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Comparison of nitric oxide-releasing NSAID and vitamin C with classic NSAID in healing of chronic gastric ulcers; involvement of reactive oxygen species

Tomasz Brzozowski[1], Sławomir Kwiecień[1], Peter Konturek[2], Stanisław J. Konturek[1], Agata Ptak[1], Małgorzata Mitis-Musiol[1], Aleksandra Duda[1], Władysław Bielański[1], Eckhart G. Hahn[2]

[1] Department of Physiology, Collegium Medicum, Jagiellonian University, Cracow, Poland
[2] 1st Department of Medicine, University of Medicine Erlangen-Nuremberg, Erlangen, Germany

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SUMMARY

Background: *Nonsteroidal anti-inflammatory drugs such as aspirin (ASA) are known to induce gastric mucosal damage including bleeding, ulceration and perforation in humans and experimental animals. These adverse effects of ASA were originally attributed to the inhibition of cyclooxygenase and the deficiency of endogenous prostaglandins induced by this drug but the role of reactive oxygen species (ROS), lipid peroxidation and antioxidizing mechanism in the pathogenesis of ASA damage has been little studied. New class of nitric oxide (NO)-releasing nonsteroidal anti-inflammatory drugs was shown to inhibit cyclooxygenase and prostaglandin generation without causing mucosal damage but it remains unknown whether these agents affect the healing process of chronic gastric ulcers.*

Material and Methods: *In this study the effect of NO-releasing aspirin (NO-ASA) and was compared with that of native aspirin applied with or without vitamin C on the healing of acetic acid ulcers. The area of gastric ulcer was determined by planimetry, the gastric blood flow (GBF) at ulcer margin was measured by H_2 gas clearance method and mucosal release of ROS was quantified by measuring the chemiluminescence before and after the treatment with ASA or NO-ASA alone and ASA combined with vitamin C. The plasma antiinflammatory cytokine such as IL-1β and oxygen radical-mediated lipid peroxidation was measured in the ulcerated gastric mucosa of ASA and NO-ASA-treated animals.*

Results: *ASA delayed significantly ulcer healing and this effect was accompanied by a marked increase in the chemiluminescence, lipid peroxidation and the fall in the GBF at ulcer margin. Vitamin C attenuated significantly both the ASA-induced gastric damage and accompanying fall in the GBF at ulcer margin and the rise in the chemiluminescence and reversed the ASA-induced lipid peroxidation. In contrast, NO-ASA failed to affect healing of gastric ulcers and failed to produce the rise in the plasma IL-1β levels and the increase of lipid peroxidation as compared to those recorded in ASA-treated animals.*

Conclusions: *1) ROS-induced enhancement in lipid peroxidation plays an important role in the mechanism of gastric damage induced by ASA, 2) vitamin C attenuates the deleterious effect of ASA on ulcer healing due to its antioxidizing activity by mechanism involving preservation of gastric microcirculation and attenuation of lipid peroxidation and cytokine release and 3) coupling of NO to aspirin fails to delay the ulcer healing suggesting that NO might compensate for prostaglandin deficiency induced by NSAID.*

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Correspondence address: Prof. Stanisław J. Konturek, Department of Physiology, Jagiellonian University School of Medicine, 16 Grzegorzecka Str, 31-531 Cracow, Poland, e-mail: mpogonow@cyf-kr.edu.pl

BACKGROUND

Non-steroidal anti-inflammatory drugs (NSAID) such as aspirin (ASA) are widely used but the major limitation of their clinical application are serious gastrointestinal side-effects, such as aggravation of stress-induced gastric damage and interference with the healing of gastric ulcerations [1–3]. Recently, a new class of NSAID has been developed by adding of nitric oxide (NO) moiety to the native NSAID [4–10]. The rationale behind this strategy is that NO released from these derivatives exerts beneficial influence on gastric mucosa by enhancing the mucosal defensive ability and preventing pathogenic events resulting from suppression of prostanoid synthesis such as the reduction in gastric microcirculation and the leukocyte-endothelial adherence [11–13]. Earlier studies revealed that endogenous NO released from vascular endothelium, sensory nerves or gastric epithelial cells cooperates with prostaglandins in the maintenance of gastric mucosa integrity and microcirculation [14,15].

