Indomethacin-induced renal damage: role of oxygen free radicals

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Abstract

Nonsteroidal anti-inflammatory drugs are used extensively in clinical medicine. In spite of their therapeutic utility, however, they are known to cause significant gastrointestinal and renal toxicities, circumstances that limit their use. The side effects produced in these organs have been attributed mainly to the inhibitory effect of these drugs on the activity of cyclooxygenase, a key enzyme in prostaglandin synthesis. In addition to this, in the small intestine it is known that reactive oxygen species also contribute to the enteropathy seen in response to these drugs. In the kidney, however, there is little information whether other mechanisms contribute to the renal toxicity. This study was designed to look at the possible biochemical mechanisms involved in indomethacin-induced renal damage. Rats fasted overnight were dosed with indomethacin (20 mg/kg) by gavage and sacrificed 24 hr later. Histology of the kidney showed abnormalities in the mitochondria in the proximal tubules. Evidence of oxidative stress was found in the kidney associated with mitochondrial dysfunction and neutrophil infiltration. The lipid composition in the mitochondria was also altered. Such effects were abolished by the prior administration of arginine, a donor of nitric oxide. This study, thus, suggests that one of the mechanisms by which nonsteroidal anti-inflammatory drugs induce renal damage is through oxygen free radicals possibly generated by activated neutrophils and mitochondrial dysfunction.

Keywords: Kidney; Indomethacin; Nitric oxide; Nonsteroidal anti-inflammatory drugs; Oxidative stress

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are used extensively in clinical medicine as analgesics, antipyretics and anti-inflammatory agents. In spite of their therapeutic utility, however, these drugs have significant adverse effects, a circumstance that limits their use. Of these, gastrointestinal and renal toxicities are of major concern [1,2].

NSAIDs are known to exert their therapeutic effects through inhibition of cyclooxygenase (COX) [3], a key enzyme in the formation of prostaglandins. The decreased physiological formation of prostanoids has also been widely held to be the basis of the toxicity caused by these drugs in the kidneys and the gastrointestinal tract. In the kidneys, prostaglandins help maintain renal blood flow and glomerular filtration rate. Decreased level of these eicosanoids is thought to compromise renal function [4,5]. Reduced levels of prostaglandins have been found to be detrimental to the stomach as well. In the small intestine, however, it has been shown that inhibition of COX alone does not fully explain the pathogenesis of toxicity by these drugs [6]. Earlier work on the effects of indomethacin on the small intestine has shown that the drug produces free radical-induced damage in the enterocytes, with the villus tip cells being particularly susceptible to these effects [7]. These cells showed evidence of oxidative stress and mitochondrial dysfunction. Oxidative stress was also seen in the intestinal brush border membranes, along with structural and functional alterations [8]. These effects appeared to be mediated through the generation of oxygen free radicals. Thus, mechanisms other than those directly related to prostaglandins appear to be involved in the pathogenesis of NSAID-induced damage in the small intestine. It is not known, however, whether such mechanisms operate in the kidney.
Nitric oxide (NO) is a potent vasodilator, an inhibitor of leukocyte activation [9] and a scavenger of free radicals produced by neutrophils [10]. NO donors have been shown to reduce gastrointestinal damage caused by the NSAIDs in animal models as well as in humans [11,12]. Similarly, such compounds have also been to protect against cyclosporine-induced nephrotoxicity [13]. Based on these observations, NO-NSAID derivatives have been developed and shown to cause less damage in the gastrointestinal tract. There is little information, however, whether this holds true for renal tissue as well.

The current study was designed to look at the possible biochemical mechanisms involved in indomethacin-induced renal damage. In addition, the amino acid L-arginine, a potential source of NO, was assessed for its ability to confer protection against indomethacin-induced changes in the kidney.

