European Journal of Pharmacology 614 (2009) 106–113

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Pulmonary, Gastrointestinal and Urogenital Pharmacology

The gastric ulcer-healing action of allylpyrocatechol is mediated by modulation of arginase metabolism and shift of cytokine balance

Sudhir K. Yadav [a], Biplab Adhikary [a], Biswanath Maity [a], Sandip K. Bandyopadhyay [a], Subrata Chattopadhyay [b,*]

[a] Department of Biochemistry, Dr. B.C. Roy Post Graduate Institute of Basic Medical Sciences and IPGME&R, 244B, Acharya Jagadish Chandra Bose Road, Kolkata-700 020, India
[b] Bio-organic Division, Bhabha Atomic Research Centre, Mumbai-400 085, India

ARTICLE INFO

Article history:
Received 25 October 2008
Received in revised form 8 April 2009
Accepted 20 April 2009
Available online 3 May 2009

Keywords:
Arginase
Cytokine
Indomethacin
iNOS
NSAID

ABSTRACT

The role of the arginine-metabolism in the healing action of the *Piper betle* phenol, allylpyrocatechol (APC) and omeprazole against indomethacin-induced stomach ulceration in mouse was investigated. Indomethacin (18 mg/kg) was found to induce maximum stomach ulceration in Swiss albino mice on the 3rd day of its administration, which was associated with reduced arginase activity (21.6%, $P<0.05$), endothelial nitric oxide synthase (eNOS) expression (72%, $P<0.001$), and IL-4 and TGF-β levels, along with increased inducible nitric oxide synthase (iNOS) (9.3 folds, $P<0.001$) expression, nitrite (2.29 folds, $P<0.001$), IL-1β and IL-6 generation. Besides providing comparable healing as omeprazole (3 mg/kg×3 days), APC (5 mg/kg×3 days) shifted the iNOS/NO axis to the arginase/polyamine axis as revealed from the increased arginase activity (73.1%, $P<0.001$), eNOS expression (67.8%, $P<0.001$), and reduced iNOS expression (65.6%, $P<0.001$) and nitrite level (53.2%, $P<0.001$). These can be attributed to a favourable anti-/pro-inflammatory cytokines ratio, generated by APC. The healing by omeprazole was however, not significantly associated with those parameters.

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1. Introduction

Stomach ulceration induced by non-steroidal anti-inflammatory drugs (NSAIDs) is a major problem ranking fourth in terms of causing morbidity and mortality (Wolfe et al., 1999). The NSAID-related gastroduodenal damage is very frequent and, the most serious complication of any drug therapy. The NSAIDs are well recognized to cause upper gastrointestinal complications, ranging from dyspeptic symptoms in up to 40% to peptic ulceration in 20–30% chronic NSAID users, and even duodenal ulcers. Currently, the use of NSAIDs accounts for approximately 25% of gastric ulcer cases with an upward trend (Tarnawski and Jones, 2003; Chan, 2006).

Mucosal defense system consists of the endogenously released prostaglandins, different growth factors, cytokines, and the antioxidants, all of which are crucial during ulcer healing (McCarthy, 1989). Many of these factors are affected by the NSAIDs such as indomethacin. Apart from the systemic activity which mainly involves inhibition of cyclooxygenases (COXs), reduced prostaglandin synthesis, and impaired prostaglandin-mediated angiogenesis, the NSAIDs also affect the COX-independent mechanisms especially the nitrogen-metabolizing enzymes that are also key contributors in wound healing (Isenberg et al., 1991; Tarnawski et al., 2001). In acute inflammatory responses, such as wound healing, arginase has been implicated as an important regulator of diverse pathways including generation of polyamines and the cytostatic free radical, nitric oxide (NO). Studies have shown that arginine itself has advantageous effects on cutaneous healing by enhancing cell proliferation and collagen synthesis as well as breaking strength (Graham et al., 1988). Further, as a mediator of macrophage function, NO, produced from arginine also plays an important role in inflammatory processes (Warner et al., 1999; Tak and Firestein, 2001). High output NO generation from iNOS during cellular stress is known to exert cytostatic/cytotoxic effects (Albina and Henry, 1991). High concentrations of NO may be detrimental by promoting inflammation via mucosal swelling and epithelial damage (Meurs et al., 2003). The temporal switch of arginine as a substrate for the inducible nitric oxide synthase (iNOS)/NO axis to the arginase/polyamine axis is subject to regulation by the inflammatory cytokines. There are reports suggestive of an intense reciprocal regulation of NOS and arginase activities *in vivo*, depending on the cytokine profile of the host (Modollel et al., 1995).

Inflammation is a complex stereotypical response of the body to cell damage and vascularized tissues. The inflammatory response is phylogenetically and ontogenetically the oldest defense mechanism that is controlled by cytokines, products of the plasma enzyme systems (complement, the coagulation, clotting, kinin and fibrinolytic pathways), lipid mediators (prostaglandins and leukotrienes) released from different cells, and vasoactive mediators released from mast cells, basophils and platelets (Ross et al., 2002). However, little is known on the interplay of the cytokines and the NO synthesis pathway during indomethacin-induced gastric ulceration. After trauma, the Th1/Th2 imbalance with Th2 predominance is reflected by the increase in the arginase-inducing cytokines such as IL-4, IL-10, and TGF-β (Shearer et al., 1997).

* Corresponding author. Fax: +91 22 25505151.
E-mail address: schatt@barc.gov.in (S. Chattopadhyay).