In contrast to native NSAID, their NO-releasing derivatives such as NO-aspirin (NO-ASA) [4,12,20] were found to exhibit lower gastric toxicity despite inhibiting both COX-1 and COX-2 activity in the gastric mucosa [20]. The major importance of NO in the prevention of mucosal damage or in preservation of ulcer healing is supported by previous studies showing that both endogenous NO released by capsaicin [15] or exogenous NO originating from L-arginine, a NO-synthase or glyceryl trinitrate exert gastroprotective activity and accelerate healing mainly by the maintenance of blood flow around the ulcer and angiogenesis [16,17].

The present study was designed; 1) to compare the effect of topical application of ASA and its NO-derivative on ulcer healing and gastric blood flow at ulcer margin; 2) to determine the role of ROS measured by the chemiluminescence in rats with gastric ulcer treated with ASA; and 3) to elucidate the lipid peroxidation, which is associated with induction of cellular oxidative stress. Since vitamin C is known to exert potent antioxidizing activity, we determined whether ASA-induced damage and the accompanying release of ROS is affected by the concurrent treatment with vitamin C.

MATERIAL AND METHODS

Male Wistar rats, weighing 180–220 g and fasted for 24 h before the study were employed in all studies.

Production of gastric ulcers

Gastric ulcers were produced in 150 fasted rats, using our modification [17] of acetic acid method originally proposed by Okabe et al [19]. With the animals under ether anesthesia, the stomach was exposed and 75 μl of acetic acid was poured through the plastic mold (6 mm diameter) onto serosal surface of anterior wall of the stomach just proximal to the antral gland area for 25 sec. This produced immediate necrosis of the entire mucosa and submucosa within the area where the acetic acid was applied, i.e, about 28 mm^2. The excess of acetic acid was then removed and the serosa was gently washed with tap saline. Our previous studies documented that these ulcers become chronic within 2–3 days and heal completely within 2–3 weeks without perforation or penetration to the surrounding organs as described in original Okabe's technique [19]. After the application of acetic acid the animals were allowed to recover from anesthesia and received only water on the day of operation (Day 0). Then, they were divided into various groups and received normal chow and water *ad libitum* for the next 3, 9 and 15 days. To evaluate the effects of native ASA and their NO-releasing derivative, the animals were anesthetized with ether and the abdomen was opened and the stomach was exposed to assess the GBF at ulcer margin, ulcer crater and in the contralateral intact mucosa using H_2-gas clearance technique as described before [16,17]. The stomach was then removed and pinned open for the determination of the area of gastric ulcers by planimetry (Morphomat, Carl Zeiss, Berlin, German) by two investigators under blinded conditions. The half of the stomach with gastric ulcer in rats with or without administration of vehicle (control), ASA or NO-ASA was taken during autopsy and immediately fixed in 10% formalin, embedded in paraffin and stained with hematoxylin and eosin for the histological assessment of the quality of ulcer healing. Coded specimens of mucosa stained with hematoxylin and eosin were evaluated at 260x magnification under blinded conditions.

Experimental groups and treatments

Several group of animals each consisting of 6–8 rats were used to determine the effect of the treatment with vehicle, ASA (50 mg/kg – d i.g.) with or without vitamin C (0.5 g/kg – d i.g.) and its NO-derivative NO-ASA (50 mg/kg – d i.g.) on the healing of gastric ulcers induced by the serosal application of acetic acid (ulcer area 28 mm^2). The NO-ASA

(NCX 4016 batch 3) was a generous gift of Mrs. Nathalie Baundry (NicOx, Valbonne, France). The animals were killed at 0, 3, 9 and 15 days after ulcer induction and the area of gastric ulcerations was determined by planimetry, the GBF was measured by H_2-gas clearance method as mentioned above and the gastric luminal NO^-_2/NO^-_3 content was assessed by Griess technique. Venous blood from *vena cava* was collected for the assessment of plasma IL-1β levels and biopsy samples were taken for determination of generation of PGE_2 by RIA and measurement of lipid peroxidation.

