Toxicology in Vitro 23 (2009) 887–896

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Activation of phospholipase A_2 is involved in indomethacin-induced damage in Caco-2 cells

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ARTICLE INFO

Article history:
Received 3 April 2009
Accepted 15 May 2009
Available online 24 May 2009

Keywords:
Anti-inflammatory agents, non-steroidal
Caco-2 cells
Cell death
Indomethacin
Methyl arachidonyl fluorophosphonate
Phospholipase A_2, cytosolic

ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs), widely used in clinical practice, cause adverse effects in the gastrointestinal tract. These effects have been attributed to mechanisms such as drug-induced cyclooxygenase inhibition, oxidative stress, mitochondrial dysfunction and changes in cell membrane lipids. Our previous study showed that indomethacin (an NSAID commonly used in toxicity studies) caused activation of cytosolic phospholipase A_2 ($cPLA_2$) in the rat small intestine. We hypothesized that activation of $cPLA_2$ is an important event in the pathogenesis of indomethacin-induced damage in enterocytes. To test this, we incubated enterocyte-like Caco-2 cells with indomethacin, with and without pretreatment with methyl arachidonyl fluorophosphonate (MAFP), an inhibitor of $cPLA_2$. Cells treated with indomethacin showed decreased viability and evidence of oxidative stress and morphological cell damage. Phospholipids were degraded in these cells, with increases in the levels of lysophospholipids and arachidonic acid. There was no evidence of apoptosis in the cells in response to the drug. Pretreatment of the cells with MAFP attenuated the drug-induced effects seen. This shows that activation of phospholipase A_2 appears to be an important event in the pathogenesis of indomethacin-induced damage in Caco-2 cells. To our knowledge, this is the first report that implicates the involvement of this enzyme in NSAID-induced enteropathy.

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are extensively used in clinical medicine as anti-inflammatory, analgesic and antipyretic agents. However, the adverse effects that these drugs produce in the gastrointestinal tract often limit their use in clinical practice (Gabriel et al., 1991). The therapeutic effects of NSAIDs result from inhibition of cyclooxygenase (Vane, 1971), a key enzyme involved in the formation of prostaglandins. Inhibition of this enzyme by these drugs is also known to contribute to adverse effects caused by NSAIDs (Whittle, 2003). However, other factors such as drug-induced mitochondrial dysfunction (Jacob et al., 2001; Nagano et al., 2005; Somasundaram et al., 1997) and oxidative stress (Basivireddy et al., 2002, 2003; Nagano et al., 2005) have also been postulated to contribute to the pathogenesis of such damage.

Phospholipase A_2 (PLA_2) is an enzyme that catalyzes the hydrolysis of the sn-2 fatty acyl bond of phospholipids to yield fatty acids and lysophospholipids (Balsinde et al., 2002). It comprises both extracellular and intracellular forms. Intracellular phospholipase A_2 is further classified into $cPLA_2$ (cytosolic calcium-dependent, group IV) and $iPLA_2$ (cytosolic calcium- independent, group VI), based on the Ca^{2+} requirements needed for basal activity. $cPLA_2$ requires micromolar concentrations of Ca^{2+} for membrane translocation, but not for catalysis, and possesses a preference for phospholipids containing arachidonic acid (AA) (Caro and Cederbaum, 2006). Lysophospholipids released by the action of PLA_2 are thought to perturb membrane homeostasis and increase membrane fluidity and permeability (Yang et al., 1999). Arachidonic acid, the other product released, is known to directly affect ion channels (Kim et al., 1995; Macica et al., 1996) and increase the mitochondrial permeability transition pore opening time (Scorrano et al., 2001). All these downstream effect of $cPLA_2$ can result in disruption of the functioning of cells.

Our earlier studies have shown that $cPLA_2$ was activated in the small intestine in response to the administration of indomethacin (Basivireddy et al., 2003). The activation of this enzyme was associated with changes in the content of phospholipids in intestinal brush border membranes isolated from these animals. Such

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doi:10.1016/j.tiv.2009.05.008

changes have been postulated to contribute to changes in barrier function of these membranes, resulting in the increased intestinal permeability commonly seen in response to the administration of NSAIDs (Bjarnason et al., 1992; Choi et al., 1995). This drug-induced increased intestinal permeability allows the entry of luminal aggressive agents, such as bacteria and bile, into the intestinal mucosa and is held to contribute to the subsequent development of NSAID-induced enteropathy (Bjarnason and Thjodleifsson, 1999). This condition is characterized by erosions and ulcers that can lead to loss of blood and protein, contributing to iron deficiency and hypoalbuminemia, respectively. Both of these conditions may contribute to the general ill-health of patients who take these drugs on a long-term basis. NSAIDs can also cause small intestinal perforation at sites of ulcers and strictures requiring surgery (Davies et al., 2000).

The current study was designed to determine the role of indomethacin-induced activation of $cPLA_2$ in the pathogenesis of indomethacin-induced damage in enterocytes. The accumulation of arachidonic acid, as a consequence of the activity of $cPLA_2$, is an event that has been shown to trigger apoptosis (Levine, 2001). In view of this, studies were also carried out to determine whether indomethacin-treated cells undergo apoptosis. The results of our studies suggest that activation of phospholipase A_2 contributes to the pathogenesis of indomethacin-induced damage in Caco-2 cells.

2. Methods

2.1. Test system used

We used Caco-2 cells, which are human small intestinal epithelial cells often used as models for enterocytes, for our studies. The cells were a gift from the laboratory of Prof. Gagandeep Kang, Wellcome Research Unit, Christian Medical College, Vellore, India. Cells were cultured in 25 cm^2 flasks with Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal calf serum, 50 U/ml of penicillin and 50 μg/ml of streptomycin, at 37 °C and 5% CO_2. After the cells reached confluence, cells obtained from passages between 20 and 35 were used for the various experiments described.

2.2. Experimental design

Methyl arachidonyl fluorophosphonate (MAFP), an inhibitor of $cPLA_2$ (Balsinde and Dennis, 1996; Slomiany and Slomiany, 2007) was used in the studies described. It was used at a final concentration of 20 μM in the cell culture medium (Slomiany and Slomiany, 2007). An equal volume of dimethyl sulfoxide (DMSO), which was the vehicle used for the drug, was added to control cells to a final concentration of 0.1% (v/v). Incubations were carried out for 30 min. This was followed by addition of indomethacin (500 μM final concentration in the medium) (Jacob et al., 2001) or equal volume of its vehicle (5% sodium bicarbonate). The cells were further incubated for an hour after this addition. These doses and time periods for incubations with MAFP and indomethacin were chosen after preliminary experiments showed that these concentrations, obtained from previously published reports, produced consistent effects at the specified time points. The various experimental groups were as follows: control (DMSO + 5% sodium bicarbonate), indomethacin (DMSO + indomethacin), MAFP + indomethacin and MAFP (MAFP + 5% sodium bicarbonate). At the end of the incubations with indomethacin or its vehicle, the cells were washed with sterile phosphate-buffered saline (PBS) pre-warmed to 37 °C and then trypsinized. The resultant cell suspension was centrifuged and the pellet of cells obtained was used for further experiments.

