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Indomethacin-induced free radical-mediated changes in the intestinal brush border membranes

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) cause small intestinal damage but the pathogenesis of this toxicity is not well established. Our earlier work has shown that villus enterocytes are most susceptible to the effects of indomethacin, a commonly used NSAID. This study looked at the acute effect of indomethacin on brush border membranes (BBM), which are present mainly in the villus cells and are in immediate contact with the contents of the small intestinal lumen. Evidence of oxidative stress was found in the mucosa of the small intestine of rats dosed with indomethacin, as indicated by increased activity of xanthine oxidase with corresponding decrease in the levels of several free radical scavenging enzymes. These changes were associated with an increase in peroxidation parameters in the BBM and a fall in the level of alpha-tocopherol. These BBM also exhibited impairment in glucose transport. Significant changes were seen in the lipid composition of these membranes, with upregulation of an 85 kDa isoform of phospholipase A_2. Pretreatment of animals with allopurinol, arginine or zinc protected against these effects of indomethacin. Thus this study suggests that in an acute model of indomethacin dosing there is impairment in structure and function of the BBM in enterocytes, with the effects possibly mediated by free radicals and phospholipases.

Keywords: Allopurinol; Indomethacin; Intestinal brush border membrane; Nitric oxide; Nonsteroidal anti-inflammatory drugs; Oxidative stress; Zinc

1. Introduction

Nonsteroidal anti-inflammatory drugs are used extensively in clinical medicine as analgesics, antipyretics and anti-inflammatory agents. In spite of their therapeutic utility, these drugs have significant adverse effects, most notably those on the gastrointestinal tract [1]. These effects have been largely attributed to their ability to inhibit the enzyme cyclooxygenase, thereby suppressing prostaglandin synthesis. However, inhibition of cyclooxygenase alone does not fully explain the pathogenesis of damage in the small intestine. Several studies have shown that other mechanisms are also likely to be involved in the process [2–4].

Our earlier work with acute indomethacin dosing has shown that the drug produces free radical-induced damage in enterocytes, with the villus tip cells being particularly susceptible to these effects [5]. The effects were most marked 1 and 2 hr after oral administration of the drug in male and female rats, respectively, while changes at 4 and 6 hr after the treatment were less significant. These cells showed evidence of mitochondrial dysfunction as seen by a decrease in oxygen uptake, increased permeability of the mitochondrial membrane and changes in the composition of mitochondrial lipids. These effects appeared to be mediated through the generation of oxygen free radicals. Increased production of these reactive molecules may result from the mitochondrial dysfunction produced by indomethacin [6,7], infiltration of neutrophils into the mucosa in response to the drug [8],

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Abbreviations: ADA, adenosine deaminase; BBM, brush border membrane; L-NAME, NG-nitro-L-arginine methyl ester; MDA, malondialdehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MPO, myeloperoxidase; NO, nitric oxide; NSAIDs, nonsteroidal anti-inflammatory drugs; SOD, superoxide dismutase; XO, xanthine oxidase; XDH, xanthine dehydrogenase.

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increased activity of xanthine oxidase [9] or a combination of these factors. Arginine (a nitric oxide donor) or zinc (a putative anti-oxidant) was found to protect against the changes produced in the enterocytes by these reactive molecules [5]. Earlier reports have shown that these compounds conferred similar protection in the gastric mucosa [10,11].

Enterocytes are highly polarized cells. Two morphologically and functionally distinct regions exist in the plasma membrane of these cells—the invaginated brush border membrane that develops during cell differentiation in the direction of the lumen and rest of the membrane called the basolateral region. The intestinal BBM are found at the apical surface of villus epithelial cells and are in immediate contact with the contents of the lumen of the small intestine. This forms an important barrier between the lumen and the internal milieu of the epithelium. Nutrients must pass through this selective barrier in their passage from the intestinal lumen into epithelial cells. Under certain pathological conditions, this barrier is adversely affected leading to an increase in permeability. Such changes have been shown to occur in a variety of conditions, including burn trauma, surgical stress [12] and following ingestion of NSAIDs [13,14].

The aim of this study, therefore, was to look at the acute effect of indomethacin on the brush border membranes of the enterocytes. Arginine, zinc and allopurinol (a known inhibitor of free radical production by xanthine oxidase) were assessed for their ability to confer protection against indomethacin-induced changes in these membranes.

2. Materials and methods

Indomethacin, allopurinol, L-arginine hydrochloride, MTT, 1-chloro-2,4-dinitro benzene (CDNB), *O*-dianisidine dihydrochloride, ethylene diamine tetraacetic acid (EDTA), adenosine, bovine serum albumin (BSA), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADPH), oxidized glutathione (GSSG), reduced glutathione (GSH), 2-thiobarbituric acid (TBA), xanthine and lipid standards were obtained from Sigma. Polyethylene glycol (PEG) 4000 was obtained from Fluka AG. C^{14} labeled glucose was obtained from Bhabha Atomic Research Center. All other chemicals used were of analytical grade. Millipore membranes (pore size 0.45 μm) were obtained from Millipore. Rabbit polyclonal antibody against $cPLA_2$ was from Santa Cruz Biotechnology Inc. Affinity purified alkaline phosphatase-conjugated goat anti-rabbit IgG was obtained from Bangalore Genei.

Male albino rats (200–250 g) were used for the experiments. All the procedures performed on the animals had been approved by the Committee for the Purpose of Control & Supervision of Experiments on Animals (CPCSEA), Government of India.

2.1. Protocol for administration of experimental compounds

Male rats were fasted overnight and dosed with indomethacin (40 mg/kg) by gavage. Control animals received an equal volume of the vehicle for the drug (5% sodium bicarbonate). One hour later, the animals were killed by cervical dislocation, their abdomens opened immediately and the entire length of the small intestine removed.

For studies on the protective effect of various compounds, the following protocols were followed. Rats were given L-arginine (300 mg/kg by intraperitoneal injection) [15], allopurinol (100 mg/kg by intraperitoneal injection) [9] or zinc sulphate (50 mg/kg by gavage) [16] at 30, 60 and 120 min, respectively, prior to dosing with indomethacin. These time intervals had been chosen after preliminary experiments had shown that the maximum effects of these compounds were seen when they were administered at these respective time periods before indomethacin. The specificity of action of nitric oxide was checked by treating the animals with L-NAME, a known inhibitor of nitric oxide synthase (NOS) (30 mg/kg by gavage for 6 days) [17], before administering L-arginine and indomethacin.

2.2. Histological studies

Intestinal tissue was fixed in 4% buffered formaldehyde and paraffin embedded. Four-micron serial sections were cut and stained with haematoxylin and eosin (H and E) and also with periodic acid Schiff (PAS) stain.