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doi:10.1016/j.ejphar.2009.04.046

Pharmacological options for the treatment of inflammatory diseases that are often chronic are associated with severe side effects. Therefore the search for less toxic, yet equally efficacious compounds is an area of intense research (Mckellar et al., 2007). The *Piper betel* plant is widely growing in the tropical humid climate of South East Asia, and its leaves, with a strong pungent and aromatic flavour, are widely consumed as a mouth freshener. The leaves are credited with diverse medicinal attributes in the indigenous Ayurvedic system of medicine (Chatterjee and Pakrashi, 1995). Very recently, we have documented (Bhattacharya et al., 2007; Banerjee et al., 2008a) impressive healing activity of allylpyrocatechol (APC, chemical structure shown in Fig. 1), the major constituent phenol of *P. betle* leaves. It was found that oral administration of APC (5 mg/kg) for three days could effectively heal the indomethacin (18 mg/kg, p. o., single dose)-induced stomach ulceration in mice. The healing activity of APC could be partly attributed to its antioxidant action as well as the ability to augment the COX isozymes improving prostaglandin synthesis, and angiogenesis.

The aim of the present study was to understand the mechanisms of the healing action of APC in terms of its capacity to regulate the arginine metabolism by modulating the balance of cytokines in the process. To this end, we have investigated the effect of APC in elevating arginase activity, and reducing NO production by altering the NOS expression. Further, the status of the pro- and anti-inflammatory, as well as regulatory cytokines during wound healing was also investigated.

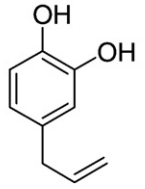
2. Materials and methods

2.1. Chemicals and reagents

APC was isolated from the ethanol extract of air-dried *P. betle* leaves as reported earlier (Bhattacharya et al., 2007). L-Arginine, indomethacin, isonitrosopropiophenone, Bradford reagent, Triton X-100, leupeptin, phenylmethylsulfonyl fluoride (PMSF), glycine, sodium dodecyl sulfate (SDS), acrylamide, bis-acrylamide, Tween 20, ethylenediaminetetraacetic acid (EDTA), 3,3′,5,5′-tetramethylbenzidine (TMB), $MnCl_2$, urea, omeprazole, Trizma base, cetyltrimethylammonium bromide (CTAB), and nitrocellulose membrane were procured from Sigma Chemicals (St. Louis, MO). Other reagents used were disodium hydrogen phosphate and sodium dihydrogen phosphate (BDH, Pool Dorset, U.K.), sulphuric acid, hydrochloric acid, phosphoric acid, sodium chloride (Thomas Becker, Mumbai, India), horseradish peroxidase (HRPO), gum acacia (Sisco Research Laboratory, Mumbai, India), rabbit polyclonal inducible NOS (iNOS) and endothelial NOS (eNOS) antibodies (Santacruz Biotechnology, Delaware, USA), peroxidase conjugated anti-rabbit IgG antibody, enhanced chemiluminescence detection kit (Roche, Mannheim, Germany), NOS and NO assay kits (Calbiochem, California, USA), TGF-β1 kit (Promega Corporation, Madison, USA) and cytokine ELISA kits (Pierce Biotechnology, Rockford, USA).

2.2. Preparation of the drugs

The drugs were prepared from APC and omeprazole as aqueous suspensions in 2% gum acacia as the vehicle, and administered to the mice orally.



Allylpyrocatechol (APC)

Fig. 1. The chemical structure of APC.

2.3. Experimental protocol for ulceration and biochemical studies

Male Swiss albino mice, bred at BARC Laboratory Animal House Facility, Mumbai, India were procured after obtaining clearance from the BARC Animal Ethics Committee (BAEC). The animals were handled following International Animal Ethics Committee Guidelines, and the experiments were permitted by BAEC. The mice (6–8 weeks old, 25–30 g) were reared on a balanced laboratory diet as per National Institute of Nutrition, Hyderabad, India, and given tap water ad libitum. They were kept at 20±2 °C, 65–70% humidity, and day/night cycle (12 h/12 h). To carry out the experiments in a blinded fashion, the animals were identified by typical notches in the ear and limbs, and randomized. The animals were deprived of food 24 h before ulcer induction, but had free access to tap water.

The mice were divided into four groups (each containing five mice), and each experiment was repeated three times. Group I mice served as normal control, while ulceration in the groups II–IV mice was induced by indomethacin (18 mg/kg, p. o., single dose) dissolved in distilled water and suspended in the vehicle, gum acacia (2%). The dose of indomethacin was standardized in our earlier studies (Bhattacharya et al., 2007; Banerjee et al., 2008a). The mice of groups I and II were given the daily oral dose of vehicle (gum acacia in distilled water, 0.2 ml) only. The groups III and IV mice were given a daily dose of APC (5 mg/kg×3 days, p. o.) and omeprazole (3 mg/kg×3 days, p. o.) respectively, starting the first dose 6 h post indomethacin administration. Four hours after the last dose of the treatments, the mice were sacrificed after an overdose of thiopental, the stomachs were opened along the greater curvature, and the wet weights of the tissues were recorded. The glandular portion from five animals was pooled, rinsed with appropriate buffer, homogenized in the same buffer under cold condition and used for studying the expression of different NOSs, and arginase and myeloperoxidase (MPO) activities. The immunological parameters were analyzed both at the tissue and serum levels. The total NOS activity and nitrite level were assayed using the serum samples. In separate experiments different doses (0.5–20 mg/kg) of APC were used to confirm the dose standardization.