2.3. Measurements made

2.3.1. Assessment of viability of cells

The cells were used for assessment of cell viability by trypan blue exclusion (Tepperman et al., 1991). For this, washed cells in suspension (approximately 0.1 ml) were made up to 1 ml with oxygenated buffer (2.5 mM calcium chloride, 5 mM glucose, pH 7.4) and centrifuged at 1000*g* at 4 °C. The pellet obtained was suspended once again in 1 ml of oxygenated buffer. Ten microlitres of cell suspension was mixed with 90 μl of 0.1% trypan blue dye. This mixture was loaded onto a Neubauer chamber and the number of viable cells counted. Viable cells were identified by their ability to exclude the dye, whereas dead cells were found to take up the dye. The percentage of viable cells was calculated by dividing the number of viable cells by the total number of cells counted.

Cell viability was also measured by using the CellTiter-Glo Luminescent cell viability assay, according to manufacturer's instructions (Promega Corporation, WI, USA). This assay measures the amount of ATP in cells, which indicates the presence of metabolically active cells. Luminescent signal was measured using a Wallac 1420 VICTOR^3 multilabel counter with fluorescence and luminescence technologies (PerkinElmer Life and Analytical Sciences, Inc., Massachusetts, USA).

The effect of indomethacin on the integrity of the cells was also determined by measuring the release of lactate dehydrogenase (LDH) from the cells into the medium. This was done by estimating the activity of the enzyme both in the cells and the medium (Moss and Henderson, 1994). For this, after the treatments described, the cell suspensions were subjected to centrifugation. LDH activity was measured separately both in the supernatant and the cells. For the latter, the cells were homogenized using Tris-EDTA-NADH buffer (Tris 56 mM, EDTA 5.6 mM, NADH 170 μM, pH 7.4)). To 2 ml of the Tris-EDTA-NADH buffer, 10 μl of cell homogenate was added. The mixture was incubated at 37 °C for 15 min. Pyruvate (200 μl of a 14 mM solution) was added to it. The absorbance of the resultant mixture was then measured at 339 nm. The results were expressed in international units (IU).

2.3.2. Assessment of cell morphology

Morphology of the treated cells was assessed by viewing them in their flasks, using a Nikon Ecllipse TE 2000-U inverted microscope at high power. Several areas in each flask were assessed. The same fields were documented before and after the various treatment protocols. More detailed assessment of cell morphology was also done. For this, the pellets of cells obtained were fixed in 2.5% glutaraldehyde, post-fixed in osmium tetroxide and embedded in araldite (epoxy resin). Once embedded, 1 μm sections were cut and stained with toluidine blue. The sections were examined by light microscopy.

Ultra thin sections were also cut from selected blocks with a Leica ultracut UCT (Wien, Austria) and a diamond knife (Diatome, Switzerland). Sections were stained with uranyl acetate and lead citrate and examined with a Philips EM 201C electron microscope.

2.3.3. Evaluation of oxidative stress

Cells were homogenized in PBS and this homogenate was used for various assays. Levels of malondialdehyde (MDA) (Ohkawa et al., 1979) and protein carbonyl (Sohal et al., 1993) were measured. Estimations were also made of the activity of the pro-oxidant enzyme, xanthine oxidase (Parks et al., 1988).

2.3.4. Analysis of lipids

Lipids were extracted from the cell homogenates. Samples corresponding to 2 mg protein concentration were used to extract lipids (Bligh and Dyer, 1959). Neutral lipids were separated on silica

gel G plate, using the solvent system hexane:diethyl ether:acetic acid (80:20:1 v/v). The separated lipids were identified by exposure to iodine vapour. Spots corresponding to standards were eluted. Cholesterol, cholesteryl esters (CE) (Zlatkis et al., 1953), triacylglycerol (TAG) and diacylglycerol (DAG) (Snyder and Stephens, 1959) levels were estimated. The separated free fatty acids were eluted, methylated and separated (Cohen and Derksen, 1969) by gas chromatography (Pye Unicom 4550 Philips). The level of arachidonic acid in the sample was quantitated by comparison with a standard.

Phospholipids were separated on silica gel H plates using the solvent system chloroform:methanol:acetic acid:water (25:14:4:2 v/v). Individual phospholipids were identified by exposure to iodine vapour. Spots corresponding to standards were eluted and quantitated by phosphate estimation after acid hydrolysis (Bartlett, 1959). Phosphatidic acid (PA) was separated on oxalic acid – impregnated silica gel G plates and quantitated by phosphate estimation (Cohen and Derksen, 1969).

2.3.5. Detection of indicators of apoptosis

Studies were carried out to determine whether indomethacin-treated cells displayed features of apoptosis. The following markers of apoptosis were assessed.

2.3.5.1. Estimation of activities of caspases 3 and 9. Activities of caspase 3 and caspase 9 in the cells were estimated using N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) and N-acetyl-Leu-Glu-His-Asp-p-nitroanilide (Ac-LEHD-pNA), respectively, as substrates, using a microtitre plate (Gurtu et al., 1997). Cells (10^7 in number) were suspended in 1 ml of lysis buffer (50 mM HEPES, 5 mM CHAPS, 5 mM DTT, pH 7.4) and incubated on ice for 20 min. The lysed cells were centrifuged at 20,000*g* for 15 min at 4 °C. The supernatants obtained were used for assays for caspase 3 and caspase 9 as described below. Protein was estimated in the supernatant using bovine serum albumin as standard (Lowry et al., 1951). An aliquot of the supernatant, corresponding to a protein concentration of 30 μg, was made up to 90 μl using the assay buffer (20 mM HEPES, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 0.002% leupeptin, pH 7.4). To this, 10 μl of specific substrate (2 mM) was added for the enzyme concerned and the mixture was incubated at 37 °C for 2 h. The absorbance of the mixture was read at 405 nm (molar absorption co-efficient of p-nitroaniline (pNA) = 10.5). The activity of each enzyme was expressed as nmol of pNA liberated per minute per millilitre.