2.3. Preparations of mucosal homogenates and assay of enzymes

The mucosa of the small intestine was scraped using a glass slide. The scrapings were used for the preparation of homogenates as described [18]. Activities of MPO [19], XO [20], XDH [20], catalase [21], glutathione peroxidase [22], glutathione reductase [23], SOD [24] and ADA [25] were measured in the homogenate.

2.4. Preparation of brush border membrane vesicles

Brush border membrane vesicles were prepared from the mucosal scrapings as described [18]. Purity of the preparation was checked by enrichment of the marker enzymes alkaline phosphatase [26], sucrase and maltase [27]. Protein was estimated using bovine serum albumin as standard [28].

2.5. Assessment of parameters of oxidative stress

Levels of MDA [29], conjugated diene [30], protein carbonyl [31] and alpha-tocopherol [32] were measured in the BBM preparation.

2.6. Measurement of D-glucose uptake

Isolated BBM were assessed for their ability to transport glucose by the rapid filtration technique as described [33].

2.7. Analysis of lipids

Total lipids were extracted from BBM by the Bliigh and Dyer method [34]. Neutral lipids were separated on silica gel G plates using the solvent system consisting of hexane:diethyl ether:acetic acid (80:20:1, v/v). The spots corresponding to the standards were identified by iodine exposure and eluted. Cholesterol, cholesteryl esters (CE) [35], triacylglycerol (TAG) and diacylglycerol (DAG) [36] were quantified. Free fatty acids were methylated and quantitated by gas chromatography [37]. Individual phospholipids were separated on silica gel H plates using the solvent system consisting of chloroform:methanol:acetic acid:water (25:14:4:2, v/v) and quantitated by phosphate estimation after acid hydrolysis [38]. Phosphatidic acid (PA) was separated on silica gel G plate impregnated with oxalic acid [37] and quantitated.

2.8. Identification of PLA_2 in BBM by immunoblotting

Phospholipase A_2 (PLA_2) in the BBM was detected by immunoblotting [39]. BBM protein (75 μg) was resolved on SDS polyacrylamide gels (7.5%) by electrophoresis for 1 hr at a constant voltage of 100 V. The samples were then transferred on to a nitrocellulose membrane (type NC, 0.45 μm pore size) using a blotting apparatus. Non-specific binding sites were blocked overnight at 4° with wash buffer containing Tween 20 (10 mM Tris pH 7.4, 150 mM NaCl, 5 mM sodium azide and 20% Tween 20) with 5% w/v fat-free dry milk powder. The membranes were incubated for 2 hr at room temperature with 1:200 diluted rabbit polyclonal IgG against $cPLA_2$. After washing in buffer containing Tween 20 for 10–15 min, the membranes were incubated for 2 hr at room temperature with 1:1000 diluted anti-rabbit IgG conjugated to alkaline phosphatase. The membranes were again washed with wash buffer containing Tween 20 before detection of the alkaline phosphatase activity using bromochloroindolyl phosphate (BCIP).

3. Results

Histological examination of the small intestine from control and drug-treated rats by light microscopy (using haematoxylin and eosin and PAS stains) showed villi of normal height and width. There was mild focal vacuolation of the epithelial cells covering the villus tip and attenuation of the brush border on PAS stained sections of tissue from indomethacin-treated rats only (Fig. 1B). There was no such attenuation seen in the tissue from control rats (Fig. 1A) as well as those that had been pre-treated with arginine, zinc or allopurinol (Fig. 1C–E) but it was evident in tissue that had been exposed to L-NAME before the administration of arginine and indomethacin (Fig. 1F).

Measurement of activity of enzymes in the mucosal homogenate indicated evidence of oxidative stress following indomethacin treatment. There was an increase in XO activity accompanied by a concomitant decrease in XDH (Fig. 2). Adenosine deaminase and MPO activities were also higher in the homogenate from these animals. Measurement of the free radical scavenging enzymes revealed that the activities of superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase were lower in the homogenate from indomethacin-dosed rats than in control preparations (Fig. 3).

In isolated brush border membranes, the marker enzymes alkaline phosphatase, sucrase and maltase showed 6–10-fold enrichment in the final preparation (data not shown), thereby confirming purity of the preparation. Measurement of indicators of oxidative stress in the BBM showed increase in the levels of MDA, conjugated dienes and protein carbonyl and a decrease in alpha-tocopherol in rats dosed with indomethacin when compared with control animals (Fig. 4).

BBM lipid analysis revealed significant changes after drug treatment. Levels of cholesteryl esters and triacylglycerol were found to be decreased with corresponding increases in the level of free cholesterol and diacylglycerol (Fig. 5). Among the phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were decreased (Fig. 6A and C) with concomitant elevations in the levels of lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE) (Fig. 6B and D) and PA (Fig. 7A). Levels of other phospholipids such as phosphatidylserine, phosphatidylinositol and sphingomyelin were also lower in the BBM isolated from drug-treated animals (data not shown). The ratio of total cholesterol to total phospholipids was higher in the indomethacin-treated BBM (Fig. 7B). Levels of free fatty acids, such as palmitic, stearic, oleic, linoleic and arachidonic acids were higher in BBM from drug-treated rats when compared with control animals (Fig. 7C). The changes in the lipid composition suggested PLA_2-mediated degradation of BBM lipids and hence the presence of PLA_2 on BBM was looked for by immunoblot. As shown in Fig. 8, the expression of $cPLA_2$ was found to be unregulated in BBM from indomethacin-treated rats as compared with BBM from control animals.

Glucose transport by these membranes was measured to check for functional alterations in the BBM. The results indicate that glucose transport by the BBM isolated from indomethacin-treated animals was impaired in comparison with control BBM (Fig. 9).