2.4. Assessment of ulcer healing

The ulcerated portions of the stomach were sectioned after fixing in 10% formol saline solution. After 24 h of fixation followed by embedding in a paraffin block, it was cut into sections of 5 μm onto a glass slide, stained with haematoxylene-eosin and the histology examined under a light microscope. One centimeter length of each histological section was divided into three fields. The damage score was assessed by scoring each field on a 0–4 scale as described previously (Dokmeci et al., 2005): 0 — normal mucosa, 1 — epithelial cell damage, 2 — glandular disruption, vasocongestion or edema in the upper mucosa, 3 — mucosal disruption, vasocongestion or edema in the mid-lower mucosa, and 4 — extensive mucosal disruption involving the full thickness of the mucosa. The experiments were performed by two investigators blinded to the group and treatment of animals. The histological sections were coded to eliminate an observer bias. Data for the histological analyses are presented as mean±S.E.M. from the review of a minimum of three sections (dividing each 1 cm section into three fields) per animal.

2.5. Determination of MPO activity

Following a reported method (Suzuki et al., 1983) with slight modifications, the MPO activity was determined immediately after sacrificing the animals. The whole process was carried out at 4 °C. The gastric tissues were homogenized for 30 s in a 50 mM phosphate buffer (pH 6.0) containing 0.5% CTAB and 10 mM EDTA, followed by freeze thawing three times. The homogenate was centrifuged at 12,000 ×*g* for 20 min at 4 °C. The supernatant was collected, and the protein content determined. The supernatant (50 μl) was added to 80 mM phosphate buffer, pH 5.4 (250 μl), 0.03 M TMB (150 μl) and 0.3 M H_2O_2 (50 μl). After

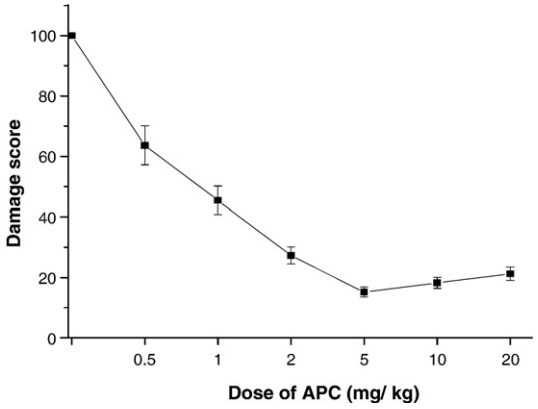


Fig. 2. Dose-dependent stomach ulcer-healing capacity of APC on the third day after indomethacin administration to mice. Stomach ulceration in mice was induced by oral administration of indomethacin (18 mg/kg). Different doses of APC were used for the experiments. The ulcer healing was assessed from the damage scores measured 4 h after the last dose of APC, and normalized considering the damage score of the third day untreated mice as 100. The values are mean ± S.E.M. of three independent experiments, each with 5 mice per group.

incubating the mixture at 25 °C for 25 min, the reaction was terminated by adding 0.5 M H_2SO_4 (2.5 ml). The MPO activity was calculated from the absorbance of the mixture at 450 nm, using HRPO as the standard. The MPO activity is expressed as μM of H_2O_2 consumed per min per mg protein at 25 °C and pH 5.4.

2.6. Arginase assay

The assay was carried out following a known method (Del Ara Rangel et al., 2002) with minor modifications. The tissue homogenate was prepared in ice-cold 25 mM Tris–HCl buffer (pH 7.5) and centrifugation at 12,000 ×*g* for 30 min at 4 °C. The reaction mixture (200 μl) containing 0.5 M L-arginine (pH 9.7), 1 mM $MnCl_2$, and the tissue extract (100 μl) was incubated for 20 min at 37.4 °C. The reaction was stopped by adding an acid mixture (800 μl, H_2SO_4–H_3PO_4–H_2O, 1:3:7) and 3% isonitroso-propiophenone, followed by heating at 100 °C for 45 min, and the absorbance at 540 nm was read. The data were quantified from a calibration curve prepared using urea (1.5–120 μg), and normalized for tissue protein. One unit (U) of enzyme activity is defined as the amount of enzyme that catalyses the formation of 1 μmol of urea/min.

2.7. Total NOS assay

The serum NOS activity was measured using a commercially available colorimetric kit following manufacturer's protocol. In this assay, the nitrite and nitrate, produced from NO are converted into nitrite and spectrophotometrically quantified using Griess reagent against KNO_3 as the standard. The NOS activity is expressed in terms of μM nitrite formed.

2.8. Estimation of nitrite

Following manufacturer's instruction, the serum nitrite concentration was measured using a commercially available colorimetric kit that measures the total nitrite concentration of the sample.

2.9. Western blot analysis of tissue eNOS and iNOS expressions

The glandular part of the gastric mucosa after being washed with PBS containing protease inhibitors was minced and homogenized in a lysis buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 ml) containing leupeptin (2 μg/ml) and PMSF (0.4 μM). Following centrifugation at 15,000 ×*g* for 30 min at 4 °C, the supernatant was collected, and the protein concentration measured. The proteins (40 μg) were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membrane was blocked for 2 h in TBST buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.02% Tween 20) containing 99% fat-free milk powder (5%) and incubated overnight at 4 °C with rabbit polyclonal iNOS or eNOS antibody (1:2000 dilution). The membrane was washed over a period of 2 h with TBST and incubated with peroxidase conjugated anti-rabbit IgG (1:2500 dilution). The bands were detected using an enhanced chemiluminescence detection kit and quantified using the Gelquant software.