2.3.5.2. Detection of cytochrome-c release. After the respective treatments, cells were separated and homogenized using a mitochondrial isolation buffer (0.3 M sucrose, 10 mM MOPS, 1 mM EDTA and 4 mM K_2HPO_4, pH 7.4). The homogenate was centrifuged at 650*g* for 15 min at 4 °C. The supernatant was collected. The pellet was re-suspended in mitochondrial isolation buffer and homogenized. The homogenate was then centrifuged at 10,000*g* for 15 min. The resultant supernatant was collected and pooled with the previous supernatant and used as the cytosolic fraction, while the pellet was used as the mitochondrial fraction (Chakrabarti et al., 2003). Cytosolic and mitochondrial samples corresponding to 75 μg protein were denatured and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.2 μm nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin in PBS and 0.1% Tween-20 for 2 h. Thereafter, the membranes were incubated with monoclonal antibodies to cytochrome-c (Cell Signaling Technology, CA, USA). A chemiluminescence detection system (Thermo Fisher Scientific Inc., USA) was used to visualize the protein bands. The bands were documented using AlphaEase FC gel documentation system (Alpha Innotech Corporation, CA) and normalized to those for beta-actin, which was used as a loading control.

2.3.5.3. Detection of poly (ADP-ribose) polymerase (PARP) cleavage. The buffer used for this part of the study contained 25 mM HEPES, 5 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 2 mM DTT, 10 μg each of pepstatin and leupeptin and 1 mM PMSF, at pH 7.5. The cell suspension was sonicated on ice for 8–10 min (Vibronics ultrasonic processor p2 250 W at 200 V) and the cell lysate was centrifuged at 160,000*g* for 20 min. The pellet obtained was solubilized in 25 mM HEPES, pH 7.5, 5 mM EDTA, 2 mM DTT, 1% Triton-X-100, 10 μg each of pepstatin and leupeptin and 1 mM PMSF and sonicated once again (Wang et al., 2000). Samples corresponding to 50 μg protein were denatured and separated by 4–20% gradient SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk in PBS and 0.1% Tween-20 for 2 h. Thereafter, the membranes were incubated with polyclonal antibodies against PARP (Cell Signaling Technology, CA, USA). The secondary antibody used was goat anti-rabbit IgG conjugated with biotin. Incubation with these was followed by the use of streptavidin-ALP conjugate to visualize the bands using BCIP/NBT as substrate and bands were documented using AlphaEase FC gel documentation system (Alpha Innotech Corporation, CA) and normalized to those for beta-actin, which was used as a loading control.

2.3.5.4. Detection of DNA fragmentation. DNA from the treated cells was isolated using TRI reagent (Sigma, USA), according to manufacturer's instructions. Isolated DNA was separated in 1% agarose gel that contained 0.05% ethidium bromide, using tris-borate-EDTA (TBE) buffer. The separated bands were visualized and documented using AlphaEase FC gel documentation system (Alpha Innotech Corporation, CA).

2.3.6. Data analysis and statistical procedures

Data were analyzed using ANOVA, with Bonferroni correction for multiple *t*-test as a post-hoc test, to look for differences in the means of the various experimental groups. A p value of less than 0.05 was taken to indicate significance. Data analysis was carried out using Statistical Package for the Social Scientist (SPSS), version 11.

2.3.7. Drugs, chemicals, reagents and other materials

4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), aprotinin, arachidonic acid, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), Eagle's minimal essential medium (MEM), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), ethidium bromide, ethylene diamine tetra-acetic acid (EDTA), ethylene glycol tetra-acetic acid (EGTA), fetal bovine serum (FBS), leupeptin, methyl arachidonyl fluorophosphonate (MAFP), 3-(N-morpholinopropanesulfonic acid (MOPS), phenylmethanesulfonylfluoride (PMSF), sodium dodecyl sulfate (SDS), thiobarbituric acid (TBA), trypan blue and xanthine were obtained from Sigma Aldrich, India. Cell culture flasks (25 cm^2) were purchased from Corning Life Science, India. Penicillin was obtained from Alembic Chemicals, Baroda, India and streptomycin from Sarabhai Chemicals, Baroda, India. Borate, 4-(2-hydroxyethyl) piperazine (2-ethanesulfonic acid) (HEPES), silica gel G, silica gel H, Tris and Triton X-100, were from Sisco Research Laboratories, Mumbai, India. Agarose and anti-rabbit and anti-mouse IgG antibodies were purchased from Thermo Fisher Scientific Inc., USA and Genei, Bangalore, India. Polyvinylidenedifluoride (PVDF) membrane (pore size 0.45 μm) was obtained from Millipore, India. Antibodies against cytochrome-c and PARP were obtained from Cell Signaling Technology, CA, USA. All chemicals used were of analytical grade.

3. Results

3.1. Effect on cell viability

Caco-2 cells treated with indomethacin showed significantly lower number of viable cells when compared with cells that had been treated with the vehicle for the drug (Fig. 1A and B). This effect was found to be dose- and time-dependent (data not shown). Pretreatment of the cells with MAFP before the addition of indomethacin resulted in significant increase in the number of viable cells. Treatment with MAFP alone did not affect the viability of the cells.

The activity of LDH was significantly higher in the medium and lower in the cells after treatment with indomethacin, when compared with cells incubated with the vehicle for the drug. These effects were attenuated when the cells were pretreated with MAFP (Fig. 2A and B). Treatment with MAFP alone did not significantly affect the extent of LDH activity, either in the medium or in the cells.

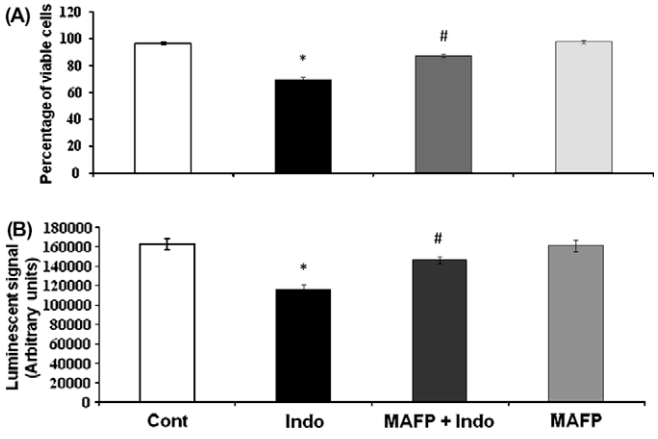

Fig. 1. Effect of MAFP on indomethacin-induced changes in cell viability. Cell viability was measured by trypan blue dye exclusion (A) and the CellTiter-Glo Luminescent cell viability assay (B), with and without pretreatment with MAFP (20 μM for 30 min), followed by incubation with indomethacin (500 μM for 1 h). Each value represents mean ± SD of 6 separate experiments. $^{*}p < 0.05$ when compared with control groups and $^{\#}p < 0.05$ when compared with indomethacin-treated group.

