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Zinc prevents indomethacin-induced renal damage in rats by ameliorating oxidative stress and mitochondrial dysfunction

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ABSTRACT

The clinical utility of non-steroidal anti-inflammatory drugs (NSAIDs) is limited by their gastrointestinal and renal toxicities. Indomethacin (an NSAID commonly used in toxicity studies) has been shown to induce significant oxidative stress and mitochondrial dysfunction in the rat kidney. The current study was designed to assess the potential of zinc, a known antioxidant, to protect the kidney against these drug-induced effects. Male Wistar rats were pre-treated with zinc sulphate (50 mg/kg) and dosed with indomethacin (20 mg/kg) by oral gavage. Rats were sacrificed 24 h after the dose of indomethacin. Parameters of oxidative stress, mitochondrial function and lipid content of the mitochondrial membranes were measured in the kidneys of these animals. It was found that zinc significantly attenuated indomethacin-induced oxidative stress, mitochondrial dysfunction and changes in the lipids in mitochondrial membranes in the kidney. The content of metallothionein, a cysteine-rich zinc-binding protein, was also determined in the tissue. There was no significant induction of metallothionein in the kidney in zinc-treated animals. Estimation of serum creatinine showed that zinc seemed to hasten functional recovery of the kidney following indomethacin administration. We conclude that pretreatment with zinc is effective in protecting against indomethacin-induced changes in the rat kidney. This protective effect does not appear to be mediated by metallothionein.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are used extensively in clinical practice for their ability to alleviate the swelling, redness and pain associated with inflammation, and to reduce fever. The principal pharmacological effects of NSAIDs as analgesics, antipyretics, anti-inflammatory and anti-thrombogenic agents are due to their ability to inhibit prostaglandin synthesis by inhibiting the enzyme cyclooxygenase (Vane, 1971). Their utility is, however, limited by the adverse effects that they produce in the gastrointestinal tract and the kidney.

While the gastrointestinal toxicity of NSAIDs is well-known and has been extensively investigated, it is becoming increasingly clear that the kidney is also an important target for untoward clinical events (Gambaro and Perazella, 2003). NSAIDs have little effect on renal function in normal human subjects, but are capable of inducing abnormalities in high-risk patients with decreased renal blood perfusion, such as in congestive heart failure, hepatic cirrhosis, chronic kidney disease, hypovolemia and other states of activation of the renin–angiotensin system (Patrono and Dunn, 1987).

Many of the adverse effects produced by NSAIDs have often been attributed to their inhibition of the eicosanoid-synthesizing enzyme, cyclooxygenase (COX). However, it is now known that other mechanisms also contribute to the toxicity produced by these drugs, both in the gastrointestinal tract and the kidney. Most important among these is drug-induced generation of oxygen free radicals and consequent oxidative tissue damage (Basivireddy et al., 2004; Vaananen et al., 1991) A large body of evidence has also clearly shown mitochondrial damage in the small intestine in response to indomethacin, a commonly used NSAID (Jacob et al., 2001; Somasundaram et al., 2000). Similarly, Basivireddy et al. (2004) have shown evidence of mitochondrial dysfunction and alterations in lipid composition of the mitochondrial membranes in the kidney following administration of indomethacin. It has thus been postulated that the mitochondrial effects produced by indomethacin contributes to the pathogenesis of damage caused by the drug.

Zinc is a transition metal with well-documented anti-oxidant properties. It has not been shown to interact directly with an oxidant species but exerts its effects in an indirect manner. The acute anti-oxidant properties of zinc are due to its ability to stabilize sulfhydryl groups and to antagonize redox active transition metals like iron and copper (Powell, 2000). The long term anti-oxidant effects are postulated to be mediated by metallothionein, a small molecular weight metal-binding protein (Maret and Vallee, 1998; Maret, 2000). Since oxidative stress has been implicated in indomethacin-induced renal effects, this study was designed to assess the ability of zinc to protect against such changes.

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2. Materials and methods

2.1. Materials

Ethylene glycol tetraacetic acid (EGTA), ferric chloride, glutathione reductase, indomethacin, o-dianisidine, reduced glutathione (GSH), sodium azide and thiobarbituric acid (TBA) were obtained from Sigma-Aldrich Chemicals, St. Louis, U.S.A. Adenosine diphosphate (ADP), arsenazo III, calcium chloride, disodium hydrogen phosphate, ethyl acetate, glacial acetic acid, guanidine hydrochloride, picric acid, hydroxylamine hydrochloride, iodonitrotetrazolium chloride (INT), mannitol, magnesium chloride, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nicotinamide adenine dinucleotide phosphate reduced (NADPH), glutathione disulfide (GSSG), ammonium molybdate, sucrose, sodium hydroxide, sodium dodecyl sulphate (SDS), sodium dihydrogen phosphate, sodium succinate, silica gel G, silica gel H, Tris HCl, trichloroacetic acid, xanthine and zinc sulphate were obtained from Sisco Research Laboratories (SRL) Pvt. Ltd., Mumbai. All other chemicals used were of analytical grade. ^{109}Cd was obtained from Board of Radiation and Isotope Technology, Department of Atomic Energy, Government of India. Mouse anti-metallothionein monoclonal antibody which detects rat MT -1 and MT -2 was obtained from Stressgen Bioreagents (cat no. #SPA-550). Anti-mouse IgG antibodies were obtained from Genei, Bangalore, India. Polyvinylidenefluoride (PVDF) membrane (pore size 0.45 μm) was obtained from Millipore, India.

