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NONSTEROIDAL ANTI-INFLAMMATORY DRUGS IMPAIR ORAL MUCOSAL REPAIR BY ELICITING DISTURBANCES IN ENDOTHELIN-CONVERTING ENZYME-1 AND CONSTITUTIVE NITRIC OXIDE SYNTHASE

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Although the use of nonsteroidal anti-inflammatory drugs (NSAIDs) is known to cause the impairment in mucosal defenses that are well recognized and clinically emphasized with respect to the gastrointestinal tract, less apparent is the extent of their interference with the repair of soft oral tissue. As the disturbances in nitric oxide generation and the release of endothelin-1 (ET-1) are the early signs of injury by NSAIDs, we investigated oral mucosal ulcer healing in the presence of NSAID administration by analyzing the expression of endothelin-converting enzyme--1(ECE-1), responsible for ET-1 generation, and the mucosal activity of inducible (NOS-2) and constitutive (cNOS) nitric oxide synthase responsible for nitric oxide production. Groups of rats with acetic-induced buccal mucosal ulcers were subjected twice daily for up to 10 days to intragastric administration of either indomethacin (5 mg/kg), aspirin (20 mg/kg), or the vehicle and their mucosal tissue subjected to macroacopic assessment of ulcer healing rate and biochemical measurements. While in the control group the ulcer healed by the tenth day, only a 57.2% reduction in the ulcer crater area was attained in the animals subjected to indomethacin and a 54.8% reduction in ulcer occurred in the presence of aspirin administration. Futhermore, by the tenth day, the delay in healing in the presence of indomethacin was manifested by a 4.9-fold higher rate of apoptosis, a 2.7-fold higher expression of ECE-1 activity, a 3.9-fold higher expression of NOS-2 activity and a 2.2-fold decline in cNOS activity, while the interference in ulcer healing by aspirin was characterized by a 5.6-fold higher rate of apoptosis, a 2.8-fold expressiom of ECE-1 activity, a 3.7-fold higher expression of NOS-2 activity and a 2.3-fold lower expression of cNOS activity. Our findings demonstrate that NSAIDs not only pose a well-known risk of gastrointestinal injury, but also interfere with soft oral tissue repair. The impairment in buccal mucosal ulcer healing by NSAID ingestion is manifested in up-regulation in the expression of ECE-1 responsible for ET-1 generation, suppression in cNOS, and amplification of apoptotic events that delay the healing process.

Key words: *Indomethacin, aspirin, impairment, oral mucosa, ulcer healing, ECE-1, cNOS, NOS-2, apoptosis.*

INTRODUCTION

Gastropathy associated with the use of nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin and aspirin poses a major threat not

only to the health of patients receiving therapy for rheumatoid diseases, but also to those using these common medications for the treatment of a variety of acute and chronic disorders ranging from headache to stroke (1—3). Although the majority of gastrointestinal side effects arise as a consequence of NSAID ingestion (2, 3), there are numerous data indicating that potentially serious complications also result from parenteral administration of NSAIDs (4—6). Indeed, the gastrointestinal effects of systemic action of indomethacin and aspirin are reflected in the potentiation of ulcerogenic response to stress, and impairment in healing of gastric and duodenal ulcers (4—6).

While the damaging effects of NSAIDs are most often ascribed to the impairment of prostaglandin synthesis and the disturbances in mucosal blood flow and superoxide generation (7, 8), more recent data also point to a detrimental action of these agents on mucosal expression of pro- and anti-inflammatory cytokies, nitric oxide production, and the process of epithelial cell apoptosis (9—12). Other cytotoxic effects of NSAIDs that affect the mucosal integrity of gastrointestinal tract are manifested by an increased release of a potent vasoactive peptide, endothelin-1 (ET-1), and the disturbances in nitric oxide synthase (NOS) activity responsible for NO generation (13—16).

Nitric oxide, a pluripotent free radical molecule, is an important biological messenger that plays a role in variety of physiological and pathophysiological conditions such as regulating blood pressure, neutrotransmission, inflammation, and septic shock (17—19). Of the three calmodulin-dependent NOS isozymes responsible for NO generation, the two constitutively (cNOS) expressed isoforms of NOS are Ca^{2+}-dependent, while the inducible isoform of NOS, known as NOS-2, is Ca^{2+}-independent and activated in response to a variety of external stimuli, including bacterial lipopolysaccharide, proinflammatory cytokines, and NSAIDs (9, 11, 17—21).