Measurement of chemiluminescence

Biopsy samples were taken from the non-ulcerated and ulcerated gastric mucosa (about 1 mm from the ulcer crater) for the measurement of lucigenin--amplified chemiluminescence. Each specimen was immediately washed in phosphate-buffered saline containing 5 mmol/l of glucose (pH 7.4) and transferred to a dark adapted scintillation vial containing 1 ml of lucigenin employed in a concentration of 400 μmol/L. All solutions were pre-oxygenated prior to use. Chemiluminescence was measured over 8 minutes in a LKB Wallac B scintillation counter (model 1409) operating in 'out of coincidence' mode. Specimens were then carefully blotted dry and weighed and chemiluminescence expressed as counts per minute per gram of tissue (cpm/g).

Determination of PGE_2 generation in gastric mucosa and luminal NO release

For *ex vivo* determination of PGE_2, the mucosa was processed, according to the method described previously [2]. Briefly, a portion of corpus mucosa (about 100 mg) excised from rats treated with vehicle, acidified ASA and NO-ASA was placed in an Eppendorf plastic tube containing 1.0 ml of 10 mM phosphate buffer (pH 7.4) and minced with fine scissors for 15 seconds. The samples were then incubated in a shaking bath (37°C) for 20 minutes and then centrifuged 30 sec at 9000 g. The supernatant was collected and frozen at –20°C for subsequent determination of PGE_2 using radioimmunoassay (PGE_2-RIA kit, Amersham, Munich, Germany).

For the measurement of NO generation, 1 ml of saline was introduced to the stomach and the luminal content was collected for the determination of total NO^-_3/NO^-_2 concentration using commercially available kit purchased from Cayman (Ann Arbor, MI, USA) as described previously [10]. This method is based on the Griess reaction and generation of chromophore absorbing at 595 nm, according to the original procedure described previously [10]. Since NO released by epithelial cells into the gastric lumen is quickly transformed into NO_3^- and NO_2^- [20], we measured photometrically the sum of both these products of NOS as an index of production of NO by the enzyme in the gastric mucosa. For this purpose, the gastric content was aspirated just before the removal of the stomach following the i.g. injection of 1 ml of saline to wash out the luminal content. After centrifugation for 10 min at 3000 rpm, the samples were mixed with Griess reagent from the commercially available kit. In all tests including those with ASA, NO-ASA and ASA combined with vitamin C, the GBF was measured in the oxyntic mucosa in each group of animals in similar manner as mentioned before and expressed as the percent control value recorded in vehicle--treated gastric mucosa.

Determination of plasma IL-1β levels and lipid peroxidation in gastric mucosa

Immediately after measurement of GBF, a venous blood sample was withdrawn from the vena cava into the EDTA containing vials and used either for the determination of plasma IL-1β concentrations by specific ELISA assay according to the manufacturer's recommendation (Endogen Inc, Cambridge, MA, USA).

Since lipid peroxidation is a well-established mechanism of cellular injury induced by reactive oxygen metabolites, we measured the changes in the malondialdehyde (MDA) as an indicator of the lipid peroxidation in gastric mucosa treated with vehicle, ASA alone or combined with vitamin C and that treated with NO-ASA. For the measurement of lipid peroxidation, the tissue was weighed, transferred to the ice-cooled test tube and homogenized in 400 μl of 20 mM Tris buffer pH=7.4 containing 5 mM butylated hydroxytoluene to prevent new lipid peroxidation that can occur during the homogenization. The homogenate was then centrifuged at 4°C for 10 min and resulted supernatant (200 μl) was stored in –80°C until an assay of lipid peroxidation (BIOXYTECH LPO-assay kit, OXIS International Inc. Portland, USA). The content of lipid peroxidation was measured at 37°C by spectrophotometer at wave length of 586 nm and compared with the absorbance of purified MDA as the standard. The results are expressed as the amount of MDA in the gastric mucosa of rats treated with

ASA with or without the combination with vitamin C and after 9 days treatment with NO-ASA as compared to vehicle-control animals.

Statistical analysis

Results are expressed as means±SEM. The significance of the difference between means was evaluated using analysis of variance followed by Duncan's test or, when appropriate, by Wilcoxon's rank sum test with a level of confidence at $P<0.05$.