Pretreatment of the experimental animals with arginine, zinc or allopurinol conferred protection against the effects

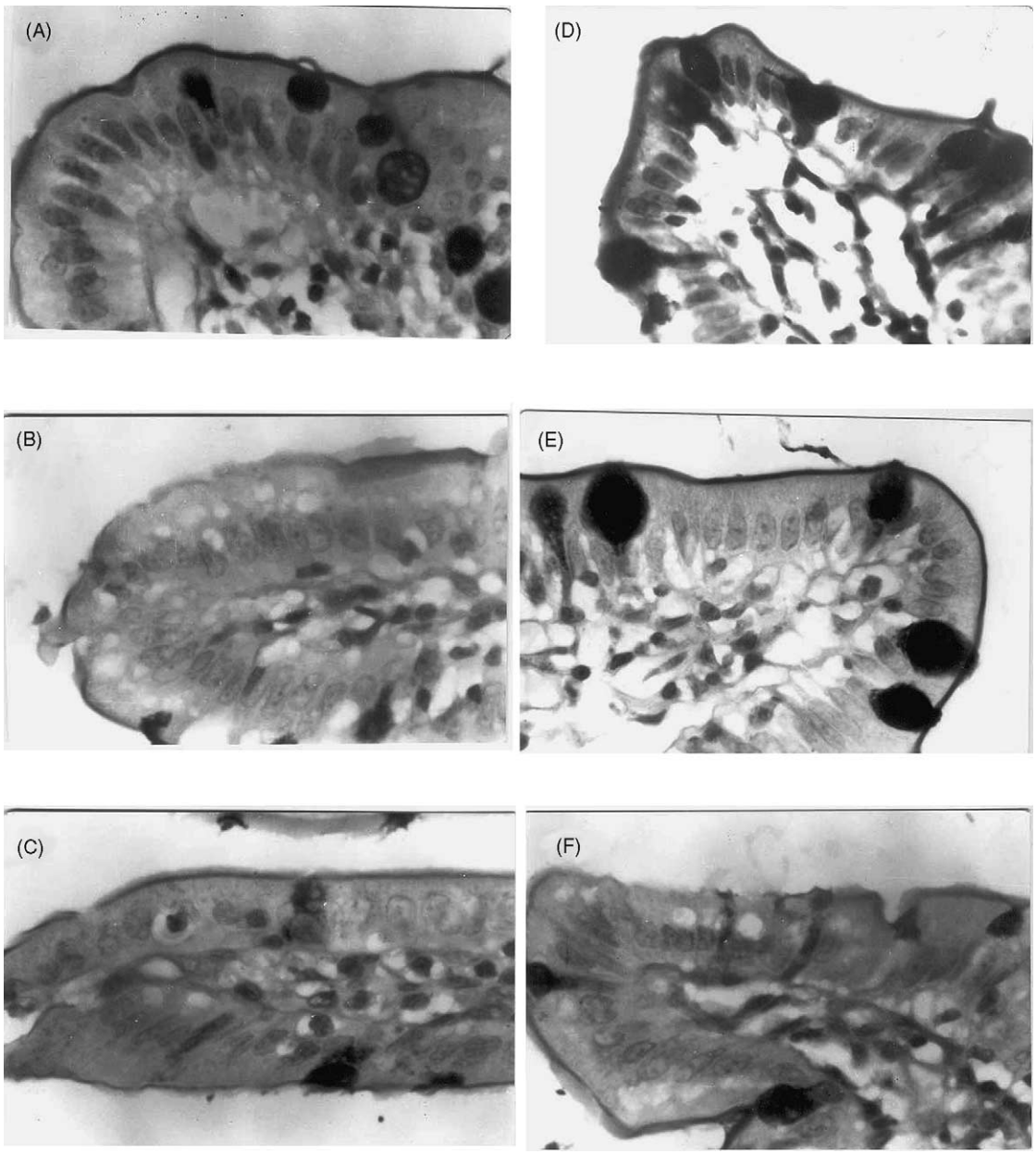


Fig. 1. Periodic acid Schiff stained histological sections of the small intestine from control (A) and indomethacin-treated rats (B). (C–E) Represent tissue from rats pretreated with arginine, zinc and allopurinol, respectively. (F) Shows tissue from rats that had been treated with L-NAME prior to the administration of arginine and indomethacin.

of indomethacin. Changes that had been observed in the parameters of oxidative stress (Figs. 2 and 4), free radical scavenging enzymes (Fig. 3), composition of membrane lipids (Figs. 5–7), glucose transport (Fig. 9) and the content of PLA_2 (Fig. 8) in BBM that were seen in response to indomethacin were all abolished by pretreatment with the agents. The histological features produced by the drug were not found in tissue obtained from animals that had received the pretreatments (Fig. 1C–E) as seen by PAS staining. The protection conferred by arginine was found to be nullified by the use of L-NAME (Figs. 1F and 2–8), thereby showing that it was the nitric oxide formed from arginine that was responsible for the protection.

4. Discussion

The enterocyte is a highly specialized cell with apparently paradoxical dual functions of facilitating absorption and maintaining an effective barrier to the entry of noxious substances from the intestinal lumen. The brush border membrane of the enterocyte, which is mainly present in the villus cells, is a distinct region of the cell, both morphologically and functionally. It forms an important component of the barrier between the contents of the lumen and the epithelium of the intestinal mucosa. Our earlier study indicated that in response to indomethacin administered by gastric gavage, the villus epithelial cells were more