Although the enhancement in NOS-2 expression provides a rapid and high nitric oxide output for host defense, its massive and sustained activation has also been identified as a culprit of transcriptional disturbances that lead to the induction of proapoptotic caspaes activity (17, 22, 23). In contrast, the cNOS isoforms provide precise pulses of NO for a fine modulation of the cellular processes, including those involved in inhibition of apoptogenic signals propagated through a caspase cascade (24, 25). Interestingly, the cytotoxic effects of NSAIDs in the gastric mucosa are manifested not only by induction of NOS-2, but also by the suppression of cNOS and up-regulation of endothelin-converting enzyme-1 (ECE-1) responsible for ET-1 release (14, 16). The presence of this membrane-bound metallopeptidase (ECE-1) in soft oral tissue has also been characterized (26).

As the elements of mucosal defense adversely affected by NSAIDs in gastrointestinal tract are also responsible for the maintenance of soft oral tissue integrity (27—29), in this study, using the acetic acid-induced buccal mucosal

ulcer model (30), we investigated the mechanism of impairment in soft oral tissue healing by NSAIDs by analyzing the effect of indomethacin and aspirin ingestion on the rate of epithelial cell apoptosis, and the mucosal expression of ECE-1, cNOS, and NOS-2 activity.

MATERIALS AND METHODS

Animals

The study was conducted with 180 to 200 g Sprague-Dawley rats cared for by the personnel of the Research Animal Facility. The animals were deprived of food and water 2 h before the procedure. 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 (27, 30). 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 ten days (27). On the second day following the procedure, designated as ulceration day 0, the animals were divided into groups and subjected twice daily for 10 days to intragastric administration of either indomethacin at 5 mg/kg, aspirin at 20 mg/kg, or the vehicle consisting of 5% gum arabic in saline. All experiments were carried out with groups consisting 10 animals per treatment. 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 the measurements of cNOS, NOS-2 and ECE-1 activity, and the assays of epithelial cell apoptosis. The rate of ulcer healing was assessed by measuring the ulcer crater area (mm^2) by planimetry (27). The protein content of samples was assayed with the BCA protein assay kit (Pierce, Rockford, IL).

Apoptosis assay

Measurements of apoptosis were carried out with epithelial cells prepared from buccal mucosal scarping (28). The cells were incubated in the lysis buffer in accordance with the manufacture's (Boehringer Mannheim) instructions, centrifuged, and the diluted supernatant containing the cytoplasmic histone-associated DNA fragments were reacted in the microtitrator wells with immobilized anti-histone antibody. Following washing, the retained complex was reacted with anti-DNA peroxidase and probed with ABTS reagent for spectrophotometric quantification (28). The inter- and intra- variability range of the assay was 5—8%.

NOS activity assay

Buccal mucosal activity of cNOS and NOS-2 was measured by monitoring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline using NOS-detect kit (Stratagene, La Jolla, CA). The specimens of buccal mucosa were homogenized in a sample buffer containing either 10 mM EDTA (for NOS-2) or 6 mM $CaCl_2$ (for cNOS), and centrifuged at 800 × g for 10 min (28). The aliquots of the resulting supernatant were incubated for 30 min at 25°C in the presence of L-[2,3,4,5-^{3}H]arginine (50 μCi/μl), 10 mM NAPDH, 5 μM tetrahydrobiperin, and 50 mM Tris-HCl buffer, pH 7.4, in a final volume of 250 μl (28). Following addition of stop buffer and Dowex-50 W (Na^+) resin, the mixtures were transferred to spin cups, centrifuged and the formed L-[^{3}H]citrulline contained in the flow through was quantitated by scintillation counting.

ECE-1 preparation

The minced specimens of buccal mucosal scrapings 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 μM leupeptin, and homogenized for 1 min in a Polytron tissuemizer (26). 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 microsomal pellet (31). The pellet was solubilized by stirring at 4°C for 30 min with 0.25 M buffered sucrose, pH 7.0, containing 0.5% Triton X-100, the mixture was centrifuged at 100,000 g for 1 h, and the supernatant used as an enzyme source.