2. Materials and methods

Adenosine diphosphate (ADP), L-arginine hydrochloride, bovine serum albumin, 1-chloro-2,4-dinitro benzene (CDNB), O-dianisidine dihydrochloride, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), ethylene diamine tetraacetatic acid (EDTA), oxidized glutathione (GSSG), reduced glutathione (GSH), indomethacin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nicotinamide adenine dinucleotide (NAD), reduced nicotinamide adenine dinucleotide phosphate (NADPH), 2-thiobarbituric acid (TBA), succinate and Tris–HCl were obtained from Sigma Chemical. All other chemicals were of analytical grade.

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

2.1. Protocol for administration of drugs

Rats were fasted overnight and dosed with indomethacin (Indo) (20 mg/kg) by gavage. Control animals received an equal volume of the vehicle for the drug (5% sodium bicarbonate). Animals were sacrificed 24 hr later, as preliminary experiments had shown that effects were most prominent at this time period. The animals were killed by cervical dislocation, their abdomens opened up immediately and the kidneys removed.

To study the effect of pre-treatment with L-arginine, the amino acid was given in drinking water (at a concentration of 1.5%) for 5 days [13] prior to dosing with indomethacin (Arg + Indo group). NG-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase (NOS), was also used. Rats were administered this compound (30 mg/kg) [14] by gavage 2 days prior to and along with L-arginine for 5 days. These rats were sacrificed on the eighth day after administration of indomethacin, as described above (L-NAME + Arg + Indo group).

2.2. Histological studies

Tissue samples were fixed in 2.5% glutaraldehyde, post-fixed with osmium tetroxide and embedded in araldite (epoxyresin). One-micron sections were cut using a glass knife and stained with toluidine blue. Well-oriented areas for ultrastructural study were chosen after examining 1-μm sections under a light microscope. Ultra-thin sections (60–90 nm) were cut on a LKBUM4 ultramicrotome using a diamond knife (Diatome). Sections were mounted on copper grids and stained with saturated aqueous uranyl acetate and Reynolds lead citrate. The grids were examined under a Philips EM201C (Eindhoven) electron microscope.

2.3. Preparation of homogenates and isolation of mitochondria

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. The homogenate was used for different assays and for isolation of mitochondria as described previously [15]. Purity of the preparation was checked by enrichment of the marker enzyme, succinate dehydrogenase.

2.4. Assessment of mitochondrial function

Mitochondrial function was assessed by the following parameters. Respiratory control ratio (RCR) was calculated by dividing the rate of oxygen uptake by mitochondria in state 3 respiration (after the addition of ADP) by that of state 4 (before the addition of ADP) [7]. MTT reduction by the isolated mitochondria was measured as described [16]. The amount of MTT formazan produced was calculated using the molar extinction coefficient, $E^{570}$ of 17,000 M$^{-1}$ cm$^{-1}$ at pH 7.4–8. The presence of mitochondrial swelling was determined by measuring the decrease in absorbance at 540 nm [17]. Calcium uptake by the mitochondria was followed by measuring the changes in absorption spectrum of arsenazo-III [18].

2.5. Assessment of parameters of oxidative stress

The homogenate of renal tissue was used for the measurement of malondialdehyde (MDA) [19], conjugated dienes [20], protein carbonyls [21], and $\alpha$-tocopherol [22], all indices of oxidative stress. These parameters, along with total thiols [23], were measured in isolated mitochondrial preparation as well. Protein was estimated by Lowry’s method, using bovine serum albumin as standard [24].
2.6. Assays of enzymes

Activities of myeloperoxidase (MPO) [25], catalase [26], glutathione peroxidase [27], glutathione reductase [28] and superoxide dismutase (SOD) [29] were measured in the homogenate.

2.7. Analysis of lipids

Lipids were extracted from mitochondria by Bliigh and Dyer method [30]. 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/v). The spots corresponding to the standards were identified by iodine exposure and eluted. Cholesterol, cholesteryl esters [31], TAG and diacylglycerol [32] were quantified. Individual phospholipids were separated on silica gel H plate using the solvent system chloroform:methanol:acetic acid:water (25:14:4:2, v/v/v/v) and quantitated by phosphate estimation after acid hydrolysis [33]. Phosphatidic acid (PA) was separated on oxalic acid impregnated silica gel G plate [34].