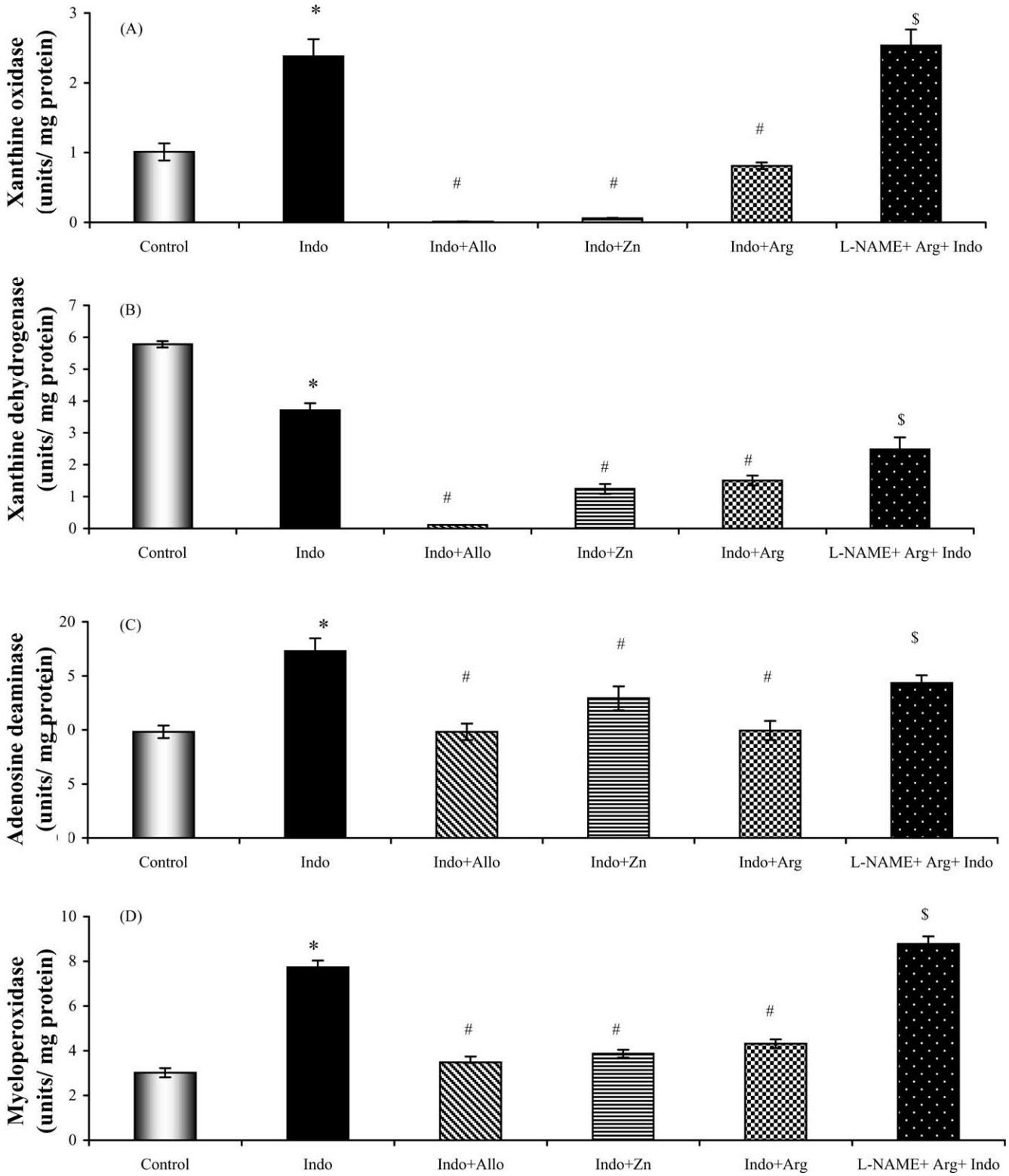


Fig. 2. Activities of various enzymes in mucosal homogenates (A–D). Homogenates were prepared from mucosal scrapings from rats in control, indomethacin-treated and different pretreatment groups. (*) $P < 0.05$ as compared with control group, (#) $P < 0.05$ when compared with indomethacin-treated group and ($) $P < 0.05$ when compared with indomethacin + L-arginine group. Each value represents mean ± SD (N = 6 rats).

susceptible to damage than other cell populations of the intestine. Since the BBM in these villus cells are in direct contact with the contents of the lumen, this study looked at the effect of this drug on these membranes.

Histological examination of the intestine showed attenuation of the glycocalyx layer as evidenced by decreased PAS staining of the brush border from the indomethacin-treated animals when compared with control

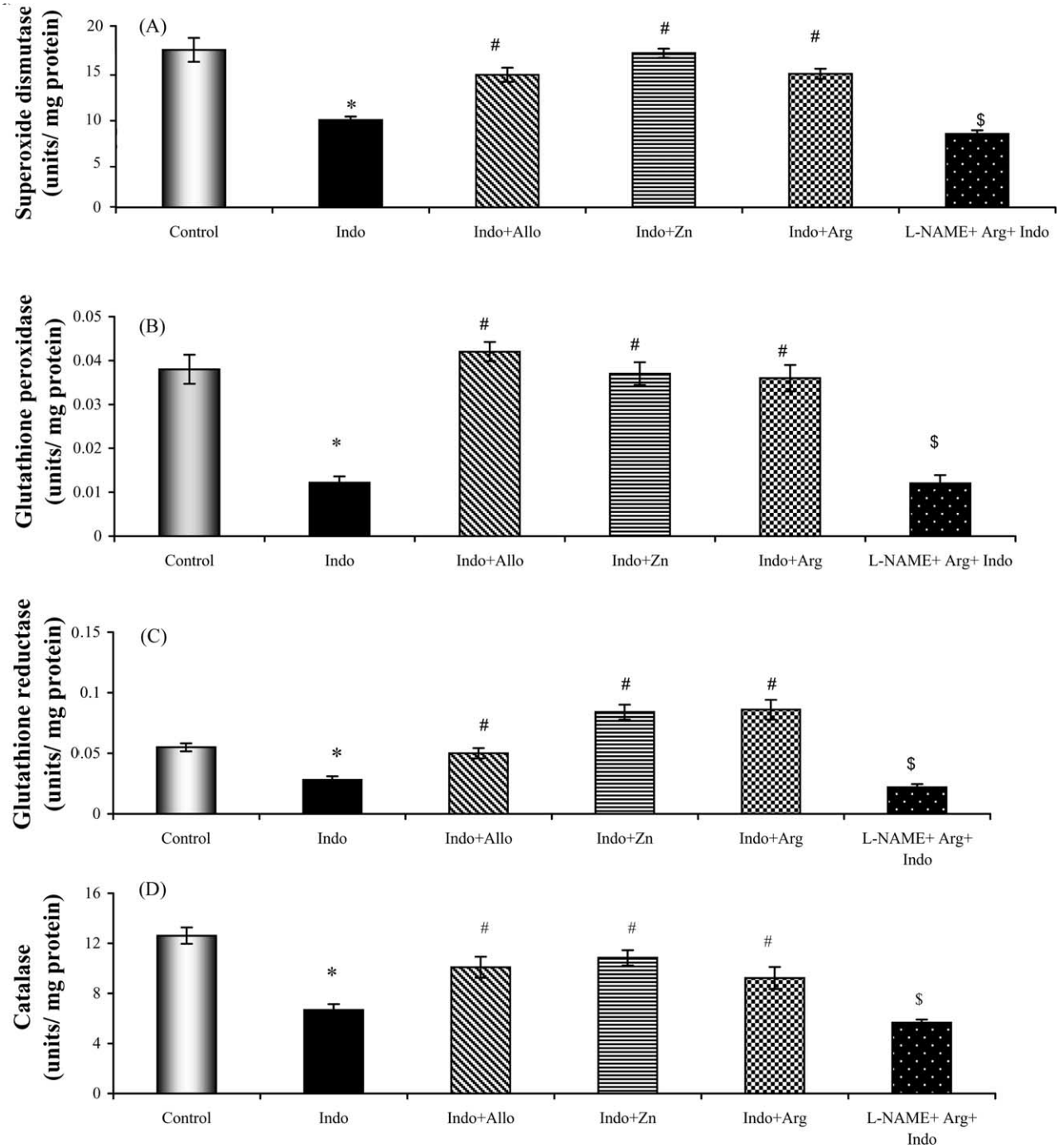


Fig. 3. Activities of anti-oxidant enzymes in mucosal homogenates (A–D). Homogenates were prepared from mucosal scrapings from rats in control, indomethacin-treated and different pretreatment groups. (*) $P < 0.05$ as compared with control group, (#) $P < 0.05$ when compared with indomethacin-treated group and ($) $P < 0.05$ when compared with indomethacin + L-arginine group. Each value represents mean ± SD (N = 6 rats).

tissue (Fig. 1), indicating possible disturbances in the glycosylation of this layer. PAS stains tissue carbohydrates and it is used to specifically stain the glycoproteins in the brush border membrane. Attenuation of the brush border in the indomethacin-treated tissue indicates loss of the glycocalyx layer. This layer has an important role to play in protecting the mucosa against damage and disruptions in the content of this layer may lead to functional impairment of this barrier.