RESULTS

Effect of vehicle, native ASA with or without combination with vitamin C and NO-releasing ASA on the area of gastric ulcers and accompanying changes in gastric blood flow (GBF) at ulcer margin and plasma IL-1β levels

Figure 1 shows the time-course of ulcer healing in rats with administration of vehicle or ASA (50 mg/kg – d i.g.), NO-ASA (50 mg/kg – d i.g.) and ASA combined with vitamin C (0.5 g/kg – d i.g.) on the area of gastric ulcers at 0, 3, 9 and 15 days upon ulcer induction. In rats treated with vehicle, a significant reduction in the area of these ulcers was observed from initial size of about 28 mm^2 to 23.8±4.3 mm^2, 16.2±3.1 mm^2 and 10.9±2.5 mm^2 at day 3, 9 and 15 upon ulcer induction, respectively. Native ASA failed to affect significantly the area of gastric ulcers at day 3 upon ulcer induction but prolonged significantly ulcer healing at day 9 and day 15 upon ulcer induction. In contrast, NO-ASA and vitamin C added to this ASA tended to accelerate the ulcer healing though this change failed to reach statistical significance.

The GBF in the non-ulcerated antro-oxyntic mucosa of rats treated with vehicle averaged 46 ml/min –100 g (taken as 100%) being significantly reduced by about 15% at the ulcer margin at day 9, when compared to that in the contralateral intact mucosa (data not shown). At day 9 upon ulcer induction, the native ASA delayed significantly ulcer healing and this was accompanied by the significant decrease in the GBF at ulcer margin and the significant rise in the plasma IL-1β levels as compared to the respective values in vehicle-treated gastric mucosa (Fig. 2). Treatment with NO-ASA resulted in a significant decrease in ulcer area and plasma IL-1β increments as compared to the respective values obtained in native ASA-treated animals and raised the GBF at ulcer margin to the extent not significantly different than that in vehicle-control gastric

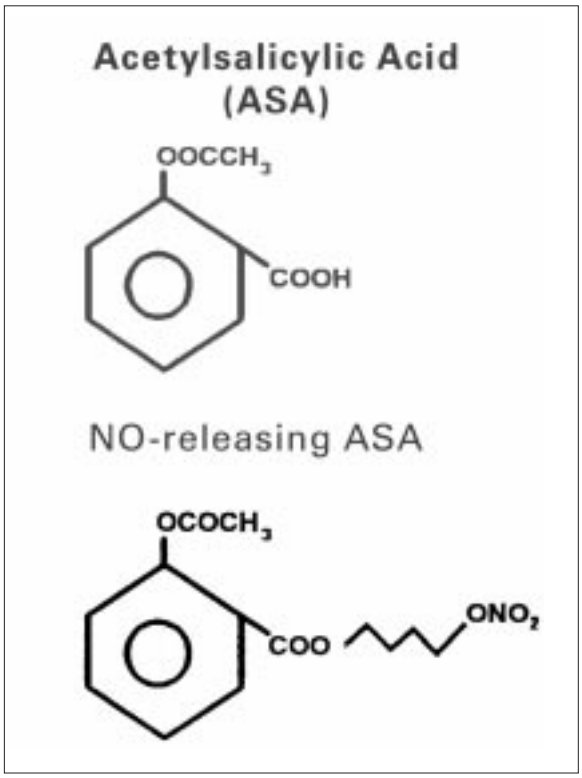

Figure 1. Chemical structures of native acetylsalicylic acid (aspirin, ASA) and nitric oxide (NO)-releasing ASA (acetylsalicylic acid 4-(nitroxy) butylester).

mucosa. When vitamin C was added to native ASA, a significant reduction in the area of gastric ulcers and plasma IL-1β levels accompanied by the increase in the GBF at ulcer margin was observed as compared to those in native ASA-treated animals (Fig. 2).