2.2. Animals

Male Wistar rats (200–250 g), exposed to 12 h light–dark cycle and fed with commercially available rat chow, were used for all experiments. All procedures performed on the animals were approved by the Committee for the Purpose of Control & Supervision of Experiments on Animals (CPCSEA), Government of India.

2.3. Protocol for administration of drugs

Rats were fasted overnight. They were allowed access to water *ad libitum*. They then were dosed with indomethacin (20 mg per kg body weight) or an equal volume of its vehicle (5% sodium bicarbonate) by oral gavage (Somasundaram et al., 1997). Studies on the adverse effects of indomethacin have used the drug at doses that vary from 20 mg/kg up to 85 mg /kg (Somasundaram et al., 1997; Basivireddy et al., 2002; Ettarh and Carr, 1993). We chose a dose of 20 mg per kg as earlier studies have shown that this dose produced significant oxidative stress in the kidney after 24 h of administration (Basivireddy et al., 2004). For zinc pre-treatment, we used zinc sulphate (50 mg per kg body weight) or an equal volume of its vehicle (water) given by oral gavage two hours prior to indomethacin treatment. Our earlier studies have shown that zinc at this dose and at this time period protected against indomethacin-induced intestinal damage (Basivireddy et al., 2002). Animals were sacrificed by cervical dislocation under halothane anesthesia 24 h after the dose of indomethacin. Their abdomens were opened, the kidneys removed and processed as described below. For estimation of serum creatinine, blood was obtained from the tail vein of animals every day for a period of five days following indomethacin administration.

2.4. Preparation of homogenates

Renal tissue was homogenized (5% w/v) in homogenization buffer consisting of 220 mM mannitol, 70 mM sucrose, 5 mM Tris and 1 mM EGTA, pH 7.4.

2.5. Assessment of parameters of oxidative stress

The homogenate obtained was used for the measurement of thiobarbituric acid reactive substances (TBARS) (Ohkawa et al., 1979) and protein carbonyl (Levine et al., 1990). These parameters were also measured in isolated mitochondrial preparations. Protein was estimated by Lowry's method using bovine serum albumin as standard (Lowry et al., 1951).

2.6. Assay of enzymes

The activities of myeloperoxidase (EC 1.11.1.7), catalase (EC 1.11.1.6), glutathione peroxidase (EC 1.11.1.9) and glutathione reductase (EC 1.8.1.7) were measured in the renal homogenates. Activity of myeloperoxidase (MPO) was estimated by a spectrophotometric assay that measures the rate of oxidation of o-dianisidine by MPO. (Krawisz et al., 1984). Catalase activity was estimated by following the rate of dismutation of hydrogen peroxide at 240 nm (Aebi, 1984). Glutathione peroxidase activity was estimated by a coupled enzymatic reaction that led to oxidation of NADPH. The rate of fall of absorbance at 340 nm was measured (Flohe and Gunzler, 1984). Glutathione reductase activity was estimated by measuring the rate of oxidation of NADPH at 340 nm (Racker, 1955).

2.7. Isolation of mitochondria

The renal homogenate was used for isolation of mitochondria (Smith, 1967). Purity of the mitochondrial preparation was checked by enrichment of the marker enzyme, succinate dehydrogenase.

2.8. Assessment of mitochondrial function

Oxygen uptake was measured using a biological oxygen monitor (YSI model 5300, Ohio, USA) which has a Clark's oxygen electrode that measures oxygen content of fluids. Respiratory control ratio (RCR) was calculated by dividing the rate of oxygen uptake by mitochondria in state 3 respiration (after addition of ADP) by that of state 4 (before the addition of ADP) (Chance and Baltscheffsky, 1958). Mitochondrial swelling was determined by measuring the decrease in absorbance at 540 nm (Takeyama et al., 1993). Calcium uptake by the mitochondria was followed by measuring the changes in absorption spectrum of arsenazo III (Scarpa, 1979). MTT formazan formation by the isolated mitochondria was also measured. The amount of MTT formazan formed was calculated using the extinction coefficient, E^{570} of 17,000 M^{-1} at pH 7.4–8 (Madesh et al., 1997a).

2.9. Analysis of lipids

Lipids were extracted from mitochondria isolated from renal tissue and separated by thin layer chromatography (Bligh and Dyer, 1959). Neutral lipids were separated on silica gel G plates using the solvent system consisting of hexane, diethyl ether and acetic acid in the ratio 80:20:1, v/v/v. The spots corresponding to the standards were identified by iodine exposure and eluted. Cholesterol and cholesteryl ester were quantified (Zlatkis et al., 1953). Individual phospholipids were separated on silica gel H plates using the solvent system consisting of choloroform, methanol, acetic acid and water in the ratio 25:14:4:2, v/v/v/v and quantified by phosphate estimation after acid hydrolysis (Barlett, 1959).