ECE-1 activity assay

Reaction mixtures to ECE-1 assay, incubated at 37°C for 60 min in a total volume of 100 μl, consisted of 0—30 μg enzyme protein, 0.5 μM big ET-1 (Sigma), and 100 mM phosphate buffrer, pH 6.8, containing 0.5 M NaCl (32). The reaction was terminated by addition of 100 mM EDTA and the mixture was boiled for 5 min, centrifuged, and the resulting supernatant applied to a Sep-Pack C-18 cartridge (26). After initial washing with 0.1% trifluoroacetic acid, the adsorbed ET-1 was eluted from the cartridge with methanol-water-trifluoroacetic acid (90:10:0.1, v/v/v). The eluates containing purified ET-1 were dried under vacuum, reconstituted in the assay buffer, and subjected to immunometric ET-1 quantitation with a double-antibody sandwich technique in accordance with the manufacturer's (Alexis Corporation, San Diago, CA) instructions. The sample aliquots were applied to the microtitrator wells coated with ET-1 capture antibody and the complex was incubated at 4°C for 16 h. After washing, the wells were probed with Ellman reagent, and ET-1 was quantitated spectrophotometrically (26).

Data analysis

All experiments were carried out in duplicate, and the results are expressed as the means ± SD. Analysis of variance (ANOVA) was used to determine significance, and the significance level was set at $p < 0.05$.

RESULTS

The acetic acid-induced buccal mucosal ulcer model was used to investigate the mechanism of interference with soft oral tissue healing by NSAID ingestion. The impairment in ulcer healing was assessed with respect to apoptosis and the changes in buccal mucosal activity of ECE-1, cNOS, and NOS-2, using rats subjected to intragastric administration of indomethacin and aspirin. As depicted in *Fig. 1*, the ulcer crater at the onset of the experiments (day, 0) averaged 12.4 mm^2, and in the absence of NSAID administration (control group) healed by the tenth day. In the animals subjected to indomethacin administration the ulcer creater by the tenth day of healing still averaged 5.3 mm^2, while in the grouo subjected to aspirin by the tenth day of healing the mean ulcer area measured 5.6 mm^2.

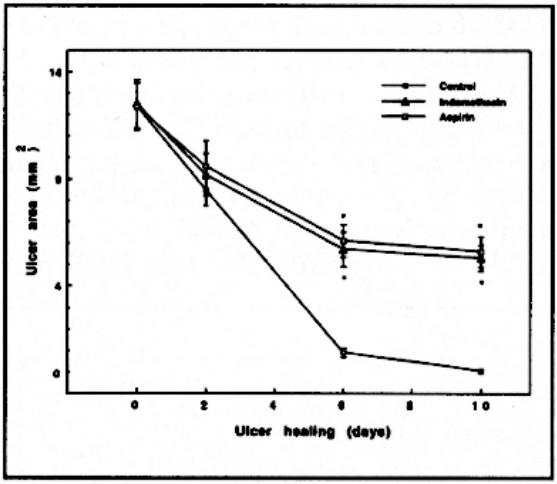

Fig. 1. Effect of intragastric administration of NSAIDs (twice daily for 10 days) on the rate of buccal mucosal ulcer healing. Administration of indomethacin (5 mg/kg) and aspirin (20 mg/kg was commenced on the day of ulcer development (day, 0). Values represent the means ± SD of duplicate analyses obtained with 10 animals in each group. * $P < 0.05$ compared with that of the control.

The apoptotic DNA fragmentation assays conducted with buccal epithelial cells of the control animals and those subjected to indomethacin and aspirin administration revealed that the delay in ulcer healing by NSAIDs was reflected in a significantly higher rate of apoptosis (*Fig. 2*). Compared with the controls, the indomethacin group showed a 2.1-fold higher rate of apoptosis by the second day of healing and a 4.9-fold higher rate of apoptosis was observed on the tenth day, while the aspirin group showed a 2.4-fold higher rate of apoptosis by the second day and a 5.6-fold higher rate by the tenth day of healing.