Effect of vehicle, native ASA with or without the combination with vitamin C and NO-releasing ASA on the lucigenin-induced chemiluminescence, lipid peroxidation, PGE_2 generation and luminal NO content

The results with the chemiluminescence in the vehicle-control mucosa as well as in those exposed to ASA alone and ASA combined with vitamin C or treated with NO-ASA are presented in Table 1. The lucigenin-dependent chemiluminescence was significantly amplified in ASA-treated mucosa as compared to that obtained in vehicle-treated animals. In contrast, co-administration of vitamin C with ASA reversed completely the rise in the chemiluminescence induced by ASA alone. NO-ASA failed to influence significantly the chemiluminescence as compared to that recorded in the gastric mucosa of rats treated with native ASA (Table 1).

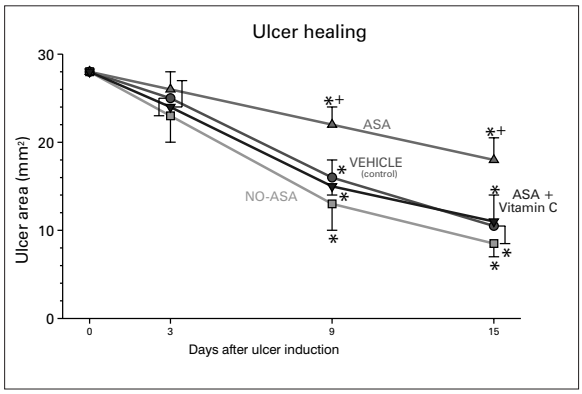

Figure 2. Mean area of gastric ulcers in rats treated throughout a period of 3–15 days with vehicle (VEH), acidified aspirin (ASA, 50 mg/kg – d i.g.), ASA combined with vitamin C (0.5 g/kg i.g.) or nitric oxide (NO)-releasing ASA (50 mg/kg – d i.g.). Mean±SEM of 6–8 rats. Asterisk indicates significant change as compared to the value obtained immediately upon ulcer induction (day 0). Cross indicates significant change as compared to the value obtained in vehicle-treated animals.

Fig. 3 shows the changes in the mucosal MDA plus 4-HNE content, measured as an index of lipid peroxidation, in the gastric mucosa of animals treated with vehicle and those treated with ASA without or with concurrent vitamin C administration or exposed to NO-ASA. In ASA-treated animals, a significant rise in the MDA was observed as compared to the value obtained in vehicle-control animals. The addition of vitamin C to ASA attenuated significantly mucosal MDA plus 4-HNE content, which reached the value not significantly different from that recorded in vehicle-treated mucosa. In contrast to native ASA, the treatment with NO-ASA failed to affect the significantly the mucosal MDA plus 4--HNE content, which reached the value not significantly different from that recorded in vehicle--treated mucosa.

The PGE_2 generation in the ulcerated gastric mucosa and the NO concentration in the gastric content in rats treated with vehicle averaged 116±8 ng/g wet tissue weight and 3.1±0.4 μmol/l, respectively, at day 15 upon ulcer induction (Table 1). ASA and NO-ASA inhibited mucosal PGE_2 synthesis in the ulcerated gastric mucosa with the extent similar to that in NO-ASA treated gastric mucosa. The ulcer healing effect of NO-ASA was accompanied by a significant rise in GBF and NO concentration in the gastric content as compared to those treated with native ASA (Fig. 2, Table 1). Mucosal content of PGE_2 was also suppressed in rats treated with NO-ASA, despite the fact that NO-ASA was found to improve the ulcer healing as compared to native ASA. (Table 1, Figs. 1 and 2).

Table 1. Effect of vehicle, acidified aspirin (ASA, 50 mg/kg-d i.g.) with or without the combination with vitamin C (0.5 g/kg-d i.g.) and the treatment with NO-ASA (50 mg/kg-d i.g.) on the mucosal generation of PGE_2 in the ulcerated gastric mucosa, the luminal NO production and chemiluminescence (CLM) in gastric mucosa of rats with gastric ulcers at day 15 upon ulcer induction. Results are mean ± SEM of 8–10 rats. Asterisk indicates a significant change as compared to the value obtained in vehicle-treated animals. Cross indicates a significant change as compared to the value obtained in ASA-treated animals.