2.10. Estimation of metallothionein by cadmium–hemoglobin assay

The metallothionein content of renal tissue was estimated by the cadmium–hemoglobin affinity (Cd–hem) assay (Eaton and Toal, 1982). Briefly, kidney tissue was homogenized (1:10 w/v) in homogenization buffer (10 mM Tris buffer, pH 8.2). The samples were

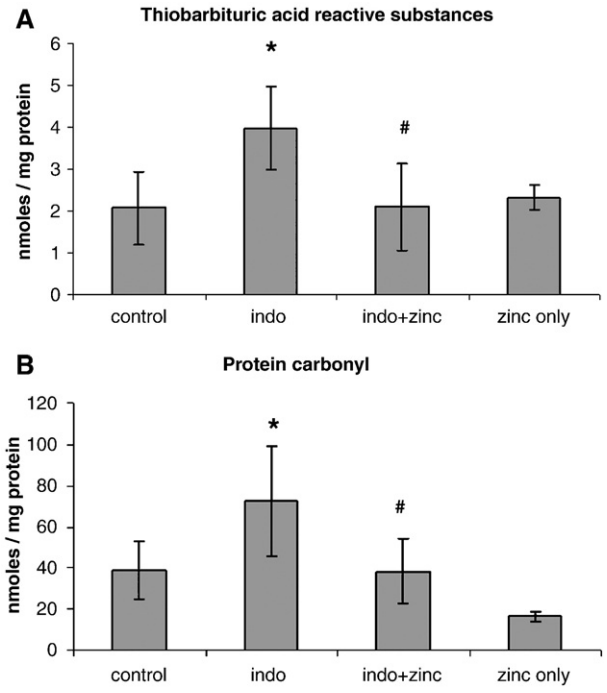

Fig. 1. Levels of peroxidation products of lipid and protein in renal homogenates from control and indomethacin treated animals with or without pretreatment with zinc. Thiobarbituric acid reactive substances (A) and protein carbonyl levels (B). Each value represents mean ± S.D. ($n = 6$). * Indicates $p < 0.05$ as compared with control group, # indicates $p < 0.05$ when compared with the indomethacin-treated group.

boiled for two minutes, cooled in ice-cold water and centrifuged at 10,000 g for 4 min. 50 µl of the supernatant was added to 200 µl of ^{109}Cd (19.6 µM) and made up to a final volume of 400 µl with homogenization buffer. The samples were incubated at room temperature for 15 min and excess ^{109}Cd was precipitated by adding 100 µl of 4% hemoglobin prepared from human blood. The samples were centrifuged at 10,000 g for 4 min. 450 µl of the supernatant was carefully removed and radioactivity of this sample was determined using a PerkinElmer 1470 gamma counter. Total metallothionein concentration was expressed as nmol of Cd bound per milligram protein.

2.11. Detection of metallothionein by western blot

Renal tissue was homogenized with homogenization buffer containing phosphate buffered saline (pH 7.4) with leupeptin, E64, aprotinin and 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) and centrifuged at 15,000 g at 4 °C for 10 min. The suspension was collected and protein concentration was determined. The sample (50 µg protein) was diluted in loading buffer (40 mmol/L Tris–HCl, pH 6.8, 1% sodium dodecyl sulfate, 50 mmol/L dithiothreitol, 7.5% glycerol, 0.003% bromophenol blue), heated at 95 °C for 5 min, and then subjected to gradient electrophoresis (4 to 20% sodium dodecyl sulfate-polyacrylamide gradient gel) at 80 V. Proteins were transferred from gels to polyvinylidene difluoride (PVDF) membrane. Membranes were rinsed briefly with Tris-buffered saline and incubated in blocking buffer (5% bovine serum albumin) for 1 h, followed by three washes with Tris-buffered saline containing 0.1% Tween 20. Monoclonal rabbit anti-metallothionein antibody (Stressgen Bioreagents) at a dilution of 1:300 was used as the primary antibody. This antibody detected metallothionein-1 as well as metallothionein-2. The secondary antibody used was conjugated with biotin. Incubation with the secondary antibody was followed by the use of streptavidin–alkaline phosphatase (ALP) conjugate to visualize the bands using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) as substrate.

2.12. Estimation of serum creatinine

Serum creatinine was estimated using the kinetic method of Jaffe (Lustgarten and Wenk, 1972). Blood was collected from the tail vein of rats every day for a period of 5 days following indomethacin/vehicle administration as a single dose on day 0. Zinc sulphate was also given as a single dose on day 0 as described above. The change in creatinine value on each day was calculated as the percentage difference between each day's value and the baseline level on day 0.