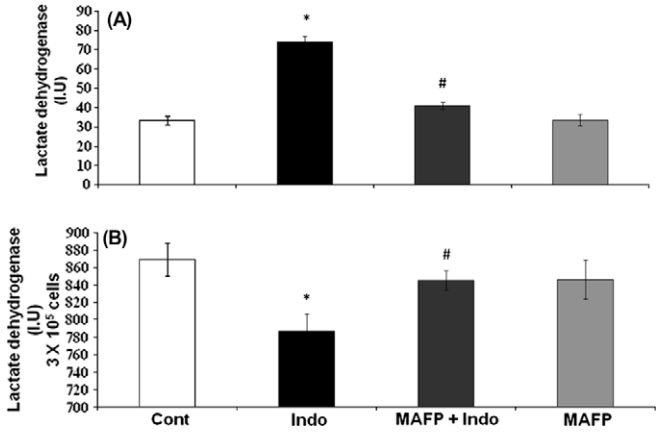

Fig. 2. Effect of MAFP on indomethacin-induced cytotoxicity in Caco-2 cells. Activity of lactate dehydrogenase was measured in the culture medium (A) and cell lysates (B) after cells were pretreated with MAFP (20 μM for 30 min), followed by incubation with indomethacin (500 μM for 1 h). Each value represents mean ± SD of 6 separate experiments. $^{*}p < 0.05$ when compared with control groups and $^{\#}p < 0.05$ when compared with indomethacin-treated group.

3.2. Effect on cell morphology

The cells that had been treated with indomethacin showed extensive detachment from most of the areas of the flask, with rounding of many of the remaining cells. Cells pretreated with MAFP showed less detachment and rounding (Fig. 3). One micron sections of the control cells showed confluent cells with vesicular nuclei and prominent nucleoli (Fig. 4A). Cells exposed to indomethacin showed cytoplasmic vacuolation and extensive detachment with rounding off of individual cells (Fig. 4B). Cells that had been pretreated with MAFP prior to indomethacin showed only partial detachment and rounding off that was less than that seen with indomethacin alone (Fig. 4C). Exposure of cells to MAFP alone induced mild cytoplasmic vacuolation but no other significant changes (Fig. 4D).

Ultrathin sections showed that the control cells and those treated with MAFP alone formed confluent sheets (Fig. 5A and F, respectively) with long microvilli and well-developed intercellular junctions (Fig. 5B). The latter were associated with dense plaques of cytoplasmic filaments (Fig. 5B). The cells treated with indomethacin showed extensive rounding with decreased intercellular junc-

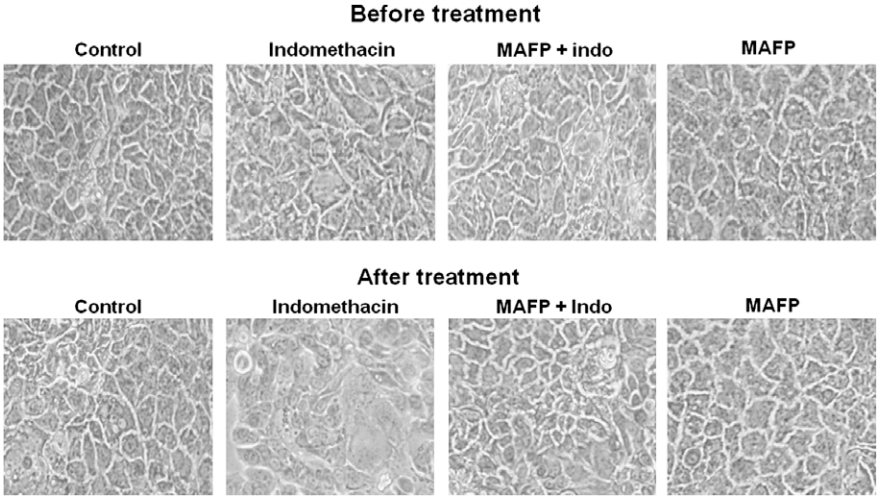

Fig. 3. Effect of MAFP on indomethacin-induced changes in morphology of cells. Cells were pretreated with MAFP (20 μM for 30 min), followed by incubation with indomethacin (500 μM for 1 h). Figures shown are at 40× magnification and are representative pictures from 3 independent sets of experiments.

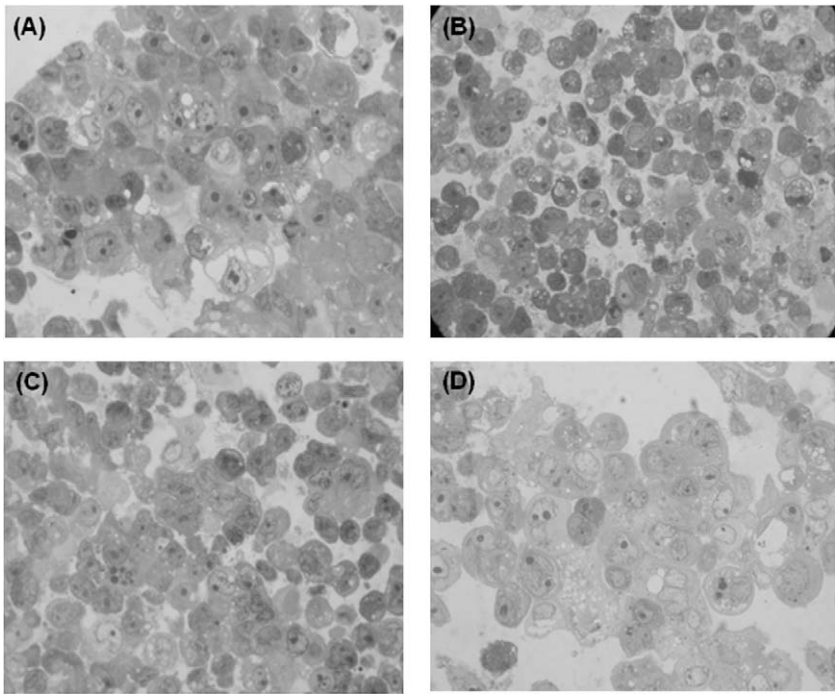

Fig. 4. One micron sections of control (A), indomethacin-treated (B), MAFP and indomethacin-treated (C) and MAFP-treated cells (D). Cells were pretreated with MAFP (20 μM for 30 min), followed by incubation with indomethacin (500 μM for 1 h). Figures shown are representative pictures from 3 independent sets of experiments.

tions, blunting of microvilli and the formation of small cytoplasmic blebs (Fig. 5D). Cytoplasmic filaments associated with the intercellular junctions were inconspicuous (Fig. 5C). The cells also showed prominent cytoplasmic vacuoles, increased phagolysosomes and dilated mitochondria and endoplasmic reticulum. Condensation of nuclear chromatin or cytoplasmic organelles suggestive of apoptosis was not seen. Cells treated with MAFP and indomethacin showed better preservation of intercellular junctions and microvilli, with less prominent phagolysosomes and dilatation of cellular organelles (Fig. 5E).