Evidence of oxidative stress in the BBM was seen following indomethacin treatment, as shown by the increase in MDA, conjugated diene and protein carbonyl with a concomitant decrease in the level of the anti-oxidant, alpha-tocopherol. Free radicals responsible for these changes were likely to be produced by XO activation, mitochondrial dysfunction and neutrophils infiltrating the mucosa. It was observed in this study that there was an increase in XO activity with a concomitant decrease in

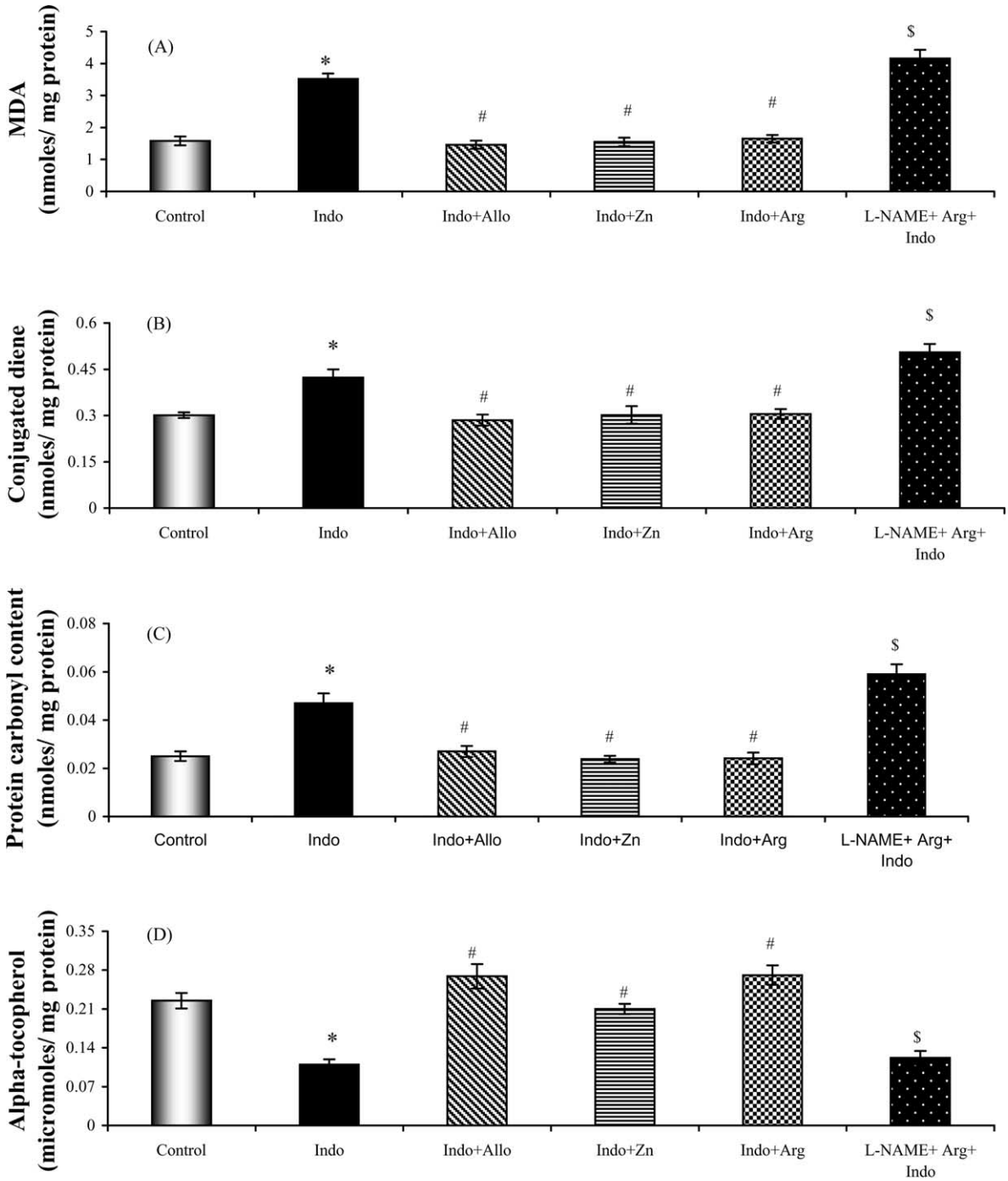


Fig. 4. Peroxidation products in BBM. Levels of malondialdehyde (A), conjugated dienes (B), protein carbonyls (C) and alpha-tocopherol (D) were measured in BBM from control, indomethacin-treated and different pretreatment groups of rats. (∗) $P < 0.05$ when compared with control group, (#) $P < 0.05$ when compared with indomethacin-treated group and ($) $P < 0.05$ when compared with indomethacin + L-arginine group. Each value represents mean ± SD (N = 6 rats).

XDH. Such a change is known to be associated with increased production of superoxide with subsequent oxidative stress [9,40]. There was also an increase in ADA activity that provides the substrate for XO. Activation of ADA may be linked to degradation of AMP by a nucleotidase to form adenosine. When there is a decline in ATP levels in the cell, as happens following its rapid utilization or inhibition of oxidative phosphorylation, excess AMP that may accumulate as a result is removed in an effort to maintain the energy charge of the cell [41,42]. A fall in energy charge has been reported in rat jejunum when exposed to indomethacin *in vitro* [7], possibly due to the mitochondrial dysfunction that has been shown to occur in enterocytes exposed to indomethacin. Such alterations could conceivably lead to increased degradation of AMP, thereby triggering many of the changes outlined