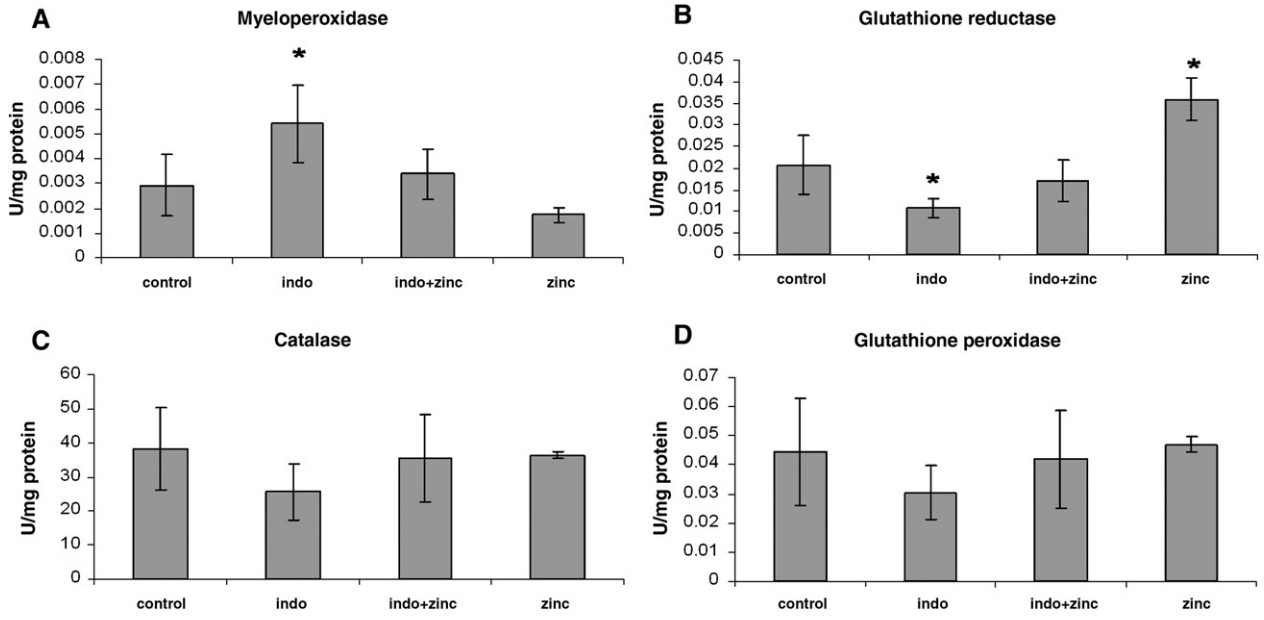

Fig. 2. Activities of myeloperoxidase and antioxidant enzymes in the kidney homogenates obtained from control and indomethacin treated animals with or without pretreatment with zinc. Myeloperoxidase (A), glutathione reductase (B), catalase (C) and glutathione peroxidase activities (D). Each value represents mean ± S.D. ($n = 6$). * Indicates $p < 0.05$ as compared with control group, # indicates $p < 0.05$ when compared with the indomethacin-treated group.

2.13. Statistical analysis

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

3. Results

3.1. Zinc prevented indomethacin-induced oxidative stress in the kidney

The results of the studies on parameters of oxidative stress in kidney homogenates 24 h following drug administration showed that indomethacin caused significant elevations of both thiobarbituric acid reactive substances (Fig. 1A) and protein carbonyl (Fig. 1B). Pre-treatment with zinc reversed these changes.

3.2. Zinc ameliorated pro- and anti-oxidant enzyme imbalance

Studies on estimation of various pro- and anti-oxidant enzymes showed significantly increased activity of myeloperoxidase (MPO) (Fig. 2A), a marker of neutrophil infiltration, and decreased activity of glutathione reductase (Fig. 2B), following indomethacin administration. The changes in the activities of other anti-oxidant enzymes, catalase (Fig. 2C) and glutathione peroxidase (Fig. 2D), were not statistically significant. Pre-treatment with zinc tended to counter these drug induced changes, but not to a statistically significant extent. We also found that zinc, when administered alone (zinc group), caused a significant increase in the activity of glutathione reductase when compared with controls (Fig. 2B).

3.3. Zinc prevented oxidative stress and mitochondrial dysfunction induced by indomethacin

The purity of the isolated mitochondrial preparation was established by showing at least a five-fold increase in the activity of the marker enzyme, succinate dehydrogenase (data not shown). Levels of thiobarbituric acid reactive substances (Fig. 3A) and protein carbonyl (Fig. 3B) in the mitochondrial preparation were elevated in response to indomethacin. These effects were reversed by zinc. Respiratory control ratio (RCR), calcium flux (Fig. 4A), mitochondrial swelling (Fig. 4B) and MTT reduction by the mitochondria (Fig. 4C) were measured as parameters of the functional integrity of the isolated organelles. The RCR was not significantly affected by the drug or zinc treatment (control -1.14 ± 0.1, indo -1.06 ± 0.05, zinc + indo -1.07 ± 0.07 and zinc -1.02 ± 0.02). Indomethacin treatment resulted in a significant decrease in the calcium flux across the mitochondrial membrane, while mitochondrial swelling and MTT reduction were both significantly increased. Administration of zinc effectively prevented these drug-induced changes and restored these parameters to control levels.

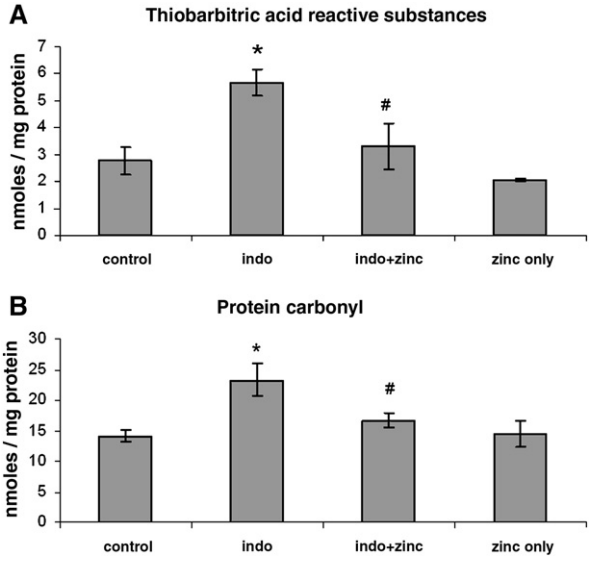

Fig. 3. Levels of peroxidation products of lipid and protein in mitochondria isolated from renal homogenates from control and indomethacin treated animals with or without pretreatment with zinc. Thiobarbituric acid reactive substances (A) and protein carbonyl levels (B). Each value represents mean ± S.D. ($n=6$). * Indicates $p<0.05$ as compared with control group, # indicates $p<0.05$ when compared with the indomethacin-treated group.