2.10. Estimation of serum and tissue cytokine levels

The IL-1β, IL-6 and IL-4 levels in the serum and tissue homogenate were estimated using commercially available ELISA kits, following manufacturer's protocol. The tissue homogenate, prepared for the western blot experiments was used for this purpose. The method of TGF-β1 estimation (Thakur et al., 2005) in sera was adopted after acidification to include the active and latent forms of the cytokine. Briefly, 96-well high binding ELISA plates were coated with anti-mouse TGF-β1 monoclonal antibody and incubated overnight at 4 °C. After blocking for 30 min at 37 °C, the wells were washed once with TBST buffer, the samples were activated by acid treatment followed by neutralization. The samples along with the standards were seeded to each well at an appropriate dilution, and incubated at room temperature for 90 min. The wells were washed (5 times), diluted polyclonal antibody (100 μl) was added, and the mixture was incubated further for 2 h at room temperature. The wells were washed, and incubated for 2 h after addition of TGF-β HRPO conjugate (100 μl). After the final wash, TMB (100 μl) was added to each well, the mixture was incubated for 15 min, the reaction was stopped by 1 N HCl, and the absorbance at 450 nm was read.

2.11. Statistical analysis

The data, expressed as the mean ± S.E.M ($n = 15$) were analyzed by a paired Student's *t* test for the paired data, or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons post hoc test. Nonparametric data (histology scoring) were analyzed using Kruskal–Wallis test (nonparametric ANOVA) followed by a Dunn's multiple comparisons post test. Bonferroni correction was also carried out for knowing the simultaneous statistical inference among the groups

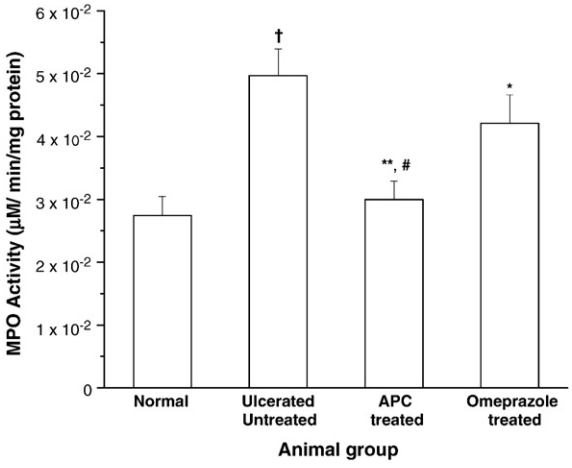


Fig. 3. Effects of APC and omeprazole in modulating the mucosal MPO level in the indomethacin-induced ulcerated mice. The supernatant of the gastric tissue homogenate was incubated with TMB in a suitable buffer and the MPO activity was assayed from the absorbance at 450 nm against HRPO as the standard. The values are mean ± S.E.M. of three independent experiments, each with 5 mice per group. $^{*}P<0.001$ compared to normal mice; $^{**}P<0.05$, $^{\dagger}P<0.01$ compared to ulcerated mice; $^{\#}P<0.05$ compared to omeprazole treatment.

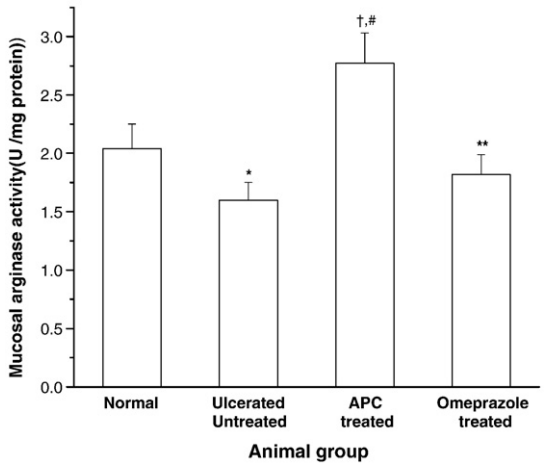


Fig. 4. Effects of APC and omeprazole in modulating the mucosal arginase activity in the indomethacin-induced ulcerated mice. The supernatant of the gastric tissue homogenate was incubated with L-arginine and $MnCl_2$ in a suitable buffer and the arginase activity was assayed from the absorbance at 540 nm. The values are mean ± S.E.M. of three independent experiments, each with 5 mice per group. $^{*}P<0.05$ compared to normal mice; $^{**}P<0.05$, $^{\dagger}P<0.001$ compared to ulcerated mice; $^{\#}P<0.01$ compared to omeprazole treatment.

under investigations. A probability value of $P<0.05$ was considered significant.

3. Results

Earlier we have found maximum ulceration in mice stomach on the 3rd day of indomethacin (18 mg/kg, p. o., single dose) administration. Under an optimized treatment regime, APC (5 mg/kg × 3 days) or omeprazole (3 mg/kg × 3 days) provided comparable (~72%) ulcer healing (Banerjee et al., 2008a). This was reconfirmed by assessing the healing in terms of damage scores (Fig. 2). Subsequently, the present experiments were carried out under the same conditions.