3.3. Effect on parameters of oxidative stress

Indomethacin-treated cells showed evidence of oxidative stress. This was evidenced by significant increases in the activity of the pro-oxidant enzyme, xanthine oxidase, and in levels of malondialdehyde and protein carbonyl in the drug-treated cells. All these effects were ameliorated on pretreatment of the cells with MAFP. Treatment of the cells with MAFP alone did not affect these parameters (Fig. 6A–C).

3.4. Effect on lipids

Significant changes were seen in the content of neutral lipids in cells treated with indomethacin. The levels of cholesterol esters and triacylglycerol showed significant decreases while those of cholesterol and diacylglycerol were significantly increased in response to the drug, when compared to control cells. Pretreatment with MAFP did not affect these indomethacin-induced changes. Treatment of the cells with MAFP alone also did not affect these parameters (Fig. 7A and B).

Significant changes were seen in the content of phospholipids in response to treatment with indomethacin. The levels of phosphatidylcholine and phosphatidylethanolamine were found to be significantly decreased on treatment with the drug, with significant increases in the content of the corresponding lysophospholipids (Fig. 8A and B). The levels of phosphatidylinositol, phosphatidylserine and sphingomyelin were significantly decreased and that of phosphatidic acid was significantly increased in the drug-treated cells (Fig. 8C). All these changes were abolished when the cells were pretreated with MAFP (Fig. 8A–C).

The total cholesterol–phospholipid (C/P) and sphingomyelin–phosphatidylcholine (SP/PC) ratios were calculated in each experimental group and found to be significantly higher in the indomethacin-treated group when compared with the control data (Fig. 9A and B). Pretreatment with MAFP prior to addition of indomethacin restored the ratios to control values. Pretreatment with MAFP alone did not significantly affect these ratios.

A significant increase was seen in the levels of arachidonic acid in cells treated with indomethacin. Pretreatment with MAFP attenuated this effect (Fig. 10). Treatment of the cells with MAFP alone has no effect on the level of this fatty acid.

3.5. Effect on indicators of apoptosis

Baseline activities of caspases 3 and 9 in the control cells were very low. There was no significant increase in the activities of these enzymes in the indomethacin-treated cells (data not shown). Similarly, there was no increased release of cytochrome-c into the cytosol or cleavage of PARP, as detected by western blots, in the drug- treated cells (Fig. 11A and B), when compared with control cells. No increase in DNA fragmentation was seen in the treated cells either (Fig. 11C). Pretreatment with MAFP, either alone or in addition to indomethacin, did not affect these parameters.

4. Discussion

NSAIDs, commonly used for treatment of arthritis and other chronic inflammatory conditions, produce adverse effects in the gastrointestinal tract. Several factors, such as drug-induced inhibition of cyclooxygenase, oxidative stress and mitochondrial dysfunction, have been shown to contribute to the pathogenesis of

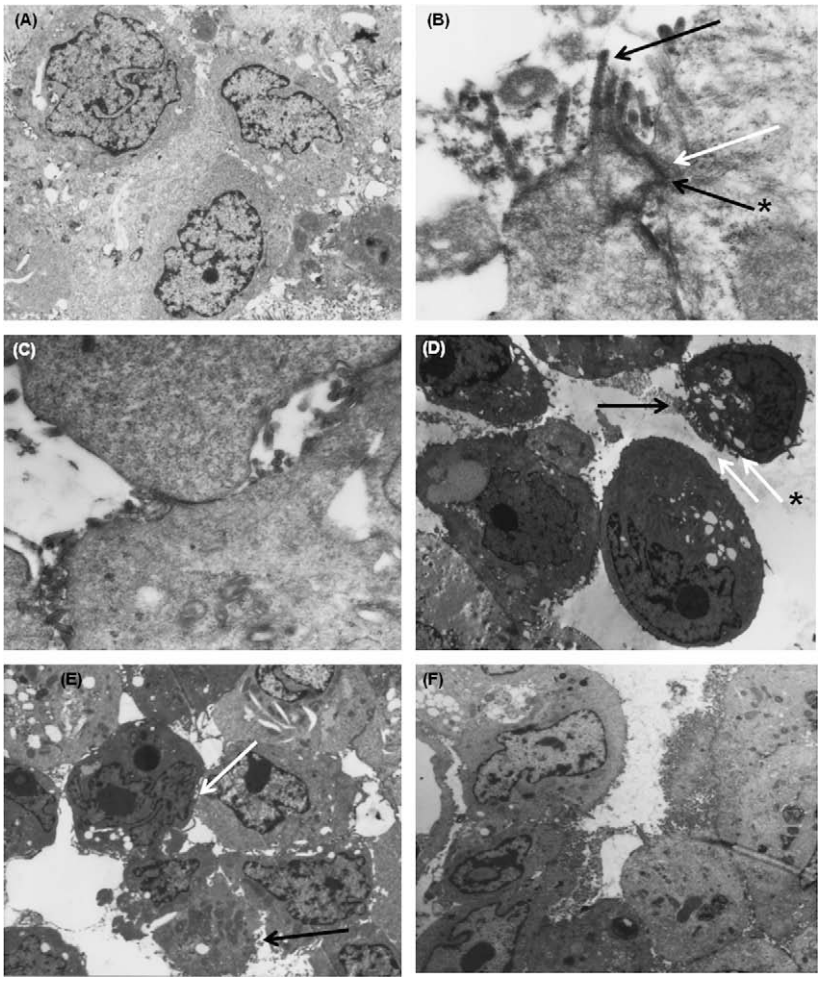

Fig. 5. Electron microscopic images of sections of control, indomethacin-treated, MAFP plus indomethacin-treated and only MAFP-treated cells. Cells were pretreated with MAFP (20 μM for 30 min), followed by incubation with indomethacin (500 μM for 1 h). Figures shown are representative pictures from 3 independent sets of experiments. Control cells (A and B) show long microvilli (black arrow), well-developed intercellular junctions (white arrow) and dense plaques of cytoplasmic filaments (black arrow with ∗). Cells treated with indomethacin (C and D) showed extensive rounding of cells (black arrow), decreased intercellular junctions (white arrow) and blunted microvilli (white arrow with ∗). Cells pretreated with MAFP (E) shows preservation of intercellular junctions (white arrow), and microvilli (black arrow). Cells treated with MAFP alone (F) shows confluent sheets and intercellular junctions.