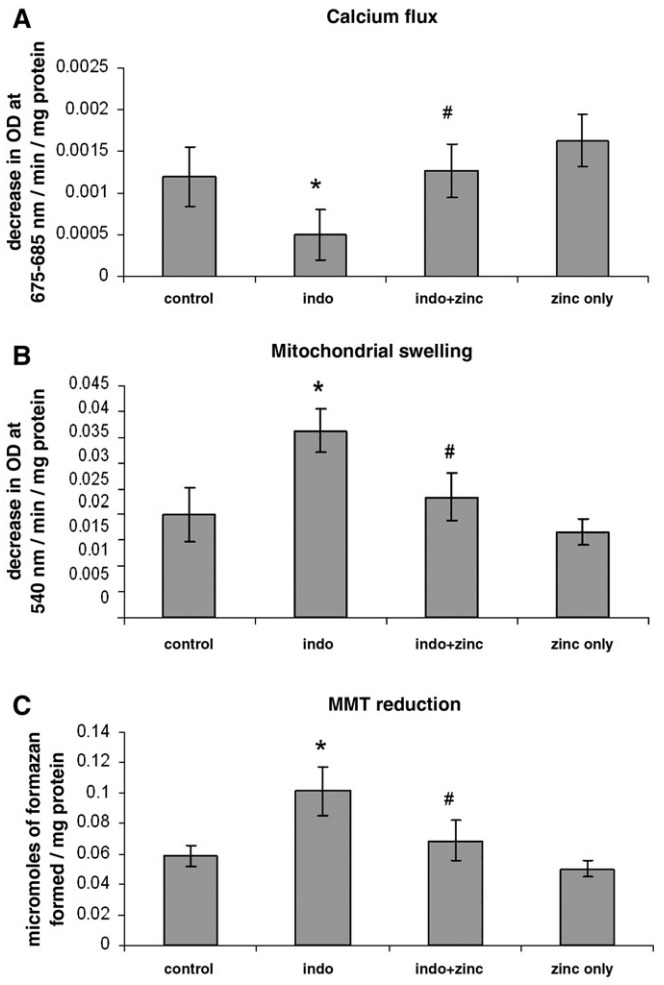

Fig. 4. Functional parameters of mitochondria isolated from renal homogenates from control and indomethacin treated animals with or without pretreatment with zinc. Changes in calcium flux across the inner mitochondrial membrane (A), mitochondrial swelling as measured by decreasing absorbance at 540 nm (B) and MTT reduction as measured by MTT formazan formation (C) in mitochondria. Each value represents mean ± S.D. ($n=6$). * Indicates $p<0.05$ as compared to control group, # indicates $P<0.05$ when compared with the indomethacin-treated group.

3.4. Zinc ameliorated indomethacin-induced changes in lipid content of mitochondrial membranes

Analysis of lipids extracted from the isolated mitochondria showed that indomethacin caused a significant decrease in the content of cholesteryl esters (Fig. 5A) and an increase in free

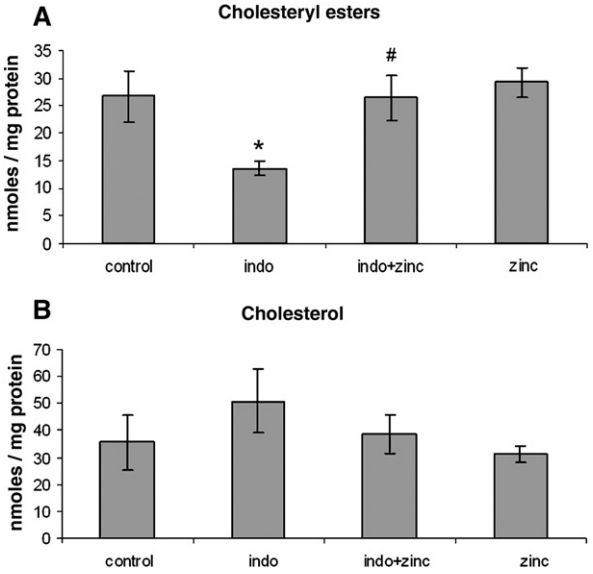

Fig. 5. Levels of cholesteryl esters (A) and free cholesterol (B) in mitochondria isolated from renal homogenates obtained from control and indomethacin treated animals with or without pretreatment with zinc. Each value represents mean ± S.D. ($n = 6$). * Indicates $p < 0.05$ as compared with control group, # indicates $p < 0.05$ when compared with the indomethacin-treated group.

cholesterol (Fig. 5B). Analysis of phospholipids revealed statistically significant decreases in phosphatidylcholine (PC) (Fig. 6A) and phosphatidylethanolamine (PE) (Fig. 6C) with corresponding increases in levels of lysophosphatidylcholine (LPC) (Fig. 6B) and lysophosphatidylethanolamine (LPE) (Fig. 6D). The content of phosphatidic acid (Fig. 6F) was also found to be elevated significantly in response to indomethacin. The drug did not have significant effects on the levels of sphingomyelin (Fig. 6E). Zinc reversed the drug-induced effect on the level of cholesteryl esters (Fig. 6A). Drug-induced changes in the content of phosphatidylcholine, lysophosphatidylcholine and lysophosphatidylethanolamine were significantly reversed by pretreatment with zinc (Fig. 6A, B and D). In the case of phosphatidylethanolamine and phosphatidic acid, the effects of zinc were not statistically significant (Fig. 6C and F). However, we found that giving multiple doses of zinc resulted in it reversing the effect of the drug on phosphatidylethanolamine and phosphatidic acid as well.