3.1. Regulation of the mucosal MPO activity

The MPO activity in the ulcerated untreated mice increased by 81.1% ($P<0.001$), compared to normal value. This was reduced by APC (39%, $P<0.01$) and omeprazole (15.3%, $P<0.05$), APC being more effect than omeprazole ($P<0.05$) (Fig. 3).

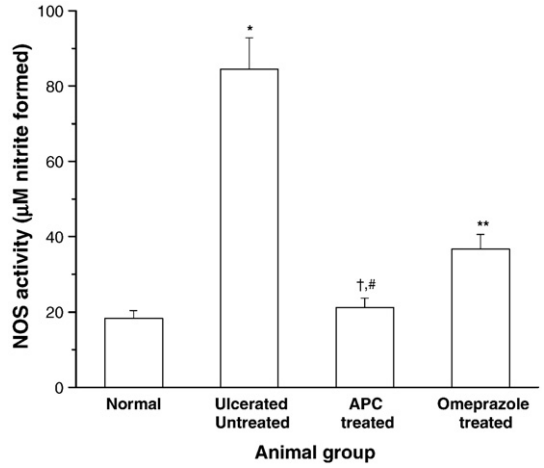


Fig. 5. Effects of APC and omeprazole in regulating the serum total NOS activity in the indomethacin-induced ulcerated mice. The NOS activity was measured using a colorimetric kit. The values are mean ± S.E.M. of three independent experiments, each with 5 mice per group. $^{*}P<0.001$ compared to normal mice; $^{**}P<0.01$, $^{\dagger}P<0.001$ compared to ulcerated mice; $^{\#}P<0.01$ compared to omeprazole treatment.

3.2. Regulation of the mucosal arginase activity

The indomethacin-mediated stomach ulceration depleted (21.6%) the arginase activity significantly, compared to normal mice (Fig. 4). Three-day treatment with APC and omeprazole enhanced the arginase activity by 73.1% ($P<0.001$) and 13.8% ($P<0.05$) respectively, compared to the untreated mice. The results of APC and omeprazole were significantly different ($P<0.01$).

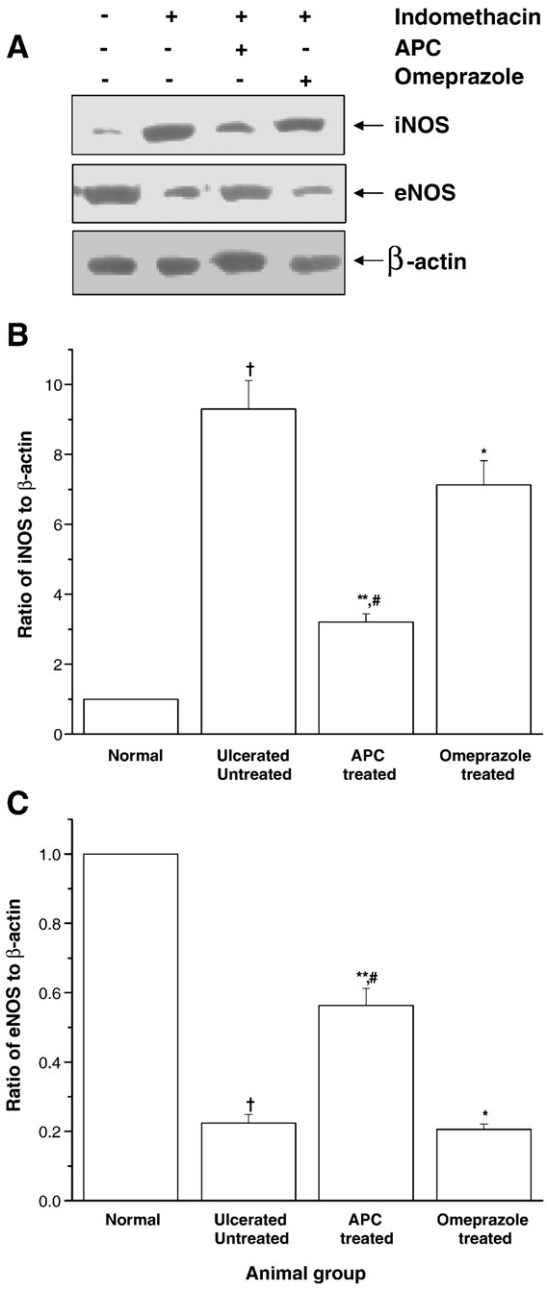


Fig. 6. The eNOS and iNOS expressions in normal, ulcerated and APC-treated gastric tissues of mice, and their quantifications. Western blots of the expressions of the enzymes (A). Ratios of the intensities of iNOS (B) and eNOS (C) bands to that of the respective β-actin bands as quantified from the western blot images, using a Kodak Gelquant software. The values (arbitrary unit, mean ± S.E.M.) are the density scanning results of three independent experiments, considering that of normal mice as 1. $^{\dagger}P<0.001$ compared to normal mice, $^{*}P<0.05$, $^{**}P<0.001$ compared to untreated mice, $^{\#}P<0.001$ compared to omeprazole treatment.