2.8. Statistical analysis

Data were analyzed by the Kruskal–Wallis test to look for differences in the means of the various experimental
groups. Tukey’s significant difference test was used as a post hoc test for pairwise 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, version 11.

3. Results

On ultrastructural examination, the proximal convoluted tubules of the kidney showed swollen mitochondria, inclusion bodies and vacuolation in tissue from indomethacin-treated animals (Fig. 1B) as compared with control tissue (Fig. 1A). Prior treatment with L-arginine showed almost complete abolition of such changes (Fig. 1C). Pre-treatment with L-NAME (inhibitor of NO synthase) along with L-arginine negated the protection offered by L-arginine (Fig. 1D).

Drug treatment resulted in increased oxidative stress in the kidney as evidenced by increased levels of MDA, conjugated dienes and protein carbonyl with concomitant decrease in $\alpha$-tocopherol in the homogenate when compared with that from control animals (Fig. 2A–D).

![Fig. 2. Levels of peroxidation products of lipid and protein and alpha-tocopherol in homogenate from control and indomethacin treated kidneys with and without pretreatment with arginine and L-NAME. Malondialdehyde (MDA) (A), conjugated dienes (CD) (B), protein carbonyls (PC) (C) and alpha-tocopherol (TC) (D). Each value represents mean ± SD ($n = 6$). Analysis by the Kruskal Wallis test showed that the experimental groups were different (MDA, $\chi^2 = 20.25$, df = 3, $p = 0.0002$; CD, $\chi^2 = 19.07$, df = 3, $p = 0.0003$; PC, $\chi^2 = 19.82$, df = 3, $p = 0.0002$; TC, $\chi^2 = 17.56$, df = 3, $p = 0.0004$). Tukeys significant difference test was then used as a post hoc test for pair-wise multiple comparisons. *$p < 0.001$, Indo vs control; # $p < 0.001$, Indo vs Arg + Indo; $\$ p < 0.001$, Arg + Indo vs NAME + Arg + Indo.](image)
Treatment with the drug also resulted in an increase in MPO activity in the homogenate (Fig. 3A) when compared with control values. Activities of some of the free radical scavenging enzymes such as catalase, SOD, glutathione reductase and glutathione peroxidase, were found to be decreased in the homogenate from indomethacin-dosed rats as compared with that from control rats (Fig. 3B–E).

Fig. 3. Activity of myeloperoxidase and antioxidant enzymes in the kidney homogenate obtained from control and indomethacin-treated animals with and without pretreatment with arginine and L-NAME. Myeloperoxidase (MPO) (A), superoxide dismutase (SOD) (B), catalase (C), glutathione reductase (GR) (D) and glutathione peroxidase (GPX) (E). Each value represents mean ± SD (n = 6). Analysis by the Kruskal Wallis test showed that the experimental groups were different (MPO, $\chi^2 = 19.11$, df = 3, $p = 0.0003$; SOD, $\chi^2 = 18.89$, df = 3, $p = 0.0003$; catalase, $\chi^2 = 18.67$, df = 3, $p = 0.0002$; GR, $\chi^2 = 19.16$, df = 3, $p = 0.0003$; GPX, $\chi^2 = 21.62$, df = 3, $p = 0.0001$). Tukeys significant difference test was then used as a post hoc test for pair-wise multiple comparisons. *$p < 0.001$, Indo vs control; #$p < 0.001$, Indo vs Arg + Indo; $\$$ p < 0.001$, Arg + Indo vs NAME + Arg + Indo.
In isolated mitochondria, the marker enzyme succinate dehydrogenase showed 6- to 10-fold enrichment in the final preparation (data not shown), thereby confirming purity of the preparation. Mitochondria from drug-treated rats showed a decrease in the RCR as compared with control preparations (Fig. 4A). These mitochondria also exhibited increased production of MTT formazan, increased permeability of the inner membrane leading to swelling of the mitochondria (as indicated by decreased absorbance at 540 nm) and decreased uptake of calcium (as evidenced by the reduced fall in optical density of the calcium–arsenazo complex) (Fig. 4B–D). Measurement of

