Indomethacin-induced mitochondrial dysfunction and oxidative stress in villus enterocytes

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) are known to cause small intestinal damage but the pathogenesis of this toxicity is not well established. Intestinal epithelial cells are thought to be affected by these drugs in the course of their absorption. These cells are of different types, viz. villus, middle and crypt cells. There is little information on which of these cells, if any, are particularly vulnerable to the effects of NSAIDs. This paper aimed to study the effects of indomethacin, an NSAID commonly used in toxicity studies, on different populations of enterocytes. Effects of the drug were assessed in terms of oxidative damage, mitotic activity, mitochondrial function and lipid composition in enterocytes isolated from the small intestine of rats that had been orally administered indomethacin. In addition, the effects of arginine and zinc in protecting against such changes were assessed. Cell viability, tetrazolium dye (MTT) reduction and oxygen uptake were significantly reduced in villus tip cells from rats dosed with the drug. Thymidine uptake was higher in the crypt cell fraction from these rats. Similarly, products of lipid peroxidation were elevated in the villus tip cells with a corresponding decrease in the level of the anti-oxidant, alpha-tocopherol. In isolated mitochondrial preparations from various enterocyte fractions, significant functional impairment and altered lipid composition were seen mainly in mitochondria from villus cells. Arginine and zinc pre-treatment were found to protect against these effects. These results suggest for the first time that the villus tip cells are more vulnerable to the damaging effects of indomethacin and that oxidative stress is possibly involved in this damage. © 2002 Elsevier Science Inc. All rights reserved.

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1. Introduction

NSAIDs, used extensively in clinical medicine, show a propensity to cause mucosal damage in the gastrointestinal tract [1], a circumstance that limits their use. They are known to exert their therapeutic effects through inhibition of cyclooxygenase [2], a key enzyme in the formation of prostaglandins. The decreased physiological formation of the prostanoids is widely held to be the basis of the gastric toxicity caused by these drugs. NSAIDs are known to have adverse effects on the small intestine also [3] but the mechanism of this damage is not well established. The inhibition of cyclooxygenase alone does not fully explain the pathogenesis of toxicity in this part of the gastrointestinal tract [4].

One of the hypotheses advanced to explain the mechanism of NSAID-induced toxicity in the small intestine involves the action of the drug in uncoupling or inhibiting oxidative phosphorylation [5]. Indomethacin, a commonly used NSAID, has been shown to have these effects in isolated liver mitochondrial preparations [6]. The drug has also been shown to produce ultra structural damage to mitochondria in rat jejunum in vitro [7] and in vivo as early as 1 hr after an oral dose [6]. This impairment of mitochondrial function along with inhibition of cyclooxygenase is thought to be necessary for the development of indomethacin-induced enteropathy.

Earlier work done has shown that jejunal tissue obtained from rats that had been dosed with indomethacin by gavage did not exhibit changes in indices of mitochondrial function [8]. Inability to demonstrate evidence of mitochondrial dysfunction in this study might be due to the fact that measurements were made in whole jejunal tissue, while the...
effects of the drug are likely to be most marked in the intestinal epithelial cells. These cells originate from the base of the crypt and migrate to the tip of the villi [9]. During the migration from crypt to villus, the cell undergoes maturation and differentiation. Thus, the villus tip cells are well differentiated and possess various proteins, which are involved in digestion and absorption. Little information is available on the effect of indomethacin on enterocytes as a whole and those at various stages of maturation. The drug is known to cause mucosal damage in the small intestine but it is not known which cells, if any, are particularly vulnerable.

Other factors have also been shown to be involved in the pathogenesis of NSAID-induced damage to the gastrointestinal tract. Oxidative stress is one of the putative mechanisms by which this process is postulated to occur. Oxygen and other drug-derived free radicals have been implicated in NSAID-induced injury to the stomach, particularly that due to indomethacin [10]. There is, however, very little information on the role of free radicals, if any, in the pathogenesis of NSAID-induced small intestinal damage.

Nitric oxide (NO) is a potent vasodilator, an inhibitor of leukocyte activation [11] and also a scavenger of free radicals produced by neutrophils [12]. NO donors have been shown to reduce gastrointestinal mucosal damage in animal models [13]. Based on these observations, NO–NSAID derivatives have been developed and shown to cause less damage to the gastrointestinal mucosa [14,15].

Recent studies have shown that zinc offers a gastroprotective effect against various ulcerogenic agents. Pretreatment with zinc monoglycerolate, zinc acetamate and zinc sulfate has been shown to significantly decrease NSAID-induced gastric mucosal injury [16–18]. However, no information is available on possible protective effects of zinc in the small intestine.