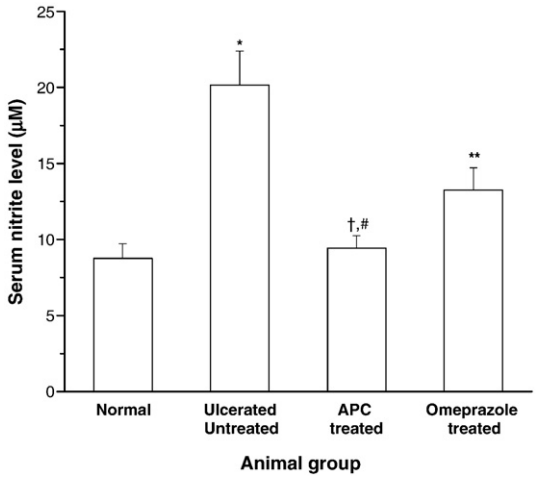


Fig. 7. Effects of APC and omeprazole in regulating serum NO level in indomethacin-induced ulcerated mice. The NO level was measured using a colorimetric kit. The values are mean ± S.E.M. of three independent experiments, each with 5 mice per group. $^{*}P<0.001$ compared to normal mice; $^{**}P<0.01$, $^{\dagger}P<0.001$ compared to ulcerated mice; $^{\#}P<0.05$ compared to omeprazole treatment.

3.3. Regulation of the NOS activity

Compared to the normal mice, a significant increase (4.61 folds, $P\times0.001$) in the total NOS activity was noticed in the ulcerated mice. APC and omeprazole reduced it by 76.1% ($P<0.001$) and 56.6% ($P<0.001$) respectively, compared to the untreated mice. APC was more effective ($P<0.01$) than omeprazole (Fig. 5).

3.4. Modulation of the mucosal eNOS and iNOS expressions

The western blots of eNOS and iNOS expressions in the gastric mucosa of the normal, ulcerated and drug (APC or omeprazole)-treated mice are shown in Fig. 6A. The eNOS expression was detected in both normal and ulcerated gastric tissues. In contrast, the iNOS expression was very high in the ulcerated tissues, but insignificant in normal gastric tissues. Quantification of the bands (Fig. 6B and C) revealed that stomach ulceration increased the expressions of iNOS (9.3 folds, $P<0.001$), while reducing that of eNOS (72%, $P<0.001$), compared to normal mice. Treatment with APC reduced the iNOS expression (65.6%, $P<0.001$) and increased the eNOS expression (67.8%, $P<0.001$), compared to the untreated mice. In contrast, omeprazole reduced the iNOS expression by 23.3% ($P<0.05$) and increased the eNOS expression by 18.2% ($P<0.05$), compared to the untreated mice. The effect of omeprazole was significantly less ($P<0.001$) than that of APC.

3.5. Regulation of the serum nitrite level

In aqueous medium, cellular NO is rapidly converted to nitrite and nitrate. However, their ratio varies substantially depending on the environment. Hence in order to investigate the effect of APC on NO production in the ulcerated mice, we assayed the total nitrite concentration, after reducing the nitrate into nitrite.

At peak ulceration, there was a significant increase (2.29 folds, $P<0.001$) in the serum nitrite level compared to the normal mice. Treatment with APC and omeprazole reduced it to 53.2% ($P<0.001$) and 34.2% ($P<0.01$) respectively, compared to the untreated mice. The effect of APC was significantly better ($P<0.05$) than that of omeprazole (Fig. 7).