Fig. 4. Functional parameters of mitochondria isolated from control and indomethacin-treated animals, with and without pretreatment with arginine and L-NAME. Respiratory control ratio (RCR) (A), amount of MTT formazan formed (B), absorbance at 540 nm (abs) (C) and rate of calcium uptake (Ca) (D). Each value represents mean ± SD (n = 6). Analysis by the Kruskal Wallis test showed that the experimental groups were different (RCR, \( \chi^2 = 18.80, df = 3, p = 0.003 \); MTT, \( \chi^2 = 17.84, df = 3, p = 0.0005 \); abs, \( \chi^2 = 16.53, df = 3, p = 0.001 \); Ca, \( \chi^2 = 23, df = 3, p = 0.0000 \). Tukey’s significant difference test was then used as a post hoc test for pair-wise multiple comparisons. *\( p < 0.001 \), Indo vs control; #\( p < 0.001 \), Indo vs Arg + Indo; $\ p < 0.001 \), Arg + Indo vs NAME + Arg + Indo.
indicators of oxidative stress in the mitochondria showed increase in the levels of MDA, conjugated dienes and protein carbonyl and a decrease in α-tocopherol and thiols in preparations from drug-treated rats when compared with control preparations (Fig. 5A–E). Mitochondrial lipid analysis revealed significant changes after drug treatment. Levels of cholesteryl ester, triacylglycerol and diacylglycerol were found to be decreased with an increase in the level of free cholesterol (Fig. 6A–D). Among the phospholipids, phosphatidylcholine and phosphatidylethanolamine

![Graphs showing levels of peroxidation products of lipid and protein, alpha-tocopherol and thiols in mitochondria from control and indomethacin treated kidneys with and without pretreatment with arginine and L-NAME.](image)

Fig. 5. Levels of peroxidation products of lipid and protein, alpha-tocopherol and thiols in mitochondria from control and indomethacin treated kidneys with and without pretreatment with arginine and L-NAME. Malondialdehyde (MDA) (A), conjugated dienes (CD) (B), protein carbonyl (PrC) (C), total thiols (TT) (D) and alpha-tocopherol (TC) (E). Each value represents mean ± SD (n = 6). MDA, $\chi^2 = 20.25$, df = 3, $p = 0.0002$; CD, $\chi^2 = 19.07$, df = 3, $p = 0.0003$; PrC, $\chi^2 = 19.82$, df = 3, $p = 0.002$; TT, $\chi^2 = 18.06$, df = 3, $p = 0.0004$; TC, $\chi^2 = 17.56$, df = 3, $p = 0.0005$. Tukeys significant difference test was then used as a post hoc test for pair-wise multiple comparisons. *$p < 0.001$, Indo vs control; §§$p < 0.001$, Indo vs Arg + Indo; $\$p < 0.001$, Arg + Indo vs NAME + Arg + Indo.
were decreased (Fig. 7A and C) with concomitant elevations in the levels of lysophosphatidylcholine, lysophosphatidylethanolamine (Fig. 7B and D) and PA (Fig. 8A). Level of other phospholipids such as phosphatidylserine, phosphatidylinositol and sphingomyelin were also lower in the mitochondria isolated from drug-treated animals (data not shown). The ratio of total cholesterol to total phospholipids was higher in the indomethacin-treated mitochondria (Fig. 8B).

Supplementation of arginine prior to indomethacin treatment resulted in abolition of the changes in the parameters of oxidative stress as well as the free radical scavenging enzymes (Figs. 2 and 3). It also protected against indomethacin-induced fall in mitochondrial RCR and calcium
uptake, and increased production of MTT formazan and membrane permeability (Fig. 4). Similarly, drug-induced peroxidation and changes in lipids in the mitochondria were also abolished by prior treatment with arginine (Figs. 5–8). Administration of L-NAME along with arginine attenuated the protective effect of arginine on all the parameters measured (Figs. 1–8).