This study was therefore designed to look at the effects of indomethacin on enterocytes. Assessments of oxidative stress and mitochondrial function in total enterocytes and in different populations of these cells were made. The possible roles of NO and zinc in protecting against these changes were also studied.

2. Materials and methods

Ethylene diamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), HEPES (4-(2-hydroxyethyl) piperazine (2-ethanesulfonic acid), trypan blue, bovine serum albumin (BSA), arsenazo-III, 3-[4,5-dimethylthia- zol-2-yl]2,5-diphenyltetrazolium bromide (MTT), indomethacin, dimethyl sulfoxide (DMSO), dithiothreitol (DTT), ADP, succinate, Tris–HCl and lipid standards were obtained from Sigma, St. Louis, USA. All other chemicals were of analytical grade.

Adult albino rats (200–250 g) of both sexes were used for all the experiments. The experiments were approved by the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), Government of India.

2.1. Protocol for administration of drugs

Rats were fasted overnight and dosed with indomethacin (Indo) (40 mg/kg) by gavage. This dose was chosen after preliminary experiments showed that at the time periods of 1 and 2 hr studied, it produced more marked and consistent effects on the parameters measured than the lower dose of 20 mg/kg. Control animals received an equal volume of the vehicle for the drug (5% sodium bicarbonate or 10% DMSO). Male animals were sacrificed 1 hr later and females 2 hr after the dose, as preliminary experiments had shown that effects were most prominent at these time intervals in the respective sexes. The animals were killed by cervical dislocation, their abdomens opened up immediately and the entire length of the small intestine removed.

For studies on the protective effects of various compounds, the following protocols were followed. Rats were given an intraperitoneal injection of l-arginine (a NO donor) at 300 mg/kg, 30 min prior to dosing with indomethacin (Indo + Arg group) [19]. When using l-NAME (NG-nitro-l-arginine methyl ester), an inhibitor of NO synthase, the rats were administered the compound (30 mg/kg) by gavage for 6 days [20]. The rats were sacrificed on the seventh day after administration of arginine and indomethacin, as described above (Indo + Arg + l-NAME group). Zinc sulfate (50 mg/kg) [17] was administered by gavage 2 hr prior to dosage with indomethacin (Indo + Zn group).

2.2. Isolation of enterocytes

Total enterocytes were isolated from the rat small intestine by the metal chelation method [21]. Enterocytes at various stages of differentiation (villus to crypt) were isolated and separated into 9 fractions as described [22]. The purity of the separated fractions was checked by the marker enzyme, alkaline phosphatase [23]. The 9 fractions obtained were pooled into 3 as follows: fractions 1–3 were pooled and consisted predominantly of villus tip cells, 4–6 formed the middle cell fraction and 7–9 were pooled to form the crypt cells.

2.3. Isolation of mitochondria

Mitochondria from various cell fractions were isolated [24] and the purity of the preparation was checked by the enrichment of the marker enzyme, succinate dehydrogenase.

2.4. Assessment of functional parameters

The isolated enterocytes (total and fractions) and mitochondria were used in the experiments described below.
The viability of isolated enterocytes was assessed by trypan blue exclusion. Oxygen uptake and MTT reduction by these cells were measured as indices of mitochondrial function. The former was determined polarographically using a Clarke type electrode [25] and the latter by using a microtire plate [26]. The amount of MTT formazan formed was calculated using the molar extinction coefficient, $E^{570}$ of 17,000 M$^{-1}$ cm$^{-1}$ at pH 7.4–8. The rate of cell proliferation was determined by measuring the rate of incorporation of $^3$H-thymidine into the DNA of different cell fractions [27] and expressed as DPM/10$^6$ cells.

Parameters of mitochondrial function in the isolated organelles were assessed as described below. Oxygen uptake and MTT reduction by the isolated mitochondria were measured as described earlier. Respiratory control ratios (RCRs) were calculated by dividing the rate of oxygen uptake by mitochondria from each enterocyte fraction in state 3 respiration (after the addition of ADP) by that of state 4 (before the addition of ADP). Permeability of the inner mitochondrial membrane was determined by measuring the decrease in absorbance at 540 nm [28]. Calcium uptake by the mitochondria was followed by measuring the changes in absorption spectrum of arsenazo-III [29].

2.5. Assessment of parameters of oxidative stress

Homogenates of enterocytes were prepared in buffer consisting of 136 mM NaCl, 6.8 mM K$_2$HPO$_4$, 2.5 mM KH$_2$PO$_4$, 1.5 mM EDTA, 0.5 mM DTT, pH 7.4. These were used for the measurements of malondialdehyde (MDA) [30], conjugated dienes [31] and alpha-tocopherol [32,33], all indices of oxidative stress. These parameters were also measured in mitochondrial suspensions. Protein was estimated by Lowry’s method, using bovine serum albumin as standard [34].