such effects (Basivireddy et al., 2002; Bjarnason et al., 1993; Vaananen et al., 1991). Oxidative stress, induced by indomethacin, has been shown to activate $cPLA_2$, causing it to translocate to the cell membrane, resulting in degradation of phospholipids in the membranes (Basivireddy et al., 2003). We, therefore, hypothesized that drug-induced activation of the enzyme is important in the pathogenesis of indomethacin-induced enteropathy. In the current study, we have shown evidence of oxidative stress in indomethacin-treated Caco-2 cells, in keeping with findings in our earlier studies that have shown such an effect of indomethacin (Basivireddy et al., 2002). We found that pretreatment of cells with MAFP, a $cPLA_2$ inhibitor (Balsinde and Dennis, 1996), at 20 μM final concentration significantly decreased indomethacin-induced damage in Caco-2 cells. This was evidenced by improved viability of the cells and a marked reduction in the morphological damage produced by indomethacin. These results correlated well with the reduced cytotoxicity seen in indomethacin-treated cells in the presence of MAFP, as evidenced by decreased release of LDH from the cells. Similar but less marked effects were seen at MAFP concentrations of 10 μM as well (data not shown). These effects were also associated with decreased degradation of phospholipids in the cells. Phospholipids form important constituents of cellular membranes. The composition of these lipids influence membrane fluidity and permeability and are prone to changes with aging and onset of disease (Bengmark, 1998). Alterations in the cholesterol/phospholipid ratio, sphingomyelin content and sphingomyelin/phosphatidylcholine molar ratio in membranes have been shown to result in changes in membrane fluidity (Dudeja et al., 1986, 1991; Ibrahim and Balasubramanian, 1995). Several studies have reported that such changes are often associated with disruption of function (Bjorkman and Jessop, 1994; Dudeja et al., 1991; Gupta et al., 1994). Similar changes have been observed in the lipids in the current study in response to indomethacin. These changes are, thus, likely to have contributed to loss of integrity of the cells. Hence, our results suggest that the activation of $cPLA_2$ appears to be an important event in the pathogenesis of NSAID-induced damage in Caco-2 cells. To our knowledge, this is the first report implicating the activity of this enzyme in NSAID-induced enteropathy and contributes significantly to a better understanding of the pathogenesis of such enteropathy (Somasundaram et al., 1997), which is commonly seen in patients who take these drugs.

We also attempted to elucidate the mechanism(s) by which indomethacin-induced cell damage occurred in this study. Indomethacin treatment was found to lead to accumulation of arachidonic acid in the cells, an effect abolished on pretreatment with MAFP. This effect of MAFP would be accounted for by its inhibition

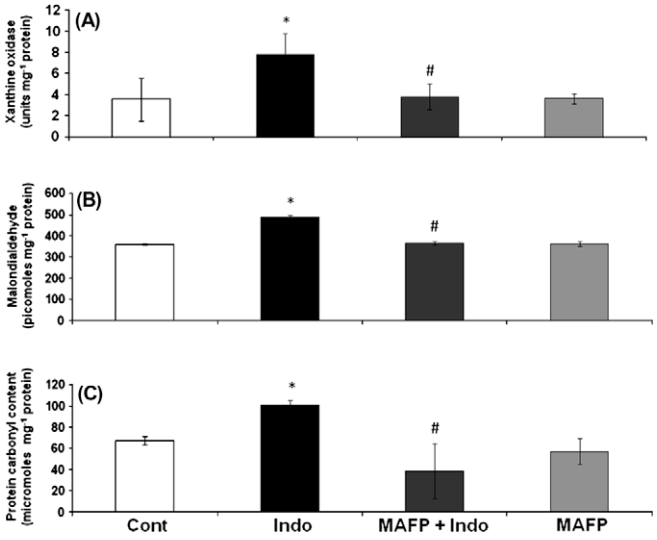

Fig. 6. Effect of MAFP on indomethacin-induced changes on parameters of oxidative stress. Xanthine oxidase (A), malondialdehyde (B) and protein carbonyl content (C) were measured after cells were pretreated with MAFP (20 μM for 30 min), followed by incubation with indomethacin (500 μM for 1 h). Each value represents mean ± SD of 6 separate experiments. $^{*}p < 0.05$ when compared with control groups and $^{\#}p < 0.05$ when compared with indomethacin-treated group.

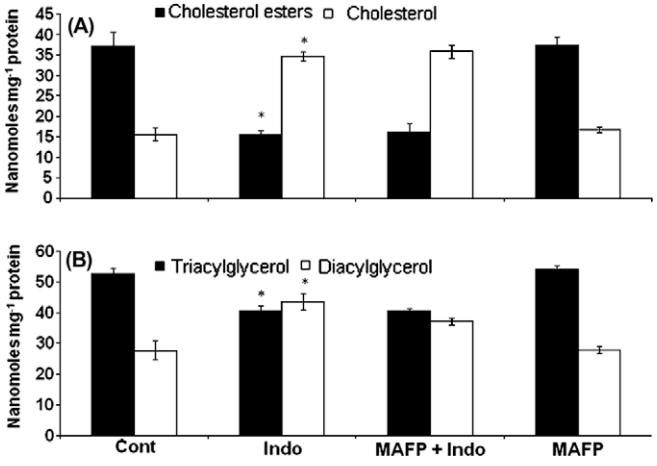

Fig. 7. Effect of MAFP on indomethacin-induced changes in neutral lipid composition. Cholesteryl esters and cholesterol (A) and triacylglycerol and diacylglycerol content (B) were measured after cells were pretreated with MAFP (20 μM for 30 min), followed by incubation with indomethacin (500 μM for 1 h). Each value represents mean ± SD of 6 separate experiments. $^{*}p < 0.05$ when compared with control groups and $^{\#}p < 0.05$ when compared with indomethacin-treated group.