Type of test	PGE_2 generation (ng/g wet tissue)	Luminal NO (μmol/L)	Chemiluminescence CLM (cpm x 1000/g)
Vehicle	116±8	3.1±0.4	5.4±0.3
ASA	35±4*	2.6±0.2	15.6±0.8*
NO-ASA	41±3*	16.4±4.5*	6.3±0.6$^+$
ASA + vitamin C	43±4*	3.8±0.7	5.9±0.4$^+$

DISCUSSION

Our study demonstrates that ASA damages the rat gastric mucosa and that this effect is accompanied by the enhancement in the ROS determined by means of chemiluminescence assay. Furthermore, we found that ASA increased mucosal MDA content in the gastric mucosa suggesting that this rise in the lipid peroxidation possible due to the activation of neutrophils as proposed originally [21], play an important role in the damaging activity of this NSAID. This increase in the neutrophil mediated-lipid peroxidation was suppressed by NO-releasing ASA and by the addition of vitamin C to native ASA, suggesting that this mechanism of new generation of so called 'safe' NSAID, namely NO--NSAID or NSAID combined with vitamin C may counteract the deleterious effect of classic NSAID on the gastric mucosa. This is supported by the fact that following the ulcer healing, the GBF was elevated and luminal NO production was enhanced in animals treated with NO-releasing ASA as compared to those treated with native ASA. Thus, our study implies that native NSAID delay ulcer healing but NO-NSAID fail to impair ulcer healing possibly due to excessive release of NO that may compensate for PG deficiency induced by these NSAIDs.

ROS were originally implicated in the gastric injury provoked in experimental animals using various

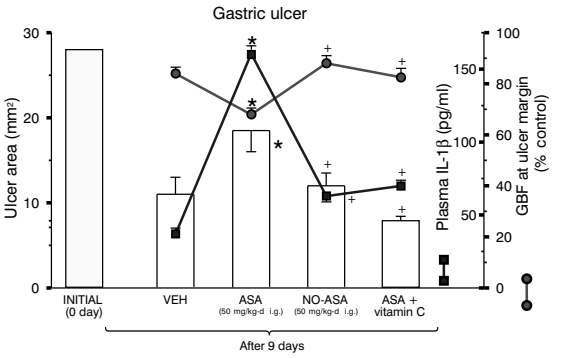

Figure 3. Mean area of gastric ulcers, the gastric blood flow (GBF) at ulcer margin and plasma IL-1β levels in rats treated with vehicle (VEH), ASA with or without the combination with vitamin C (0.5 g/kg – d i.g.) or treated with NO-ASA applied in a dose of 50 mg/kg – d i.g. Mean±SEM of 6–8 rats. Asterisk indicates significant change as compared to the value obtained in vehicle-treated gastric mucosa. Cross indicates significant change as compared to the values recorded in ASA-treated animals.

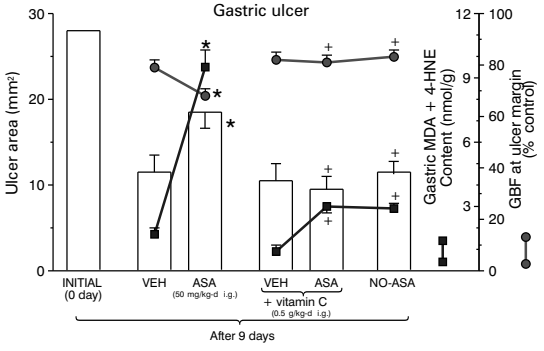

Figure 4. Mean area of gastric ulcers, the gastric blood flow (GBF) at ulcer margin and gastric malonyldialdehyde (MDA) plus 4-HNE content in rats treated with vehicle (VEH), ASA with or without the combination with vitamin C (0. 5 g/kg – d i.g.) and those treated with NO-ASA applied in a dose of 50 mg/kg – d i.g. Mean±SEM of 6–8 rats. Asterisk indicates significant change as compared to the value obtained in vehicle-treated gastric mucosa. Cross indicates significant change as compared to the values recorded in rats treated with native ASA.

experimental models including ischemia reperfusion [24], stress [23], aspirin and indomethacin [24]. Our study are in keeping with the previous observations in experimental animals showing that NSAID such as ASA and indomethacin are capable to induce focal ischemia that leads to the enhanced generation of free radicals. ROS may play an important role in the pathophysiology of acute ulceration induced by NSAIDs and ischemia-reperfusion because the treatment with potent scavengers of free radicals such as superoxide dismutase (SOD), catalase or dimethyl sulfoxide (DMSO) conferred mucosal protection and significantly reduced the severity of these lesions [25]. In another study the development of the stress-induced gastric lesions was accompanied by the rise in the lipid peroxidation and neutrophil-dependent myeloperoxidase activity as well as a decrease in nonprotein sulfhydryl levels in the gastric mucosa [26].