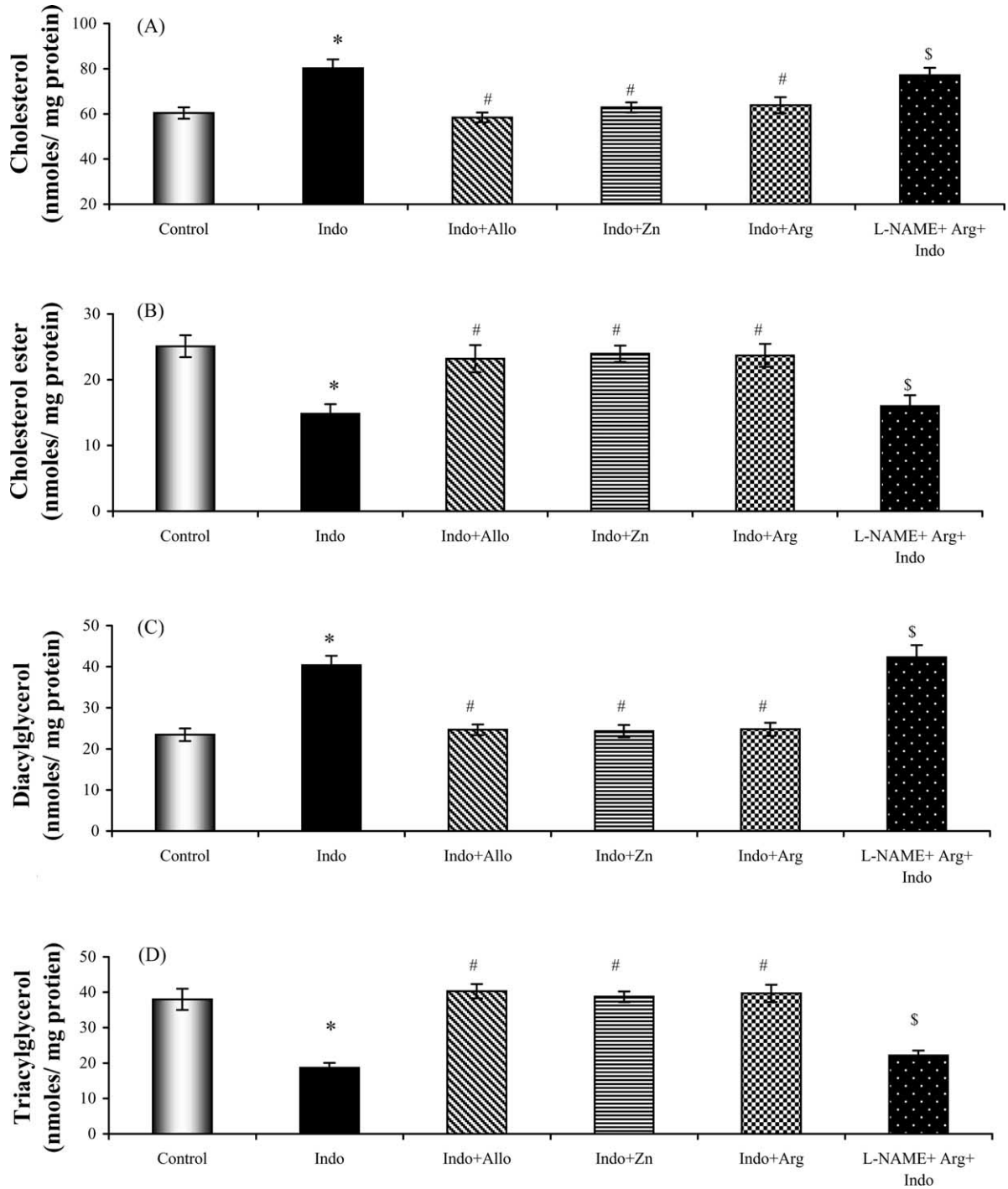


Fig. 5. Neutral lipids in BBM (A–D). The composition of neutral lipids in BBM from control, indomethacin-treated and different pretreatment groups of rats was determined. (*) $P < 0.05$ when compared with control group, (#) $P < 0.05$ when compared with indomethacin-treated group and ($) $P < 0.05$ when compared with indomethacin + L-arginine-treated group. Each value represents mean ± SD (N = 6 rats).

above. The mitochondrial dysfunction produced by indomethacin [6,7] is also likely to contribute to elevated levels of free radicals. In addition, there was also evidence of infiltration of neutrophils into the intestinal mucosa as assessed by increased myeloperoxidase activity following indomethacin treatment, a finding in keeping with earlier reports [8] and a circumstance contributing to increased oxidative stress.

Lipids are important components of the biomembrane; hence any alteration in their composition may bring about structural and functional changes in the membrane. Treatment with indomethacin resulted in changes in both the neutral lipid and phospholipid composition of the BBM, leading to alterations in the cholesterol/phospholipid ratio. Changes in this ratio in biomembranes are known to alter fluidity of the membrane [43,44] and this may cause