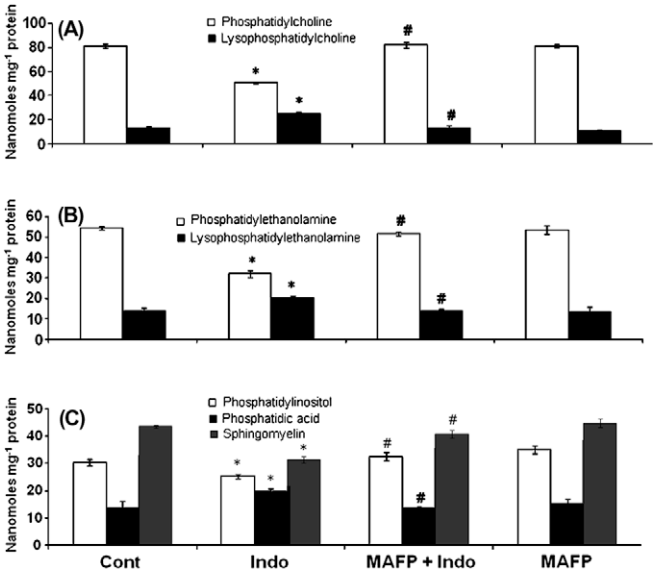

Fig. 8. Effect of MAFP on indomethacin-induced changes in phospholipid composition. Phosphatidylcholine and lysophosphatidylcholine (A), phosphatidylethanolamine and lysophosphatidylethanolamine (B), phosphatidylinositol, phosphatidic acid and sphingomyelin (C) were measured after cells were pretreated with MAFP (20 μM for 30 min), followed by incubation with indomethacin (500 μM for 1 h). Each value represents mean ± SD of 6 separate experiments. $^{*}p < 0.05$ when compared with control groups and $^{\#}p < 0.05$ when compared with indomethacin-treated group.

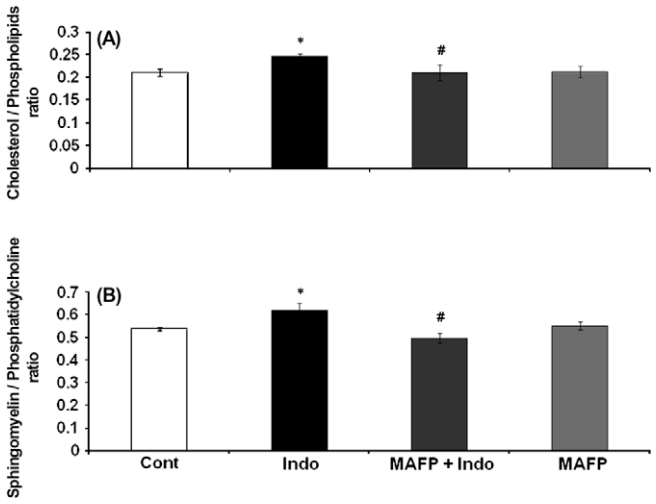

Fig. 9. Effect of MAFP on indomethacin-induced changes in total cholesterol-phospholipid (C/P) (A) and sphingomyelin-phosphatidylcholine (SP/PC) (B) ratios. Each value represents mean ± SD of 6 separate experiments. $^{*}p < 0.05$ when compared with control groups and $^{\#}p < 0.05$ when compared with indomethacin-treated group.

of PLA_2, thereby decreasing the release of arachidonic acid from membrane phospholipids. Arachidonic acid has been reported to be cytotoxic at physiological and supraphysiological concentrations, causing apoptosis and necrosis, respectively (Pompeia et al., 2002). During arachidonic acid metabolism, reactive oxygen species are formed. These generate lipid peroxides and cytotoxic products such as 4-hydroxynonenal and acrolein, which induce cellular damage (Nanda et al., 2007). Accumulation of arachidonic acid has been shown to be accompanied by an increase in the glutathione disulfide/reduced glutathione ratio, indicating oxidative stress (Higuchi et al., 2007). Arachidonic acid has also been shown to reduce levels of intracellular nicotinamide adenine dinucleotide (NAD), ATP and membrane potential in mitochondria, all indicators of mitochondrial dysfunction (Higuchi et al., 2007). In addition, an increase in levels of lysophosphatidylcholine has been documented to disrupt RBC membranes, causing cell lysis (Bierbaum et al., 1979; Weltzien, 1979). It is likely that such effects, produced by increased levels of arachidonic acid and lysophospholipids in the cells, may have been operational in the experimental model we have studied, leading to the cell damage seen. Further studies are warranted to determine the definitive biochemical mechanisms that are involved in producing indomethacin-induced damage in these cells.

Cell death, mediated via stress, can be either due to apoptosis or necrosis. Studies have shown that NSAIDs induce apoptosis in vivo

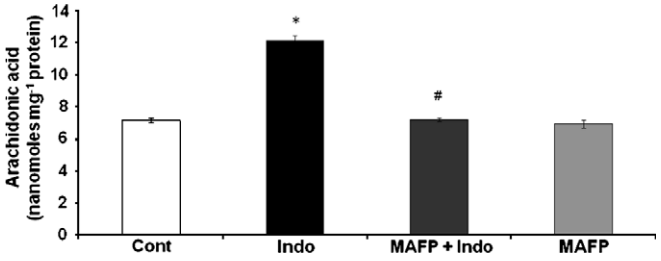

Fig. 10. Effect of MAFP on indomethacin-induced changes in arachidonic acid levels. Levels of the fatty acid were measured after cells were pretreated with MAFP (20 μM for 30 min), followed by incubation with indomethacin (500 μM for 1 h). Each value represents mean ± SD of 6 separate experiments. $^{*}p < 0.05$ when compared with control groups and $^{\#}p < 0.05$ when compared with indomethacin-treated group.