Since the NSAID-induced gastric damage is neutrophil-dependent process [21], the protective action of these scavengers was attributed to the inhibition of neutrophil adherence in the microvessels and their activation. We tested this hypothesis in rats treated daily with ASA by determination of ROS by direct chemiluminescence assay in ASA-treated mucosa and we found that this chemiluminescence was significantly higher when compared to control mucosa treated with vehicle. This rise in free radical production as determined by lucigenin-amplified chemiluminescence in gastric mucosa of ASA-treated rats was completely suppressed by the addition of vitamin C to ASA. >From this finding, we conclude that activation of neutrophils seems to be crucial for the development of ASA damage and that neutrophils act as a major source of free radicals in ASA-treated stomach. This notion is supported by our observation that ulcerogenic properties of ASA and ASA-induced rise in the free radical production were remarkably suppressed by addition of vitamin C to native ASA and by NO-ASA.

It is of interest that ASA produced elevation of plasma IL-1β concentration during ulcer healing while NO-releasing ASA or the addition of vitamin C to native ASA failed to alter significantly the plasma IL-1β levels as compared to vehicle-treated animals. This indicates that NO-ASA can differ from native ASA in limitation of inflammatory response around the ulcer, which is an important event taking place during the prolongation of ulcer healing by classic NSAID such as ASA.

Experimental evidence suggests that lipid peroxidation on the cell membranes may play a significant role in ROS-mediated cell injury by the destruction and damage to cell membranes that consequently leads to mucosal injury [27]. Lipid peroxidation is an autocatalytic ROS mediated destructive process whereby polyunsaturated fatty acids in cell membranes undergo degradation to form lipid hydroperoxides. These latter compounds spontaneously re-

arrange to produce multiple degradation products, including malondialdehyde (MDA) [28].

It was demonstrated that pretreatment of gastric mucosa with ROS scavengers such as SOD, DMSO or taurine exhibited the protective activity against NSAID-induced gastric mucosal injury due to the suppression of an increase in thiobarbituric acid-reactive substances (TBA) measured as an index of lipid peroxidation and neutrophil activation [29]. In the study of Tanaka and Yuda [27], indomethacin produced an increase in the lipid peroxidation and markedly decreased the SOD and GSH-x activity in the gastric mucosa. These authors [27] concluded that decrease in the SOD and GPx activity contributes to an increase in lipid peroxidation and aggravation of the injury induced by free radicals.

In agreement with these studies we have shown, for the first time, in rats with preexisting gastric ulcerations that exposure of the rat gastric mucosa to ASA resulted in the elevation of gastric mucosal MDA content. This remains in agreement with experimental evidence in laboratory animals that severe oxidative stress caused by focal ischemia or indomethacin is manifested by the excessive lipid peroxidation and depletion of SOD activity in the gastric as well as pancreatic tissue [28–30]. Further studies are needed to clarify whether prolonged treatment with ASA influences the activity and expression of potent antioxidizing enzymes such as SOD and GSH-x during the process of healing of preexisting gastric ulcers.

In summary, we have demonstrated that ASA produces an increase in ROS and that this event contributes significantly to the delay in ulcer healing induced by this NSAID. The increase in free radical metabolites depends possibly upon neutrophil activation and is associated with the significant increase in lipid peroxidation, the fall in the GBF at ulcer margin and the excessive release of proinflammatory cytokine such as IL-1β. This increase in lipid peroxidation and plasma IL-1β levels as well as the deleterious effect on the GBF at ulcer margin were, in part, abolished by topical supplementation with vitamin C or the treatment with NO--ASA suggesting that the treatment with these new generation of NSAID or vitamin C could limit the adverse effects such prolongation of ulcer healing induced of classic NSAID.

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