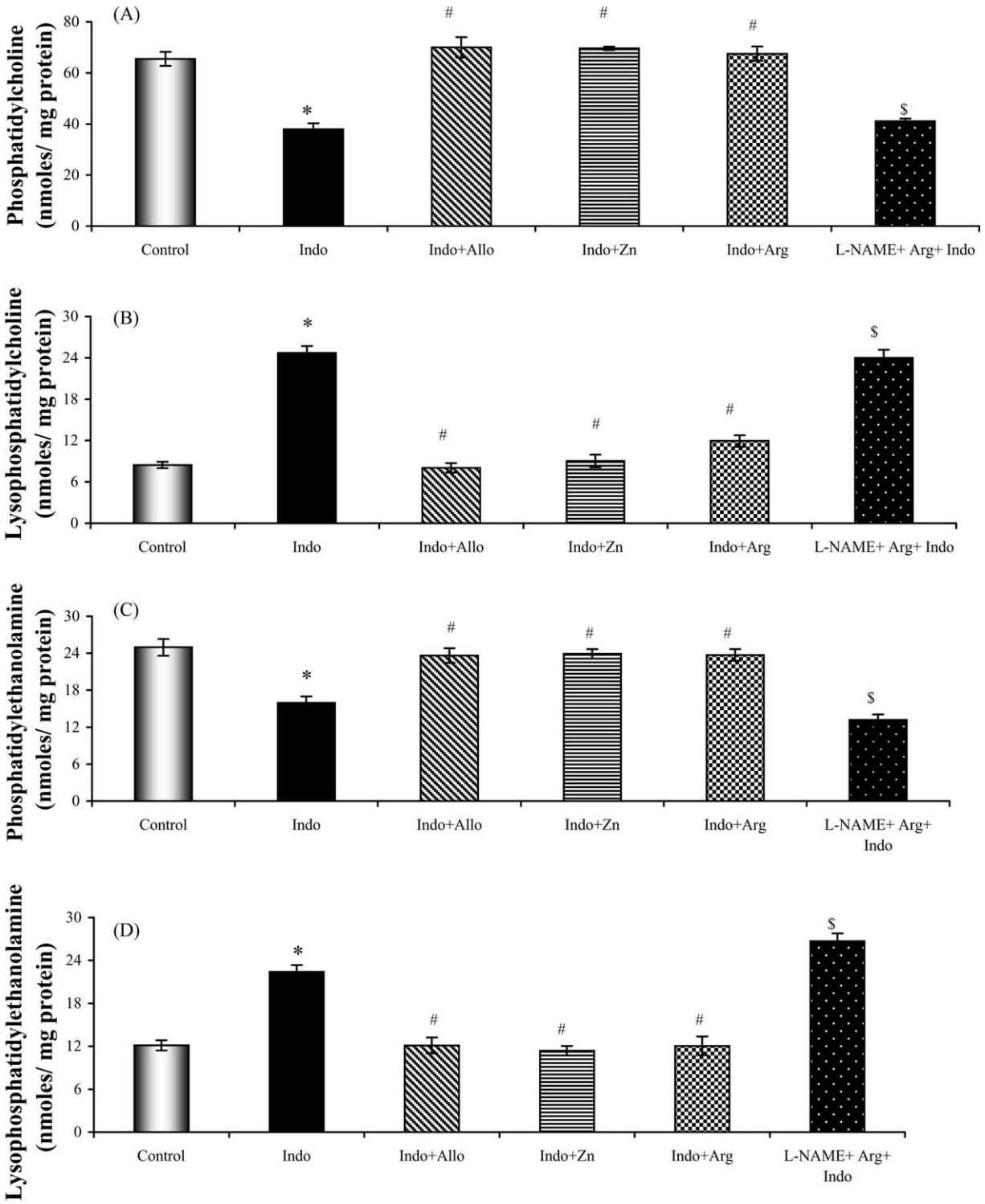


Fig. 6. Phospholipids in BBM (A–D). The composition of phospholipids in BBM from control, indomethacin-treated and different pretreatment groups of rats was determined. (*) $P < 0.05$ when compared with control group, (#) $P < 0.05$ when compared with indomethacin-treated group and ($) $P < 0.05$ when compared with indomethacin + L-arginine-treated group. Each value represents mean ± SD (N = 6 rats).

changes in barrier function. It is possible that these effects may contribute to the increased intestinal permeability seen in experimental animals and humans in response to orally administered indomethacin [45,46]. The increase in permeability as a consequence of exposure to NSAIDs allows the entry of luminal aggressive factors such as bile, enzymes and bacteria, and is postulated to be an important event in the pathogenesis of NSAID-induced enteropathy [3,4].

The decreases in PC and PE with concomitant increases in LPC, LPE and free fatty acids, especially arachidonic

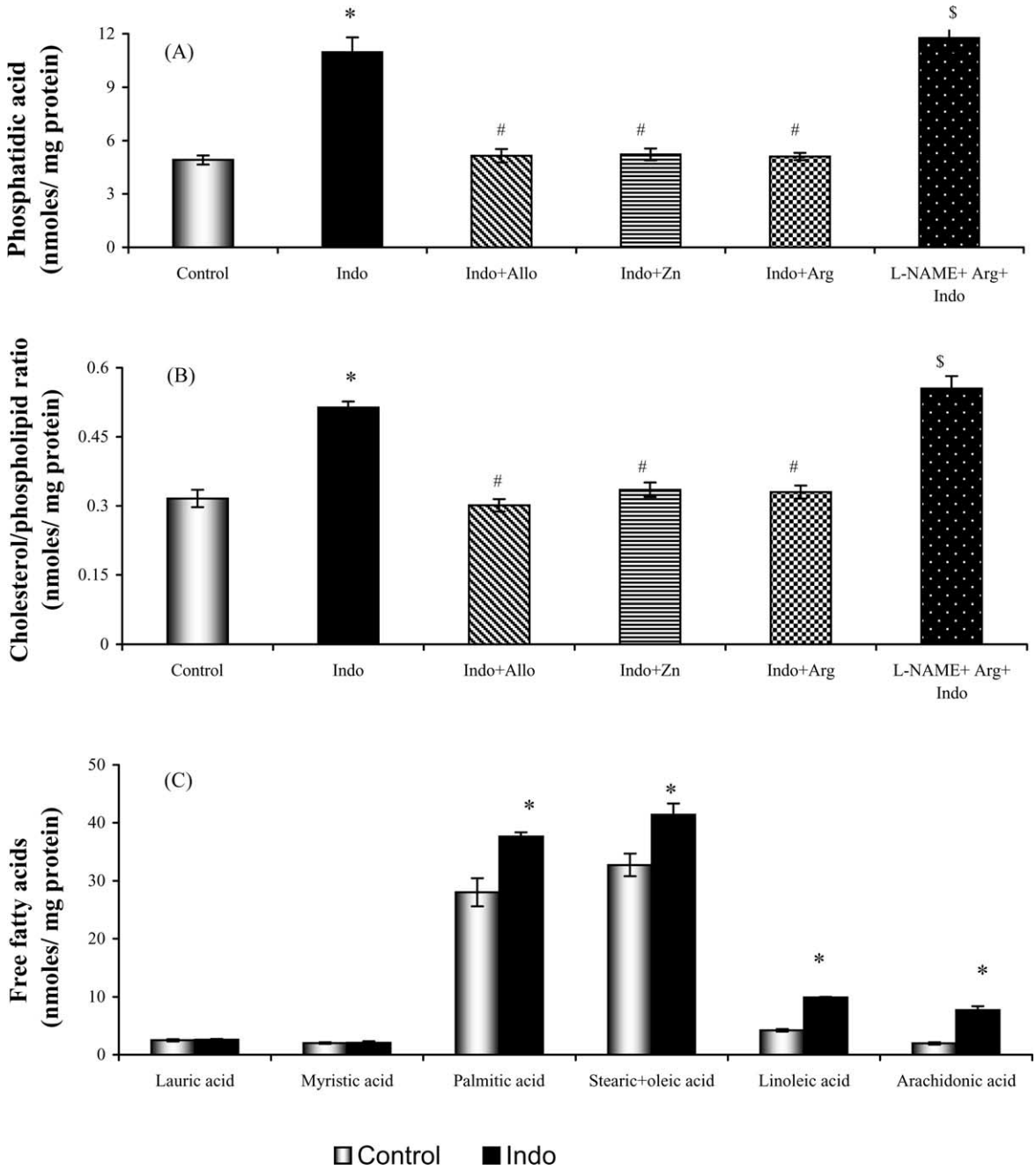
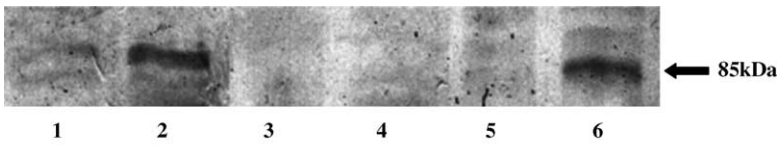