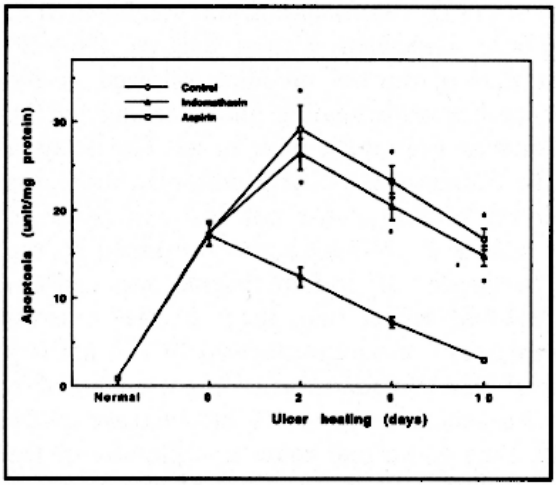

Fig. 2. Effect of intragastic administration of indomethacin and aspirin on the rate of epithelial cell apoptosis during buccal mucosal ulcer healing. Values represent the means ± SD of duplicate analyses performed with 10 animals in each group. * $P < 0.05$ compared with that of the control.

The data on buccal mucosal expression of cNOS during ulcer healing in the presence of NSAIDs administration are presented in *Fig. 3*. Compared with the values for normal mucosa, the ulcer onset (day, 0) was characterized by an

87.4% reduction in the expression of cNOS activity, followed in the absence of NSAIDs by a gradual rise with healing. Nevertheless, the cNOS activity in ulcerated buccal mucosa by the tenth day of healing still remained about 52% lower than that of normal. Thea delay in healing in the presence of NSAIDs was reflected in a significantly slower rise in buccal mucosal cNOS activity, which by the tenth day of healing in the presence of indomethacin administration was a 2.2-fold lower than that of the control group, while a 2.3-fold lower values for the cNOS were attained in the aspirin group.

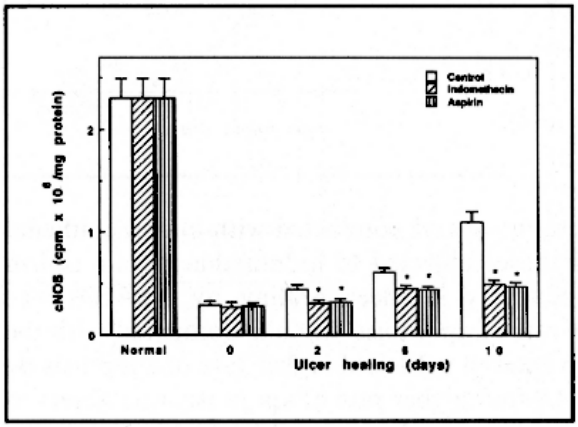

Fig. 3. Effect of intragastric administration of indomethacin and aspirin on the expression of cNOS activity in buccal mucosa during ulcer healing. Values represent the means ± SD of duplicate analyses performed with 10 animals in each group. * $P < 0.05$ compared with that of the control.

The pattern of changes in buccal mucosal expression of NOS-2 activity during ulcer healing in the presence of NSAID administration is summarized in *Fig. 4*. The ulcer onset (day, 0) was associated with a massive (86-fold) induction in NOS-2 activity over that of normal mucosa, followed in the absence of NSAIDs by a gradual decline with healing and reaching by the tenth day a value of an 80.3% lower than that at the ulcer onset. The delay in buccal mucosal ulcer healing in the presence of NSAID administration was characterized by a significantly slower decline in the mucosal expression of NOS-2. As a result, the respective values for NOS-2 activity attained by the tenth day of healing in the presence of indomethacin and aspirin administration were 3.9-fold and 3.7-fold higher than those of the controls.

The data on the time-course expression of buccal mucosal ECE-1 activity during ulcer healing in the presence of NSAIDs administration are presented in *Fig. 5*. The ulcer onset (day, 0) was associated with a 4.3-fold increase in the expression of ECE-1 activity over that of normal mucosa, followed in the absence of NSAIDs by a gradual decline with healing and reaching by the tenth day a value of a 66.2% lower than that at the ulcer onset. The ulcer healing in the presence of indomethacin and aspirin administration was reflected by the second day in a marked increase in buccal mucosal ECE-1

activity over that of day 0, followed by a decline thereafter. However, the values attained for ECE-1 activity by the tenth day of healing in the presence of indomethacin and aspirin administration were 2.7-fold and 2.8-fold higher than those of the respective controls.