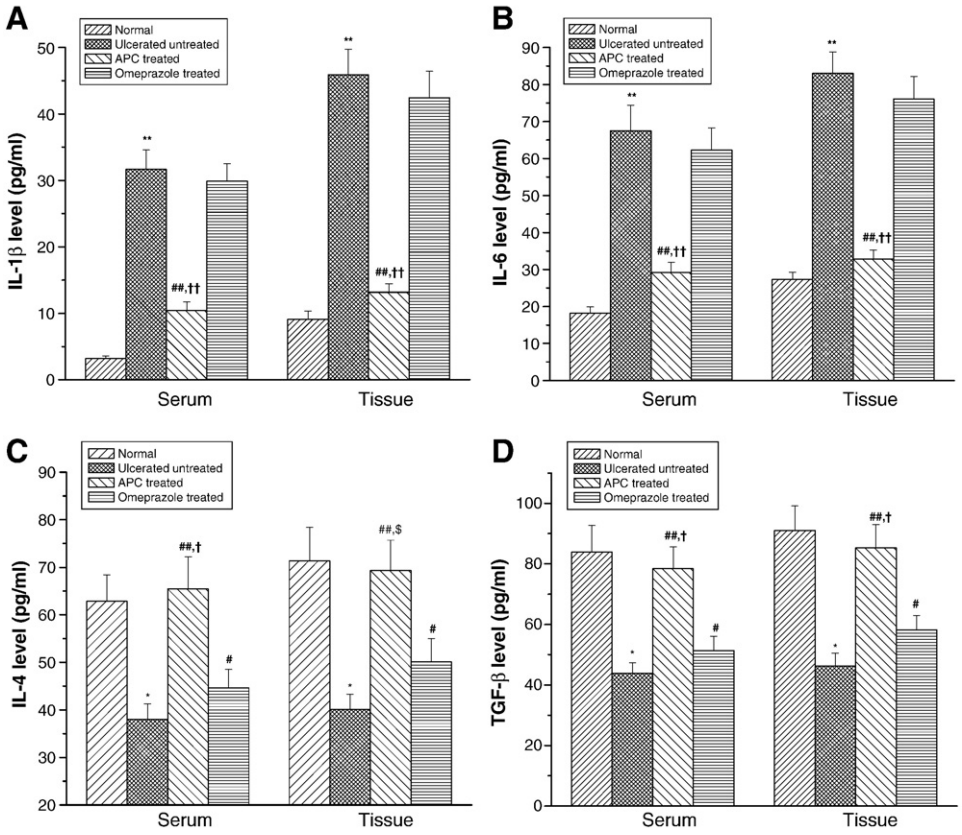


Fig. 8. Modulation of the serum and tissue levels of different pro- and anti-inflammatory cytokines by APC and omeprazole after indomethacin administration. (A) IL-1β; (B) IL-6; (C) IL-4; (D) TGF-β. The cytokine levels were assayed by ELISA. The values are mean ± S.E.M. of three independent experiments, each with 5 mice per group. $^{*}P<0.01$, $^{**}P<0.001$ compared to normal mice; $^{\#}P<0.05$, $^{\#\#}P<0.001$ compared to ulcerated mice; $^{\$}P<0.05$, $^{\dagger}P<0.01$, $^{\dagger\dagger}P<0.001$ compared to omeprazole treatment.

3.6. Regulation of the serum Th1 (IL-1β and IL-6) and Th2 (IL-4) cytokines

Compared to the normal value, ulceration drastically increased the serum and mucosal IL-β levels (Fig. 8A) by 9.9 and 5 folds respectively ($P<0.001$). APC suppressed both these parameters by 67.1% and 71.3% ($P<0.001$), compared to the untreated mice. Omeprazole, however, reduced the serum and mucosal IL-β levels by 5.7% and 7.5% respectively, which were much less than that of APC ($P<0.001$).

Likewise, ulceration also increased the serum and mucosal IL-6 levels (Fig. 8B) by 3.6 and 3 folds respectively ($P<0.001$), compared to the normal value. APC suppressed these by 55.4%, and 60.4% ($P<0.001$) respectively, compared to the untreated mice. Omeprazole reduced the serum and mucosal IL-6 by 4.9% and 8.3% compared to the untreated mice. The effect of APC was significantly better than that of omeprazole ($P<0.001$).

In contrast, the serum and tissue IL-4 levels (Fig. 8C) in the ulcerated mice were reduced by 39.5% and 43.8% ($P<0.01$) respectively, compared to the normal mice. Treatment with APC improved it appreciably both at the serum and tissue levels by 72.1%, and 73.2% ($P<0.001$) compared to the untreated mice. Omeprazole increased the serum and tissue IL-4 levels by 17.4% and 25.1% ($P<0.05$) respectively, compared to the untreated mice. The effect of APC was significantly better than that of omeprazole, both at the serum ($P<0.01$) and tissue ($P<0.05$) levels.

3.7. Regulation of the mucosal TGF-β level

Compared to the normal value, ulceration reduced ($P<0.01$) the levels of serum and mucosal TGF-β1 (Fig. 8D) by 47.7% and 49.2% respectively. However, treatment with APC and omeprazole increased it by 78.8% ($P<0.001$) and ~17% ($P<0.05$) respectively at the serum level, compared to the untreated mice. At the mucosal compartment, APC and omeprazole augmented TGF-β1 by 84.1% ($P<0.001$) and 25.7% ($P<0.05$), compared to the untreated mice. The better potency of APC over omeprazole was pronounced at both the serum and mucosal levels ($P<0.01$).

4. Discussion

Besides causing gastric ulceration, the NSAIDs including indomethacin also delay ulcer healing (Fiorucci et al., 2001), wherein several factors such as enzymes, cytokines, and soluble mediators, liberated due to the inflammatory response play crucial roles. The impressive healing capacity of APC against the indomethacin-induced gastric ulceration in mice encouraged us to investigate its probable modulatory effect on arginase and NOS as well as the Th1/Th2 cytokines profiles since these are some of the established mediators of wound healing. It is worth noting that earlier, we have carried out an elaborate study on the treatment regime with APC (Banerjee al., 2008a). Hence, presently, we confined the dose optimization study up to the 3rd day of ulceration to confirm our previous results. Although better healing was observed by extending the treatment period, a major part of it was due to natural healing. Thus, the adopted treatment regime provided a better understanding of the drug action.

The MPO activity, a marker of neutrophil aggregation at the site of inflammation is frequently increased in ulcerated conditions, and reduced during wound healing (Souza et al., 2004). Our studies depicted that while indomethacin administration enhanced the gastric mucosal MPO activity, treatment with APC (5 mg/kg × 3 days) and omeprazole (3 mg/kg × 3 days) reduced it almost equally. These results are consistent with our damage score results, where both APC and omeprazole produced comparable ulcer healing at the designated doses (Banerjee et al., 2008b).

Metabolism of arginine that can be catalyzed by arginase, and NOS, plays a vital role in gastric ulceration and its healing. Upregulation of arginase increases the level of polyamines, which play a significant role in wound healing. The regulatory role of arginase in acute intestinal inflammation and tissue repair has been demonstrated (Bernard et al., 2001; Satriano, 2004). On the other hand, catabolism of L-arginine by NOS produces NO, which can play dual roles in gastric mucosal defense and injury. NOSs exist as constitutive (cNOS), and inducible isoforms (iNOS). The low concentration of NO, produced by the endothelial NOS (eNOS), one of the cNOS isoforms helps wound healing by increasing blood flow (Whittle, 1994) and angiogenesis (Ma and Wallace, 2000; Ziche et al., 1994; Konturek et al., 1993) in the damaged gastric mucosa. However, the enhanced generation of NO by the iNOS may contribute to the pathogenesis of various gastroduodenal disorders including peptic ulcer (Souza et al., 2004; Jaiswal et al., 2001). An increase in iNOS activity and a decrease in eNOS activity in the gastric mucosa are closely related to the development of gastric mucosal lesions. Thus, the temporal switch between the i-NOS and arginase activities *in vivo* decides ulceration and healing (Shearer et al., 1997; Modollel et al., 1995).