Fig. 7. Phosphatidic acid (A) and cholesterol/phospholipid ratio (B) of BBM from control, indomethacin-treated and different pretreatment groups of rats. Free fatty acid (C) composition of the BBM from control and indomethacin-treated groups. (∗) $P < 0.05$ when compared with control, (#) $P < 0.05$ when compared with indomethacin-treated rats and ($) $P < 0.05$ when compared with indomethacin + L-arginine-treated groups. Each value represents mean ± SD (N = 6 rats).

acid, following treatment with indomethacin indicates involvement of PLA_2. This was confirmed by identification of $cPLA_2$ in the BBM by immunoblot. Release of arachidonic acid suggests involvement of $cPLA_2$ that is specific for the degradation of phospholipids containing arachidonic acid. This activation of phospholipase (possibly the cytosolic isoform [$cPLA_2$] that has translocated from the cytosol to the membrane) may have occurred as a result of the oxidative stress produced in the cell [47]. The accumulation of free arachidonic acid, due to both PLA_2 activity and inhibition of cyclooxygenase by indomethacin, may induce apoptosis in the enterocytes [48], thereby contributing to the enteropathy produced by the drug.

Decreased transport of glucose by the indomethacin-treated BBM vesicles was seen when compared with control vesicles. This may be an indication of functional impairment of the BBM. Tiruppathi *et al.* [33] have shown similar dysfunction in transport systems of the small intestinal brush border membrane, in response to alterations in lipids.



1 - Control
2 - Indo
3 - Indo + Allo
4 - Indo + Zn
5 - Indo + Arg
6 - L-NAME + Arg + Indo

Fig. 8. Western blot of PLA_2. PLA_2 content in BBM from control, indomethacin-treated and different pretreatment groups was measured by immunoblot.

Oxidative stress, produced by treatment with indomethacin, and its consequent effects on the structure and function of the BBM was prevented by pretreatment with allopurinol, L-arginine or zinc. Allopurinol is a known inhibitor of XO [49] and it reduces the amount of free radicals generated in the tissue in response to the drug. This probably accounts for its protective effect. L-Arginine is the substrate for NOS and this releases NO, which can react with the superoxide radical and thus prevent oxidative stress. This protective effect was lost by pretreatment with L-NAME (inhibitor of NOS) along with L-arginine, indicating that the effect was mediated by the production of NO. This observation is in agreement with those reported earlier [50]. Zinc is known to be a membrane stabilizing anti-oxidant [51,52] and this effect may be responsible for the protection that pretreatment with zinc offered. The effectiveness of these agents in abolishing the changes produced in the BBM by indomethacin testifies to the important role played by free radicals in initiating and sustaining the process of damage induced by the drug.

In conclusion this study has shown that the oral administration of an acute dose of indomethacin results in oxidative stress in the small intestine and this leads to structural and functional alterations in the BBM. We postulate that

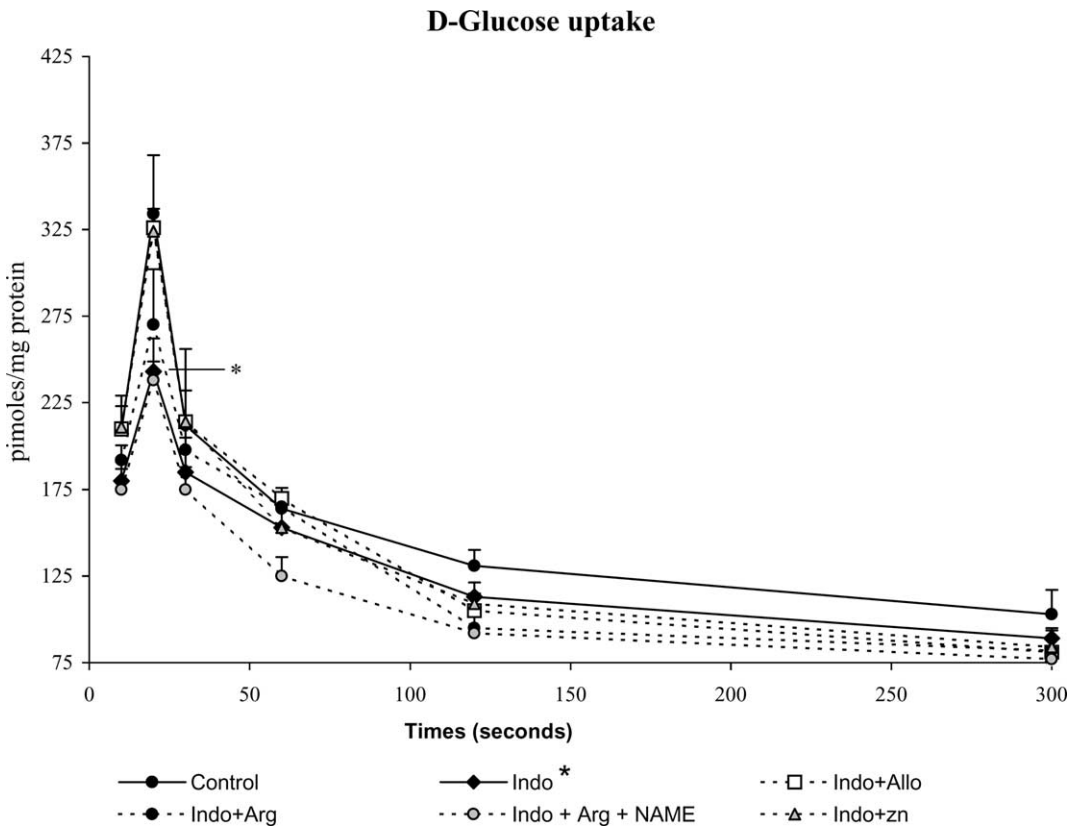


Fig. 9. D-Glucose transport by the BBM. D-Glucose uptake by the BBM from control and indomethacin-treated groups of rats was determined. (*) $P < 0.05$ when compared with control group. Each value represents mean ± SD (N = 6 rats).

such alterations may contribute to the increased intestinal permeability that occurs in response to the drug with subsequent progress to macroscopic damage. Since the BBM is the first line of contact with the luminal contents of the intestine, which include bacteria, bacterial products and bile, BBM damage may facilitate entry of these substances leading to NSAID-induced ulceration. Inhibitors of free radical generation or free radical scavengers appear to prevent the initiation of these effects.

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