4. Discussion

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used classes of drugs for the management of acute and chronic pain. The use of repeated doses of NSAIDs, however, is known to produce significant gastrointestinal and renal toxicity. While the gastrointest-
inal toxicity of these medications is better known, it is becoming increasingly apparent that the kidney is also an important target for untoward clinical events [4,5].

The renal effects of NSAIDs include electrolyte imbalance, acute renal failure, nephrotic syndrome associated with interstitial nephropathy and papillary necrosis. Electrolyte disorders and acute renal failure are observed more frequently in patients with risk factors, such as those with age-related declines in glomerular filtration rate, those with hypovolemia, particularly patients taking loop diuretics and those with congestive heart failure, cirrhosis, or nephrosis. Abnormalities have also been reported in the ultrastructure of the kidney in response to administration of NSAIDs [35]. These include intercellular edema, changes in the configuration of cells, decreased electron density of hyaloplasm, swollen mitochondria and dilated cisternae of the golgi complex and endoplasmic reticulum. Such changes may be the result of pharmacologic depression of prostaglandin synthesis produced by the NSAIDs, causing decreased blood supply to the kidneys.

Two isoforms of COX are found in cells. These are COX1 and COX2. COX1 is constitutively expressed in most tissues and performs a housekeeping function to synthesize prostaglandins that regulate normal cell activity. COX2 is the inducible form of the enzyme, which is expressed in settings of inflammation. In the kidney, however, COX2 is constitutively expressed in the basement membrane. Studies have shown that renal side effects produced by NSAIDs are associated with the inhibition of COX2 [4,36]. Indomethacin is a non-specific COX inhibitor and it inhibits both COX1 and COX2, but the degree of affinity for COX2 is much less than it is for COX1 [37,38]. Thus, the degree of inhibition of COX2 by indomethacin is relatively less. Hence, the effects seen in the kidney are not likely to be only due to the inhibition of COX2. This lends credence to the possibility that mechanisms other than the inhibition of COX may be important in causing the renal toxicity produced by the drug.

Another possible mechanism that may be operational is inhibition of oxidative phosphorylation by NSAIDs, which may depress renal function [39]. In addition, other factors may also be operational in producing the effects of these drugs on the kidney. For example, it has been shown that diclofenac-induced nephrotoxicity may involve production of reactive oxygen species leading to oxidative stress and massive genomic DNA fragmentation, and these free radical-mediated events may ultimately translate into apoptotic cell death of kidney cells [40].

The current study showed increase in oxidative stress in the kidney following indomethacin administration. The free radicals responsible for these changes may be produced by neutrophils infiltrating the kidney and/or due to mitochondrial dysfunction. The possibility of the former occurring is supported by the finding that there was an increase in the activity of MPO, a marker enzyme for neutrophils, in the kidneys following indomethacin treatment. Neutrophil infiltration in response to NSAID administration has been shown to occur in the gastrointestinal tract and has been implicated in pathogenesis of damage in

![Fig. 8. Phosphatidic acid (PA) content (A) and cholesterol/phospholipid ratio (CPR) (B) of mitochondria isolated from control and indomethacin-treated animals, with and without pretreatment with arginine and L-NAME. Each value represents mean ± SD (n = 6). PA, $\chi^2 = 17.82$, df = 3, $p = 0.0005$; CPR, $\chi^2 = 17.58$, df = 3, $p = 0.001$. Tukeys significant difference test was then used as a post hoc test for pair-wise multiple comparisons. *$p < 0.001$, Indo vs control; #$p < 0.001$, Indo vs Arg+Indo; $\$ p < 0.001$, Arg + Indo vs NAME + Arg + Indo.](image-url)
these tissues [41,42]. Activated neutrophils generate oxygen free radicals and are proposed to be a major cause of the cell and tissue damage associated with many chronic inflammatory diseases [43]. Lipid peroxides that accumulate due to peroxidation in membrane lipids brought about by free radicals are known to be potentially harmful to cells and tissue [44]. If cells are exposed to a large amount of free radicals beyond a certain period of time, lowered intracellular levels of ATP may result in apoptotic changes of the cell [45].