2.6. Assays of enzymes

Arginase activity was measured in homogenates of various enterocyte fractions [35]. For the measurement of myeloperoxidase activity, a marker of neutrophil infiltration, the small intestine was removed from drug-treated rats and its entire length opened along the anti-mesenteric border. The mucosal surface was scraped with a glass slide. Homogenates of these scrapings were prepared and used for the assay of myeloperoxidase [36].

2.7. Analysis of lipids

Lipids were extracted from the enterocyte fractions and mitochondria by Bligh and Dyer method [37]. Neutral lipids were separated on silica gel G plates using the solvent system hexane:diethyl ether:acetic acid (80:20:1, v/v). Free fatty acids were quantitated by gas chromatography after separation on a 5% EGSS-X column. Individually phospholipids were separated on silica gel H plate using the solvent system chloroform:acetic acid:water (25:14.4, v/v) and quantitated by phosphate estimation after acid hydrolysis [38]. Phosphatidic acid (PA) was separated on oxalic acid impregnated silica gel G plate [39].

2.8. Statistical analysis

Data were analyzed by the Mann–Whitney $U$-test. A $P$-value of less than 0.05 was taken to indicate statistical significance.

3. Results

3.1. Effects of indomethacin on total enterocytes

Indomethacin at a dose of 40 mg/kg was chosen, as preliminary experiments had shown that it produced more consistent results than the lower dose of 20 mg/kg. MTT reduction by total enterocytes from indomethacin-treated male rats was significantly lower 1 hr after the dose (117.6 ± 35.3 nmoles/min/10$^7$ cells against a control value of 230.1 ± 31.1 nmoles/min/10$^7$ cells, $P < 0.001$). Oxygen uptake by these enterocytes was also significantly lower (52.1 ± 8.5 nmoles/min/10$^7$ cells against a control value of 88.2 ± 17.3 nmoles/min/10$^7$ cells, $P < 0.001$). Similar results were obtained with female animals 2 hr after dosing with the drug (data not shown).

3.2. Effects of indomethacin on enterocyte fractions

Viability counts done by trypan blue exclusion in the different enterocyte fractions revealed that the number of viable cells was significantly lower in the drug-treated villus cells only (Fig. 1A). MTT reduction and oxygen uptake were also found to be less only in this fraction (Fig. 1B and C). $^3$H-thymidine incorporation studies showed increased uptake only by crypt cells from indomethacin-treated rats when compared with corresponding controls (Fig. 1D).

Measurement of parameters of lipid peroxidation showed that MDA and conjugated dienes were elevated and alpha-tocopherol was decreased in the villus tip cells from indomethacin-dosed rats (Fig. 2A–C). These changes were not found in the middle and crypt cell fractions.

3.3. Effects of indomethacin on mitochondrial function and lipid composition

The RCR of mitochondria from drug-treated villus cells was lower than that of controls (Fig. 3A). These mitochondria also showed increased swelling as evidenced by decreased absorption at 540 nm (Fig. 3B). Similarly, there was less uptake of calcium into the indomethacin-treated
villus cell mitochondria in comparison with control preparations (Fig. 3C). These effects were not apparent in mitochondria from the middle and crypt cells.

Mitochondria from villus cells from indomethacin-dosed rats also showed elevated levels of MDA and conjugated dienes with a decrease in alpha-tocopherol (Fig. 4A–C). Phosphatidylcholine and phosphatidylethanolamine levels were lower in these, with corresponding increases in lysophosphatidylcholine and lysophosphatidylethanolamine, respectively (Fig. 5A–D). PA content was also higher in these preparations (Fig. 5E), as were the levels of free fatty acids (Table 1). These changes were not seen in mitochondria from the middle and crypt cells.

3.4. Effect of indomethacin on enzymes

Myeloperoxidase activity in mucosal scrapings from indomethacin-treated rats showed higher values (7.80 ± 1.52 units/mg protein) when compared with control animals (3.25 ± 0.28 units/mg protein; P ≤ 0.01). Arginase activity was higher in the villus cell homogenate after indomethacin treatment (0.348 ± 0.016 units/mg protein) as compared...
with that from control animals (0.242 ± 0.0136 units/mg protein; \( P \leq 0.01 \)). There was no significant effect of the drug on the activity of arginase in the middle and crypt cell homogenates (data not shown).