3.5. The protective effects of zinc were not mediated by metallothionein

Metallothionein levels were measured in the kidney by the Cadhem assay as well as by western blots. Both methods showed that there were no significant differences in the levels of metallothionein in the kidney among the various treatment groups (Fig. 7).

3.6. Zinc hastened functional recovery of the kidney following indomethacin-induced damage

Serum creatinine was estimated every day over a period of five days after drug administration in the various treatment groups. Following indomethacin administration, serum creatinine levels appeared to be elevated on day 1 and tended to return to baseline levels by day 4. In animals pre-treated with zinc, creatinine levels

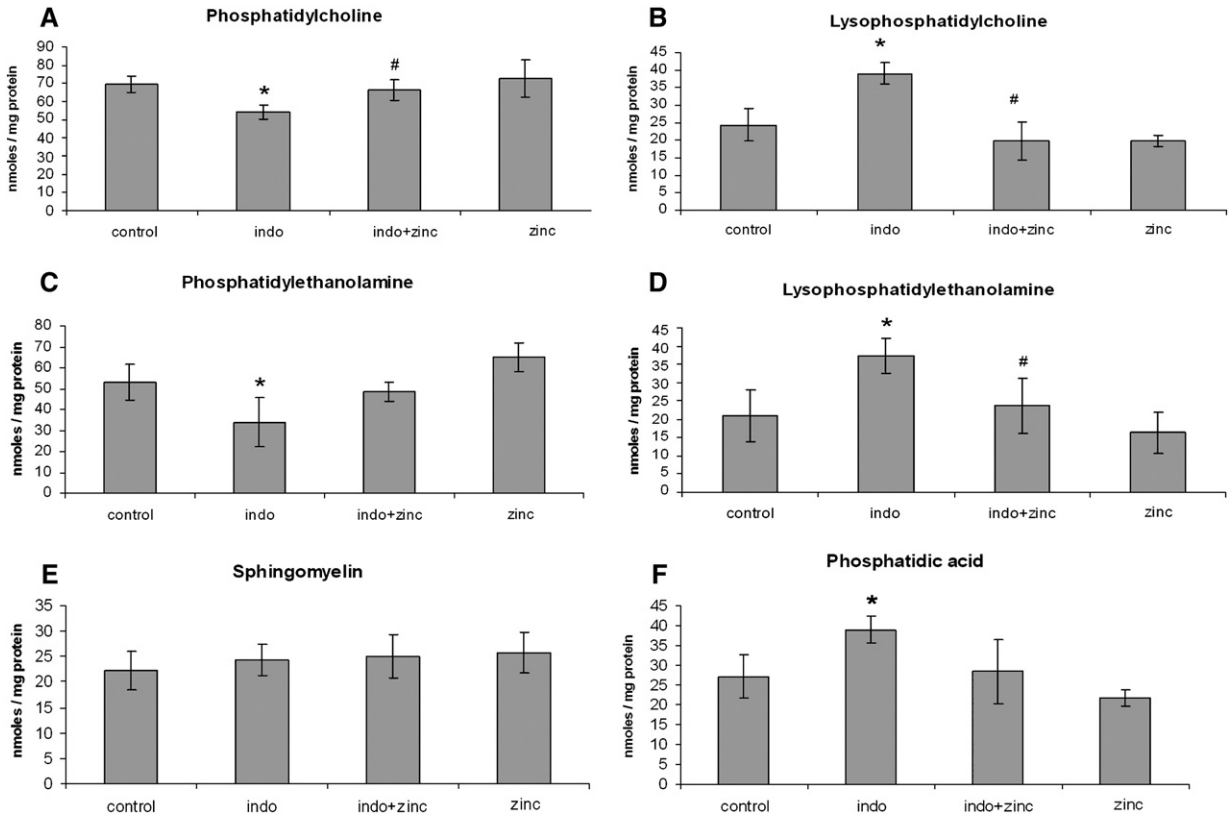

Fig. 6. Phospholipid content of mitochondria isolated from renal homogenates obtained from control and indomethacin treated animals with or without pretreatment with zinc. Phosphatidylcholine (A), lysophosphatidylcholine (B), phosphatidylethanolamine (C), lysophosphatidylethanolamine (D) sphingomyelin (E) and phosphatidic acid (F) content of renal mitochondria. Each value represents mean ± S.D. ($n = 6$). * Indicates $p < 0.05$ as compared with control group, # indicates $p < 0.05$ when compared with indomethacin-treated group.