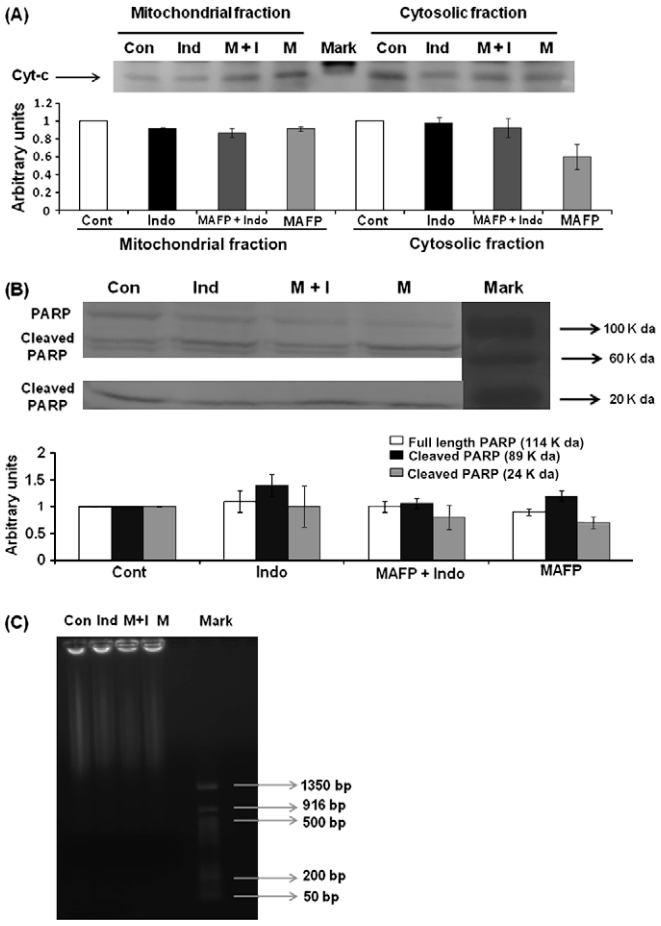

Fig. 11. Effect of indomethacin and MAFP treatments on cytochrome-c levels in mitochondria and cytosol (western blots) (A), on PARP cleavage (western blots) (B) and DNA fragmentation (agarose gel electrophoresis) (C), were determined after cells were pretreated with MAFP (20 μM for 30 minutes), followed by incubation with indomethacin (500 μM for 1 hour). The intensities of the bands obtained were normalized to those for beta-actin, which was used as a loading control. These are representative images obtained from 3 independent experiments.

(Alderman et al., 2000). Apoptosis is a mode of cell death that occurs under normal physiological conditions, where the cell is an active participant in its own death. Cells undergoing apoptosis show characteristic morphological and biochemical features (Cohen, 1993). Our studies on the morphology of the cells did not show changes characteristic of apoptosis. The light microscopy studies on cell morphology showed that the cells treated with indomethacin were damaged, with resultant detachment from the flask, when compared with control cells. This was confirmed by the ultrastructural studies done, where the changes of cellular rounding were associated with dilatation of cellular organelles and prominent lysosomes. Significant nuclear or cytological changes suggestive of apoptosis, such as margination of chromatin or segmentation of nuclei or cytoplasm, were not seen. These features, associated with reduction in the number of viable cells, indicate that the cells were undergoing some kind of cell death, which appeared to be non-apoptotic in nature. We confirmed this by looking for biochemical indicators of apoptosis in the model used. We found no evidence of apoptosis occurring in the cells as indicated by no increased release of cytochrome-c, no increase in the activities of caspases, no PARP cleavage and no DNA fragmentation in the indomethacin-treated cells. Tomisato et al. (2001) have shown that treatment of primary gastric cells with indomethacin results in necrosis or apoptosis, depending on the concentration of drug used and the period of treatment. Their results have shown that 0.5 mM indomethacin (the concentration of indomethacin that we have used in our study) did not produce apoptosis after 1 h of incubation (the time period we have used) but produced necrosis under these conditions. Our results showed the occurrence of non-apoptotic cell death and are, thus, in keeping with this report. Our study also showed that Caco-2 cells seem to be more sensitive to the effects of indomethacin than the primary gastric cells used by Tomisato et al. (2001), as we observed a greater degree of damage in our model when using similar concentrations of the drug. On the other hand, Huang et al. (2006) have reported that treatment with indomethacin at 0.5 mM for 1 h resulted in apoptosis in colon cancer cells. Their studies were done after serum starvation for 24 h before the indomethacin treatment, unlike in our study where the cells were not serum starved. Serum depletion of cells has been shown to activate p38 mitogen-activated protein kinase pathways and predispose them towards apoptosis (Kummer et al., 1997). It is likely that this could account for the difference between the findings of Huang et al. (2006) and our study.

Cell and organelle swelling, ATP depletion, increased plasma membrane permeability and macromolecule release result in cell necrosis. It has been proposed that PLA_2 activity is enhanced during necrosis and leads to accelerated hydrolysis of membrane phospholipids. This, in turn, increases plasma membrane permeability and results in cell lysis (Cummings et al., 2000). In keeping with this, Sapirstein et al. (1996) have reported that LDH release induced by hydrogen peroxide or menadione was significantly greater in kidney epithelial cells that over-express PLA_2 as compared with control cells. The findings in our study, of drug-induced cytotoxicity and loss of viability, are in keeping with these reports.

One of the limitations of our study is that MAFP is an inhibitor of both $cPLA_2$ (Ca^{++} dependent) and $iPLA_2$ (Ca^{++} independent). We have not investigated the latter. It would be necessary to assess the role of iPLA2 also in indomethacin-induced damage in our model. We have also used a high concentration of the drug in this study, at a level that is higher than is likely to be encountered in clinical practice. However, such concentrations and high doses have been used in previous studies on the pathogenesis of NSAID-induced gastrointestinal toxicity (Ettarh and Carr, 1993; Jacob et al., 2001; Somasundaram et al., 1997). The use of supratherapeutic doses in such studies is a compromise to attempt to study the cumulative effect of therapeutic doses over prolonged periods (given that NSAIDs are drugs used over years and their effects are cumulative). Such prolonged exposure is difficult to reproduce in experimental settings and hence supratherapeutic doses are often used over short periods in studies on the pathogenesis of adverse drug effects. It would, nevertheless, be necessary to repeat these studies using lower concentrations of the drug over longer periods of time and to document the effect of the inhibitor under these

conditions as well. It would also be useful to study the effect of other inhibitors of $cPLA_2$ in this context.

In conclusion, we present preliminary evidence that inhibition of $cPLA_2$ protects Caco-2 cells from indomethacin-induced cell damage. The cell damage that occurs in response to treatment with the drug appears to be mediated by non-apoptotic mechanisms. We postulate, therefore, that activation of $cPLA_2$ may be an important event in the pathogenesis of enteropathy caused by this drug and possibly other NSAIDs. To our knowledge, this is the first report that implicates the involvement of this enzyme in NSAID-induced enteropathy. Further work in this area is warranted to further elucidate the nature of this relationship.

Acknowledgements

The authors would like to acknowledge the Council for Scientific and Industrial Research (CSIR), New Delhi, India [Grant No. 37/1182/04 EMRII] for financial assistance for the study. Both Nageswaran Sivalingam and Jayasree Basivireddy were Senior Research Fellows funded by the CSIR.

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