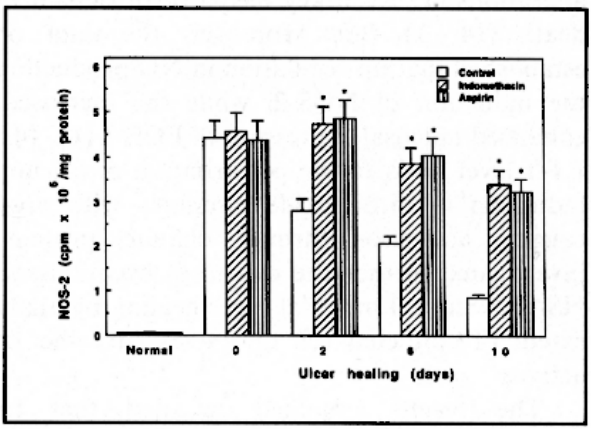

Fig. 4. Effect of intragastric administration of indomethacin and aspirin on the expression of NOS-2 activity in buccal mucosa during ulcer healing. Values represent the means ± SD of duplicate analyses performed with 10 animals in each group. * $P < 0.05$ compared with that of the control.

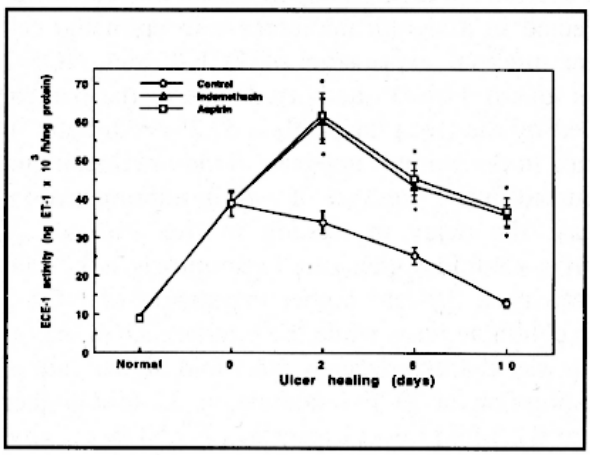

Fig. 5. Expression of ECE-1 activity in buccal mucosa during ulcer healing in the presence of intragastric administration of indomethacin and aspirin. Values represent the means ± SD of duplicate analyses performed with 10 animals in each group. * $P < 0.05$ compared with that of the control.

DISCUSSION

Although the wide range of systemic benefits makes NSAIDs one of the most widely used classes of drugs in the world, the agents are known to cause potentially serious impairments in mucosal defenses that are most apparent and clinically emphasized with respect to the gastrointestinal tract (1—3). Recent studies with an animal model of experimentally-induced buccal mucosal ulcer, however, revealed that intragastric administration of NSAIDs interferes also with the repair of soft oral tissue (30). The interference in buccal

ulcer healing was associated with enhancement in the mucosal expression of TNF-α, perturbation in the process of programmed cell death and up-regulation in the release of a potent vasoactive peptide ET-1 (30), known for its stimulatory effect on proinflammatory cytokine gneration and the alterations in NO, a key mediator of signaling events linked to apoptotic cell death (14, 33—36). Moreover, the data obtained with gastric mucosa established that up-regulation in NO production by NSAIDs is associated with the induction of NOS-2, while the increased ET-1 generation reflects the enhanced mucosal expression of ECE-1 (12, 14, 15, 16). As the enhancement in ET-1 level leads to the perturbation of calcium channel activation, while the induction in nitric oxide interferes with postreceptor pathways related to calcium activation through channel protein S-nitrosylation (25—29), we investigated further the course of events associated with the impairment by NSAIDs in oral mucosal ulcer healing by analyzing the interplay between the extent of epithelial cell apoptosis, and the expression of ECE-1 and NOS activity.