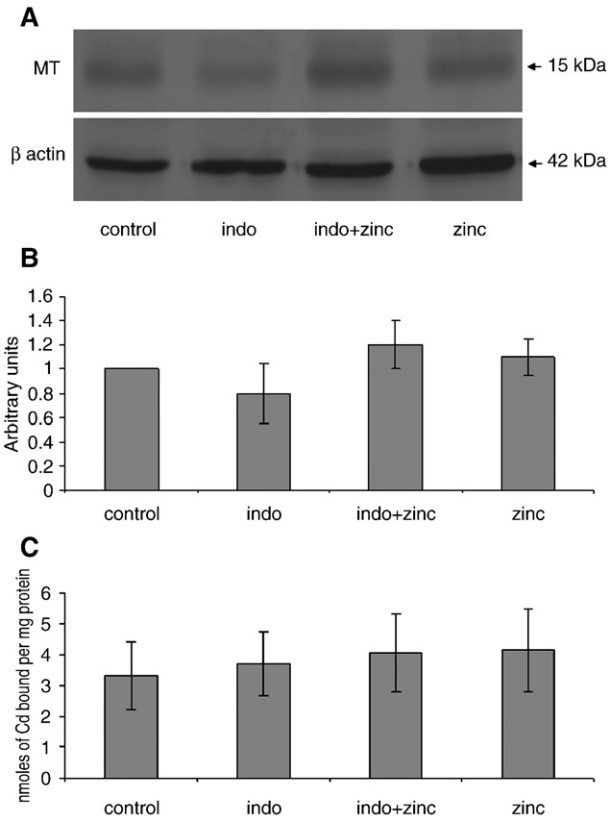

Fig. 7. (A) Representative western blot for metallothionein in rat kidney homogenates from control, indo, indo + zinc and zinc rats. (B) Graphical representation of densitometric analysis of western blot for metallothionein ($n = 3$). (C) Levels of metallothionein estimated by the Cd–hem assay in renal homogenates from control, indo, indo + zinc and zinc rats. Each value represents mean ± S.D. ($n = 8$).

returned to control levels by day 2 and remained so over the next 3 days (Fig. 8).

4. Discussion

The kidneys are important targets for the adverse effects of NSAIDs. Prostaglandins are known to help maintain glomerular filtration, especially in conditions where effective renal perfusion is compromised (Patrono and Dunn, 1987). Indomethacin non-specifically inhibits the synthesis of all prostaglandins by inhibiting the enzyme cyclooxygenase (COX). However, the initial contention that indomethacin-induced adverse effects are solely due to the inhibition of COX has now been refuted (Ligumsky et al., 1983; Langenbach et al., 1995). Several studies in the gastrointestinal tract and kidney, have shown that indomethacin also produces other effects in tissue, such as induction of oxidative stress and mitochondrial dysfunction (Basivireddy et al., 2002; Basivireddy et al., 2003; Basivireddy et al., 2004). It has therefore been postulated that these effects, in addition to inhibition of COX, contribute to indomethacin-induced organ dysfunction.

Our results confirm that indomethacin induces oxidative stress in the kidney as evidenced by increased levels of thiobarbituric acid reactive substances, protein carbonyls and pro-oxidant enzyme activities and decreased anti-oxidant enzyme activities in the renal homogenates. The elevation in activity of MPO, a pro-oxidant enzyme, showed that there was significant infiltration of neutrophils into the kidney. Indomethacin has been shown to induce the expression of intercellular adhesion molecule — 1 (ICAM-1) and pro-inflammatory cytokines (Okada et al., 1998), resulting in infiltration of activated neutrophils into tissues. Activated neutrophils are an important source of free radicals and have been implicated in the pathogenesis of NSAID-induced gastrointestinal damage (Wallace et al., 1990). These alterations in the balance between free radical production and scavenging ability appear to contribute to the oxidative stress produced.

Administration of zinc tended to reverse most of the drug-induced effects seen. In the case of the pro- and anti-oxidant enzymes, this tendency was observed when zinc was given as a single dose but the effects appeared to be more pronounced when zinc was given in multiple doses. For example, when our preliminary work used multiple doses, zinc significantly reduced drug-induced MPO activity [0.0024 ± 0.0004 U/mg protein compared to 0.0054 ± 0.0016 U/mg protein in indomethacin-treated rats ($p = 0.011$)] and increased drug-induced glutathione reductase activity [0.029 ± 0.0027 U/mg protein compared to 0.011 ± 0.002 U/mg protein in indomethacin-treated rats ($p = 0.039$)]. However, these preliminary observations require confirmation. One of our findings was the observation that rats administered only zinc (zinc group), showed a significant increase in the activity of glutathione reductase when compared to the control animals. This is in contrast to recent studies in isolated liver mitochondria (Gazaryan et al., 2007) and in primary astrocyte culture (Bishop et al., 2007) which show that zinc, in relatively high concentrations (~150 μM), inhibits glutathione reductase activity. On the other hand, it is also known that zinc stabilizes sulfhydryl groups at the active site of enzymes and may thus increase their activity when present in low concentrations (Bray and Bettger, 1990). Glutathione reductase has critical sulfhydryl groups in its active site (Pai and Schulz, 1983). Therefore, the effect of zinc on glutathione reductase activity is probably concentration-dependent and may also be influenced by other factors *in vivo*. A limitation of our study is that we were not able to measure zinc levels to ascertain what concentrations had been achieved in the kidney and in blood. However, Blalock et al. (1988) have reported that there was no significant rise in serum levels of zinc in animals that had received dietary zinc supplementation.

NSAIDs have been shown to uncouple and inhibit oxidative phosphorylation in mitochondria in the rat kidney (Mingatto et al., 1996; Basivireddy et al., 2004) and also in enterocytes (Somasundaram et al., 1997; Jacob et al., 2001). These studies and other indirect evidence suggest that uncoupling of oxidative phosphorylation and/or inhibition of electron transport by NSAIDs may play a role in free radical generation and subsequent toxicity of these drugs. Results from our study confirm that indomethacin causes mitochondrial damage in the kidney. An important factor that determines the structural and functional integrity of the mitochondria is the lipid composition of its membranes. Treatment with indomethacin resulted in changes in the neutral lipids and phospholipids of the mitochondrial membranes. Such changes have been shown to affect fluidity of the membranes (Matthys et al., 1984). Analysis of the phospholipids in our study has shown that there were significant decreases in the levels of phosphatidylcholine (PC) and phosphatidylethanolamine (PE), with corresponding increases in the levels of lysophosphatidylcholine