The other possible source of free radicals in the cell is the mitochondrion, the sub-cellular organelle involved in energy production and calcium homeostasis [46]. It is known that indomethacin and other NSAIDs uncouple and inhibit oxidative phosphorylation, both in isolated mitochondrial preparations and jejunal tissue in vitro [47,48]. Such an effect would result in mitochondrial dysfunction with subsequent decrease in intracellular ATP and increased free radical formation, both of which render a cell vulnerable to damage.

Normally small amounts of oxygen free radicals are produced endogenously as a by-product of electron transport, and mitochondria have their own scavenging system to neutralize these radicals [49]. Alteration in the balance between free radical production and scavenging ability is thought to lead to mitochondrial injury. In addition, organelle integrity is important for normal cellular function. The fall in absorbance seen at 540 nm is a consequence of swelling of the mitochondria and is due to increased permeability of the inner mitochondrial membrane to small molecular weight solutes. This finding is supported by current ultrastructural studies that showed swollen mitochondria on drug treatment. This may also contribute to the mitochondrial dysfunction shown to occur in the present study as evidenced by increased MTT reduction, reduced uptake of calcium and decreased RCR.

Increased levels of lipid and protein peroxidation products and a decrease in the antioxidant, α-tocopherol, in the mitochondria after indomethacin treatment is evidence of oxidative stress in the kidney. It is likely that peroxidation of lipids can lead to degradation of membrane lipids, and studies have shown that interaction of these degradation products with intra- and extracellular targets can produce new reactive species during the course of chain reactions resulting in further damage to cells and tissues [50]. Similarly, the decreased levels of free radical scavenging enzymes such as catalase, SOD, glutathione reductase and glutathione peroxidase and α-tocopherol in the homogenate as well are further evidence of oxidative stress produced in the tissue. This is in agreement with earlier studies done in the small intestine [51].

Functional alterations in the mitochondria may be also induced by structural changes in the organelle. Analysis of the composition of lipids in the mitochondrial membrane revealed that drug treatment resulted in changes in the composition of lipids in these membranes. These changes include degradation of phospholipids and generation of lysophospholipids. These degradation products are membrane lytic agents and are probably the result of phospholipase A₂ activation [52]. In addition to these products, the level of PA was also increased accompanied by decreased phosphatidyl ethanolamine and phosphatidyl choline, suggesting activation of phospholipase D. Earlier work in our laboratory has shown the presence of phospholipase D in intestinal mitochondria, which can be activated by oxygen free radicals and divalent metal ions, thereby resulting in mitochondrial damage [53]. The changes in the content of neutral lipids and phospholipids in the mitochondria probably account for the altered cholesterol–phospholipid ratio in these organelles. Changes in this ratio in biomembranes are known to alter the fluidity of membrane [54] and this may contribute to the increased permeability of the inner mitochondrial membrane seen in the present study.

L-Arginine is the substrate for NOS and this releases NO. Nitric oxide has the capacity to inhibit reactive oxygen metabolites, including superoxide anion, and can prevent the cellular damage attributable to hydrogen peroxide [10]. In the present study, pre-treatment with arginine prior to indomethacin protected against indomethacin-induced changes. This protective effect was nullified by pre-treatment with L-NAME (inhibitor of NOS) along with L-arginine, indicating that the effect was mediated by the production of NO. These observations thus emphasize the importance of NO in renal defenses against indomethacin-induced injury and are supported by an earlier study [55]. Another protective role of NO may lie in its ability to ameliorate vasoconstriction and improve organ blood flow in the kidney [56].

Earlier studies have also shown that the renal side effects occur at a later period as compared to that seen in the intestine [57]. The current study supports this observation as the renal effects of indomethacin were most marked at 24 hr after the administration of the drug rather than at earlier time periods as seen in the intestine [7,8].

In conclusion, this study has shown that various biochemical abnormalities are produced in the kidney in response to the administration of indomethacin. These effects include oxidative damage and impairment of structure and function of mitochondria and appear to be mediated through the production of free radicals. Pre-treatment with arginine conferred protection against such changes.

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References