Our results showed down-regulation of the mucosal arginase activity along with an increased expression of the iNOS due to ulceration. This suggested a shift of the arginine metabolism towards the NO/iNOS pathway during ulceration. The elevated expression of iNOS accounted for the increased total NOS activity as well as serum nitrite level during ulceration. Treatment with APC improved the arginase activity and raised the eNOS/iNOS ratio to a level favourable for efficient ulcer healing. This would generate more polyamines at the expense of the iNOS-derived nitrite that may be a key contributing factor in the anti-ulcer effect of APC. The reduction of the total NOS activity and nitrite level by APC was primarily due to suppression of the iNOS expression. Earlier, using eNOS deficient mice, the importance of eNOS and eNOS-derived NO in regulating microvascular structure during acute inflammation has been demonstrated (Luo et al., 2003). Our results suggested that the eNOS-derived NO contributed maximum to the ulcer-healing property of APC, although a role for neuronal NOS-derived NO cannot be excluded.

In contrast, despite showing less effect on modulating eNOS/iNOS expressions and NO production, omeprazole provided excellent healing. This may be due to other operative mechanism in its healing action as observed by us and others (Banerjee et al., 2008a,b; Ng et al., 2008). Factors such as control of intragastric pH (Goldstein et al., 2007) and stimulation of epithelial cell proliferation through increased serum gastrin level (Takeuchi et al., 2003) are attributed to its healing property.

Stimulation of inflammatory cytokines is extremely important in mucosal defense. One of the most prominent modes of mediation of indomethacin-induced gastropathy is the increased expression of the pro-inflammatory cytokines (Yoshikawa et al., 1993; Brzozowski et al., 2001), which also correlate with the extent of ulceration. The cross-talk amongst NOS/NO and arginase/polyamine is guided by the cytokine profile of the host (Satriano, 2003; Jenkinson et al., 1996). In view of this, the immune response due to ulceration, and its modulation by APC and omeprazole was monitored. This enabled us to associate the inflammatory response with a better prognosis.

Indomethacin administration raised the levels of pro-inflammatory cytokines (IL-1β and IL-6) while reducing the anti-inflammatory cytokines (IL-4 and TGF-β) both at the mucosal and serum levels. These led to a cytokine imbalance. We selected IL-1β for this study, since depending on its concentration in different loci of the gastrointestinal track, this cytokine modulates ulcer healing via the COX-2 pathway. Likewise, IL-4 that remains under the influence of NO, controls the expression of growth factors, and production of the pro-inflammatory cytokines such as TNF-α. These important factors govern the ulcer onset and healing.

The increased levels of Th1 cytokines due to ulceration would augment the iNOS/NO pathway to produce excess NO, which is likely to promote oxidative stress and result in ulceration (Chatterjee et al., 2006; Murphy, 1999). This was also associated with reduction in the

IL-4 level as reported earlier (Slomiany et al., 1999). Treatment with APC, however, reversed the imbalance by reducing the Th1 cytokines drastically, and restoring the levels of IL-4 and TGF-β almost to normalcy. The upregulation of the anti-inflammatory cytokines by APC is likely to inhibit the stimulatory effect of indomethacin on the level of pro-inflammatory cytokines release in blood and gastric mucosa. The immunosuppressive Th2 cytokine, TGF-β has a direct role in stimulating epithelial restitution (Kaviratne et al., 2004). Besides suppressing the IFN-γ-induced iNOS gene expression and thereby NO generation, it also increases arginase activity during inflammatory processes (Shearer et al., 1997; Modollel et al., 1995; Mitani et al., 2005). The altered arginase activity and iNOS expressions observed by us during ulceration, and APC treatment are consistent with their respective effects in modulating the mucosal TGF-β status. The enhanced IL-4 level by APC would trigger the TGF-β-SMAD-signaling pathway to stimulate the extracellular remodeling and subsequent tissue repair. In contrast, except for the TGF-β, the other cytokines were not affected significantly by omeprazole, as reported earlier (Slomiany et al., 1999). This was also reflected in its marginal effect in regulating arginase.

The results, taken together suggested a direct topical anti-inflammatory effect of APC in the stomach. This is reflected in the changes in the cytokines and arginase/NOS. A combination of these and the improved eNOS expression caused by APC might tilt the balance in favour of the repair mechanisms, explaining its ulcer-healing action. The bimodal nature of general immune responses is explained by the Th1/Th2 paradigm (Abbas et al., 1996). The regulatory T cells and Th2 cytokines often collaborate to suppress the Th1 response. Perhaps even more importantly, they strongly promote the mechanism of wound healing. However, the role of cytokine imbalance in gastropathy has not been adequately emphasized. Our results highlighted that the balance of the pro- and anti-inflammatory, as well as regulatory cytokines could play a significant role in the NSAID-induced gastric mucosal injury.

Acknowledgement

One of the authors (SKB) gratefully acknowledges the financial grant from the Department of Science and Technology (DST), Government of India for carrying out the work.

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