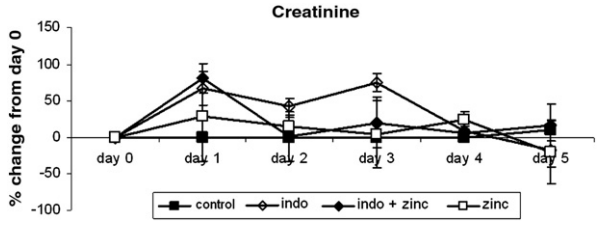

Fig. 8. Changes in serum creatinine levels in control, indo, indo + zinc and zinc rats measured every day for a period of 5 days following indomethacin / vehicle administration as a single dose on day 0. Blood sample on day 0 was taken just prior to drug treatment. Subsequently, blood samples were obtained from the tail vein at intervals of 24 h. Each value represents mean ± S.D. ($n = 3$).

(LPC) and lysophosphatidylethanolamine (LPE). This pattern of change suggests activation of phospholipase A_2, which can result in membrane dysfunction and apoptosis (Penzo et al., 2004). In addition, the finding of elevated levels of phosphatidic acid suggests the activation of phospholipase D. Phospholipase D has been shown to be activated by oxygen free radicals in the intestinal mitochondria (Madesh et al., 1997b). The results obtained in our study suggest that similar mechanisms may be operational in the kidney as well. Administration of zinc was shown to reverse the changes induced by indomethacin, thus diminishing the drug-induced mitochondrial membrane damage. We postulate that zinc produces these effects by alleviating the oxidative stress produced by the drug.

Zinc is a redox inactive metal ion. Despite its lack of redox capacity, it can exert anti-oxidant actions (Powell, 2000). The role of metallothionein in mediating the anti-oxidant functions of zinc has been extensively investigated (Maret, 2000). Metallothionein is a protein in which the redox properties originate from its unique structural architecture. Since metallothionein is proposed to be a link between cellular zinc and the redox state of the cell, it is likely that the induction of this protein by zinc plays a role in mediating its anti-oxidant effects. In the current study, we found that there were no significant differences in renal metallothionein levels in the different treatment groups (Fig. 7). Metallothionein was estimated by the cadmium–haem assay as well as by western blotting. Our results are consistent with previous studies that have shown that, in the rat kidney, zinc had no effect on metallothionein after 24 h (Suzuki et al., 1990). In addition, it is known that zinc is a poor inducer of metallothionein in the kidney compared to cadmium or mercury (Durnam and Palmiter, 1981).

It is therefore likely that the anti-oxidant effects of zinc observed in the kidney in our study are mediated by mechanisms other than those involving metallothionein. Our results are supported by other studies that have shown potent anti-oxidant properties of zinc that are independent of metallothionein (Kang and Zhou, 2005, Zhou et al., 2002). It is known to inhibit transition metal-mediated oxidative stress. Transition metals like iron and copper are powerful oxidants because they catalyze hydroxyl radical- producing Fenton reactions, causing oxidative damage to protein, lipids and DNA. Copper and iron are present intracellularly in association with high- or low-molecular-weight cellular components, such as nucleotides, peptides, polypeptides, proteins or DNA (Chevion, 1988). Zinc has been shown to effectively displace these metal ions from their intracellular binding sites (Powell et al., 1999), thus antagonizing their oxidant effects.

Zinc also protects sulfhydryl groups against oxidation It plays an important role in maintaining the intracellular levels of reduced glutathione (Bray and Bettger, 1990). It alters the modulation of the redox-sensitive transcription factors like activator protein-1 (AP-1) and nuclear factor kappa B (NFκB) (Oteiza and Mackenzie, 2005). These transcription factors are known to alter the expression of genes of antioxidant enzymes like glutathione reductase, glutathione peroxidase and catalase (Winyard et al., 2005). We postulate that these are possible mechanisms that may explain the changes in antioxidant enzyme levels noted in this study.

Zinc sulphate is a relatively non-toxic compound when given orally (Fosmire, 1990). At a dose of 50 mg/kg body weight (approximately equivalent to 20 mg of elemental zinc per kg body weight), zinc sulfate has been shown to be effective in preventing indomethacin-induced gastric damage and no toxic effects were reported (Joseph et al., 1999). Hence it appears to be a safe alternative that can be used in this setting.

Our study has also shown that administration of indomethacin caused elevated serum creatinine levels on day 1 (at 24 h) following drug administration, irrespective of zinc pre-treatment (Fig. 8). This is likely to be due to the alterations in the glomerular filtration induced by inhibition of prostaglandin synthesis by indomethacin as prostaglandins are known to be critical in the maintenance of normal glomerular filtration rate (Patrono and Dunn, 1987). We therefore measured creatinine levels over a period of five days after indomethacin administration (given as a single dose on day 0) to ascertain if the antioxidant actions of zinc led to an improvement in renal function during this period. Zinc pre-treatment resulted in the creatinine levels returning to baseline levels by day 2, while in rats treated with indomethacin alone, creatinine tends to remain elevated till day 4. It is possible that oxidative stress-induced renal damage may be responsible for prolonged elevation of creatinine in animals given indomethacin alone, with pre-treatment with zinc ameliorating these effects. This may account for the quicker functional recovery of the kidney with zinc. However, the mechanism by which this recovery occurs is not entirely clear and warrants further study.

5. Conclusion

The results of this study show that zinc protects against indomethacin-induced renal damage. It appears to do so by ameliorating drug-induced oxidative stress and mitochondrial dysfunction in the kidney.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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