The results obtained revealed that intragastric administration of indomethacin as well as aspirin exerted a marked delay in buccal mucosal ulcer healing and the effect was reflected in a significant increase in epithelial cell apoptosis, up-regulation in the mucosal expression of ECE-1 and NOS-2 activity, and suppression in the mucosal cNOS activity. While in the control group the ulcer essentially healed by the tenth day, only a 57.2% reduction in the ulcer creater area was attained in the animals ubjected to indomethacin and a 54.8% reduction in ulcer occurred in the presence of aspirin administration. Furthermore, by the tenth day, the delay in healing in the presence of indomethacin was manifested by a 4.9-fold higher rate of apoptosis, a 2.7-fold higher expression of ECE-1 activity, a 3.9-fold higher expression of NOS-2 activity and a 2.2-fold decline in cNOS activity, while the interference in buccal mucosal ulcer healing by aspirin was characterized by a 5.6-fold higher rate of apoptosis, a 2.8-fold higher expression of ECE-1 activity, a 3.7-fold higher expression of NOS-2 activity and a 2.3-fold lower expression of cNOS activity. These findings, together with our results on the enhanced expression of ET-1 during buccal mucosal ulcer healing in the presence of NSAID administration (30), and the evidence on ET-1 induced apoptosis (37), underscore the role played by ECE-1, through ET-1 generation, in controlling the severity of oral mucosal inflammatory reaction to injury. The above data also point strongly towards ECE-1 as a key mucosal target of the systemic action of NSAIDs that leads to up-regulation of the processes that interfere with the mechanisms controlling the soft oral tissue repair. Indeed, enhanced expression of ECE-1 activity and the elevated levels of ET-1 have been singled out as culprits implicated in pathogenesis of a number of pathologic wound healing diseases, including pulmonary and cardiac fibrosis, and hepatic cirrhosis (38—40).

Our finding that the delay in buccal mucosal ulcer healing in the presence of NSAID administration, associated with a marked amplification of NOS-2 expression and a pronounced drop in cNOS, coincided with prolongation of oral mucosa epithelial cell apoptosis provides a strong indication as to the importance of synchronized expression of cNOS and NOS-2 in regulation of the events involved in programmed cell death. Apparently, the enhanced expression of NOS-2 results in the formation of NO-related species such as nitrosthiols, peroxynitrate and dinitrosyl iron complexes which exert a direct inhibitory effect on NFκB (22), and hence cause transcriptional disturbances that lead to up-regulation of proinflammatory cytokie production and the enhanced rate of apotosis (17—19). On the other hand, the cNOS plays an active role in the inhibition of apoptogenic signals through S-nitrosylation of cysteine residue on the catalytic site of caspase-3 and interference with the activation of ETA receptor by ET-1 which leads to the suppression of TNF-α biosynthesis (17, 22—24). Moreover, cNOS is also involved in the inhibition of caspase enzymes through a cGMP mechanism, associated with phosphorylation-dephosphorylation events, that function on the level of caspase zymogen activation that requires cleavage adjacent to aspartates (23, 25). Hence, the reported herein amplification in the suppression of cNOS and the reduction of NOS-2 activity during buccal mucosal ulcer healing in the presence of indomethacin and aspirin administration may well be an important factor in NSAID interference with the repair of soft oral tissue injury. This interpretation of our findings is supported by the recent results obtained with gastric mucosa and indicating that the expression of cNOS activity plays a vital role in the maintenance of mucosal integrity and remains under a direct control of ET-1, while the induction of NOS-2 by indomethacin appears to be independent of ET-1 and may be associated with up-regulation of inflammatory responses to NSAIDs in general (41).

While the importance of intracellular Ca^{2+} level maintenance in the mucosal protection against NSAIDs is of paramount significance, it should be noted that the activity of cNOS, aside of calcium/calmodulin influence, is also dependent on the cellular level of cofactors such as NADPH, FAD, FMN, and tetrahydrobiopterin which stabilizes the active homodimetric NOS and acts as a redox cofactor of L-arginine oxidation (42, 43). Equally deserving consideration for optimal cNOS activation, is the effect of ECE-1 inhibition on the proteins that interact with NOS and limits its activity within the specific compartments of the cell (19). Indeed, studies show that N-myristoylation is essential for targeting cNOS to the plasma membrane and that the translocation of cNOS to cytosol is associated with phosphorylation event and results in a decrease of the enzyme activity (44, 45). Interestingly, according to most recent data, the phosphorylation of endothelial NOS by cAMP- and

cGMP-dependent kinase lowers the enzyme senstitivity for Ca^{2+} requirement or even leads to its Ca^{2+}-independent activation (44—46).

In conclusion, our data demonstrate that NSAIDs not only pose a well-known risk of gastrointestinal injury and interfere with healing of gastric and duodenal ulcers, but also exert untoward impact on the process of soft oral tissue repair. We also show that the impairment in buccal mucosal ulcer healing by the ingestion of indomethacin and aspirin is associated with up-regulation in the expression of ECE-1 responsible for ET-1 generation, suppression of cNOS, and amplification of apoptotic events that delay the healing process.

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