluble TNF- $\alpha$  elicited by aspirin was obtained with SB 203580 at 5 mg/kg, a 73.6% reduction in TNF- $\alpha$  occurred with the agent at 10 mg/kg, and a 74.8% reduction in TNF- $\alpha$  was attained with SB 203580 at 20 mg/kg.

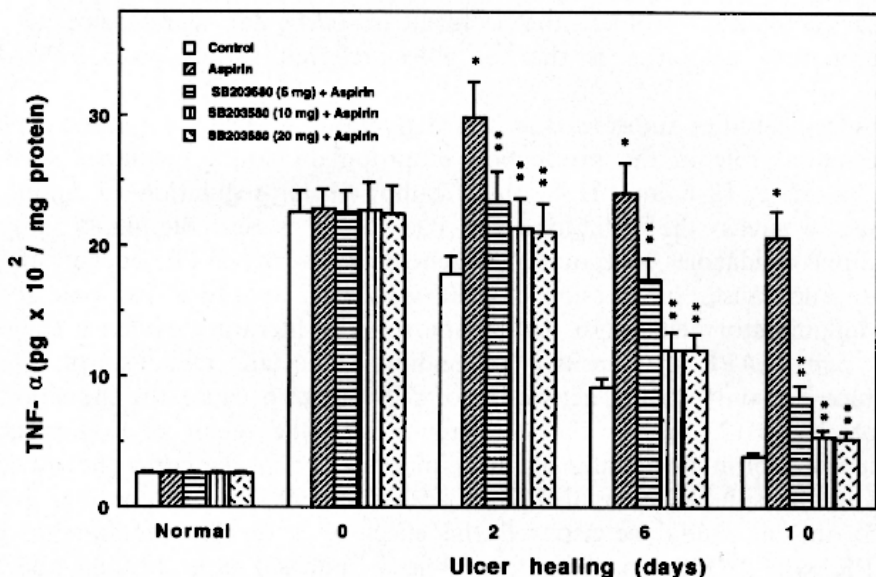


Fig. 3. Effect of pretreatment with SB 203580, twice daily for 10 days, on the mucosal level of soluble TNF- $\alpha$  during buccal mucosal ulcer healing in the presence of intragastric administration of aspirin (twice daily at 20 mg/kg). The bars presented under "Normal" represent the level of TNF- $\alpha$  prior to ulcer induction. Values represent the means  $\pm$  SD of duplicate analyses performed with 10 animals in each group. \*P < 0.05 compared with that of the control. \*\*P < 0.05 compared with that of aspirin.

## DISCUSSION

Recent studies with an animal model of mucosal tissue repair following injury demonstrated that the use of NSAIDs not only impairs healing of gastric and duodenal ulcers (2, 3), but also interferes with soft oral tissue repair (4, 5). Moreover, it became apparent that the interference in buccal mucosal ulcer healing by aspirin is associated with a marked enhancement in epithelial cell apoptosis triggered by rise in the mucosal level of the soluble form of TNF- $\alpha$  (4). The cellular responses to this proinflammatory cytokine involve the activation of several types of mitogen-activated protein kinase (MAPK) pathways of which signaling through the p38 MAPK subfamily has been shown to play a major role in inflammation, cell growth and differentiation, cell cycle progression, and programmed cell death (16, 17). While the p38 group of

kinases consists of four ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ) closely related 38 kDa proteins, exhibiting over 60% identity in their amino acid sequence and displaying similar activation profiles, the isoform of particular significance to the inflammatory responses is that of p38 $\alpha$ , referred simply as p38 MAPK (16).

Indeed, studies indicate that the activation of the p38 pathway plays an essential role in the production of proinflammatory cytokines such as TNF- $\alpha$ , IL-1, IL-6 and IL-8, thus leading to up-regulation of apoptotic events, whereas the inhibition of p38 by a series of highly specific pyridinyl imidazole compounds which bind to the ATP pocket of the kinase and causes suppression of TNF- $\alpha$ -induced apoptosis, exerts a potent anti-inflammatory effect (16, 18). Moreover, the literature evidence suggests that p38 MAPK is sensitive to sodium salicylate and aspirin, which produce not only strong activation of p38 but also cause the induction in apoptosis (8, 12, 16). As the interference with the repair of oral mucosal tissue by aspirin is manifested by a marked enhancement in the mucosal level of the soluble form of TNF- $\alpha$  and up-regulation of apoptotic events (4, 5), in this study we assessed the effect of a specific inhibitor of p38 MAPK, SB 203580, on the rate of buccal mucosal ulcer healing and the apoptotic processes in rats subjected to intragastric administration of aspirin.

The results obtained revealed that the detrimental influence of aspirin on the rate of ulcer healing, characterized by a significant increase in epithelial cell apoptosis, was reflected in up-regulation in buccal mucosal level of soluble TNF- $\alpha$ . While in the control group the ulcer essentially healed by the tenth day, only a 54.8% reduction in the ulcer area was observed in the presence of aspirin administration. Moreover, by the tenth day, the delay in ulcer healing caused by aspirin was reflected in a 5.6-fold higher rate of apoptosis and a 5.2-fold higher level of soluble TNF- $\alpha$ . Treatment with SB 203580 produced dose-dependent reduction (up to 74.8%) in aspirin-induced increase in the mucosal level of soluble TNF- $\alpha$ , caused up to 69.7% decline in the rate of epithelial cell apoptosis, and led to a marked reversal (up to 73.9%) in aspirin-induced delay in ulcer healing. These findings, together with our recent results on the aspirin-induced increased processing of the membrane-anchored TNF- $\alpha$  to its mature 17-kDa soluble form in buccal mucosal injury (4), and the evidence that up-regulation in the level of soluble TNF- $\alpha$  is a characteristic feature of many chronic inflammatory diseases (6, 19), lend further support to the concept of persistent TNF- $\alpha$  activation as a culprit responsible for the aspirin-induced delay in oral mucosal repair.

Indeed, there are strong indications that the variations in soluble TNF- $\alpha$  level and the resulting fluctuation in the extent of NF- $\kappa$ B activation account for the variable pattern of inflammatory disease progression (6), and that salicylate



and aspirin, through the p38 MAPK pathway activation, cause the inhibition of NF- $\kappa$ B nuclear translocation and hence promote the up-regulation in apoptosis (10–12). Moreover, several recent reports demonstrated that the p38 MAPK inhibitor, SB 203580, can prevent both salicylate induced apoptosis and inhibition of TNF- $\alpha$  signaling, thus indicating that the p38 MAPK signal transduction is the critical arm of the TNFR1 signaling pathway involved in the regulation of TNF- $\alpha$  gene expression (6, 8, 10, 12). The results of our study are certainly consistent with the above interpretation, as we have shown herein that treatment of the animals with SB 203580 significantly reduced the aspirin-induced increase in TNF- $\alpha$  and epithelial cell apoptosis, and markedly countered the delay in ulcer healing.

While the results of our study link the delay in buccal mucosal ulcer healing caused by aspirin ingestion to the disturbances in the p38 MAPK activation, it is important to note that salicylate and its pro-drug aspirin are also potent inhibitors of cellular proliferation and cell cycle progression, and cause G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M cell cycle arrest (9, 20, 21). The interference with any of these processes poses major threat to the efficiency of the mucosal repair, as it affects an orderly processing of signaling cues that propel the mucosal cells to proliferation, differentiation, and migration to the site of injury.

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