3.5. Effect of pre-treatment with arginine and zinc on indomethacin-induced changes in the enterocyte fractions

Pre-treatment with arginine improved the viability of villus tip cells from animals dosed with indomethacin (87 ± 1.7% in the group pre-treated with arginine and then given indomethacin as against 72 ± 2% in the rats given only indomethacin). This pre-treatment also protected against indomethacin-induced inhibition of MTT reduction and oxygen uptake (Fig. 6A and B). The increase in thymidine uptake in crypt cells was also blocked by arginine (Fig. 6C). Similarly, arginine conferred protection against the increase in indomethacin-induced lipid peroxidation seen in the villus enterocytes (Fig. 7A–C). Prior administration of L-NAME abolished the protective effect of arginine on all the parameters measured. Pre-treatment with zinc also conferred protection against impairment of cellular respiration, prevented the proliferation seen in the crypt cells in response to indomethacin and also the accompanying lipid peroxidation (Figs. 6A–C and 7A–C). When zinc alone was administered to animals, all the parameters measured showed findings similar to those seen in control animals (data not shown).
4. Discussion

Indomethacin, an NSAID commonly used in toxicity studies, is known to affect oxidative phosphorylation. Such an effect has been demonstrated in mitochondrial preparations in vitro and in jejunal tissue, both in vitro and in vivo [6,7]. This change is postulated to be an important initiating event in the development of NSAID-induced enteropathy. In the intestine, enterocytes are found at different stages of maturation, with the crypt cells at the base being least differentiated and the villus tip cells most highly differentiated. Little is known about the effects of indomethacin on enterocytes at these different stages of maturation and differentiation. In the current study, parameters of cellular respiration (MTT reduction and oxygen uptake) were found to be significantly depressed in indomethacin-treated villus cells. Impairment of cellular respiration is evidence of the deleterious effects of indomethacin on mitochondria and is indicative of mitochondrial dysfunction. Evidence of lipid peroxidation was also seen in the indomethacin-treated villus cells, showing that oxidant-induced damage had occurred. In the middle and crypt cells, no such changes were seen. These results indicate that the differentiated villus tip cells appear to be most vulnerable to the effects of indomethacin. To our knowledge, this is the first such report showing differential sensitivity of different populations of enterocytes to the effects of indomethacin. This increased susceptibility of the villus cells to the deleterious effects of the drug may possibly be mediated through mitochondrial dysfunction and oxidative stress. Mitochondrial membrane damage due to oxidative stress is known to occur [40,41]. Such damage to these membranes due to increased generation of reactive oxygen species has been shown to lead to apoptosis [42,43]. In the current study, the damage produced in the villus cells appears to stimulate the proliferation of the crypt cells, evidenced by increased levels of thymidine incorporation into the crypt cells from indomethacin-treated rats. These results are consistent with other studies, which have shown changes in the villi in response to orally administered indomethacin. The changes documented in these other studies include slowing of villus blood flow, microvascular distortion, villus shortening and epithelial disruption [44,45]. The observations in the current study...
cells appear more predisposed to the development of apoptosis and this may explain the greater vulnerability of the well-differentiated villus tip cells to the effects of indomethacin in the current study.

Mitochondrial preparations from enterocyte fractions from indomethacin-treated rats also showed changes similar to those seen in the enterocytes. Impairment of function of mitochondria from villus cells is evidenced by the lowered RCR, fall in absorbance at 540 nm and decreased influx of calcium into the mitochondria (Fig. 3A–C). Decrease in RCR is indicative of a disturbance of mitochondrial function. The fall in absorbance at 540 nm is a consequence of swelling of the mitochondria that is in turn due to increased permeability of the inner mitochondrial membrane to small molecular weight solutes. Inability to take up calcium also indicates functional impairment of the mitochondria, as these organelles normally sequester calcium. Thus, there appears to be definite evidence of mitochondrial dysfunction in response to the administration of indomethacin. This impairment in function may be a consequence of a direct effect of indomethacin on the organelle, an indirect action of the drug causing free radical production that affects mitochondrial function or a combination of these factors.

Lipid peroxidation shown to occur in the villus cells is probably a consequence of increased generation of free radicals in the cells. Mitochondrial dysfunction can result in increased generation of these reactive molecules. Another possible source is from neutrophils, which are known to infiltrate the intestinal mucosa following administration of indomethacin [47]. The current study showed increased levels of myeloperoxidase activity in the mucosal scrapings from drug-treated animals, a finding indicative of neutrophil infiltration, which suggests that these inflammatory cells also possibly contribute to the oxidant-induced damage.

The increased lipid peroxidation in the drug-treated mitochondria is consistent with similar findings in the enterocytes. Analysis of the composition of lipids of the mitochondrial membranes from indomethacin-treated villus cells showed significant changes in the content of various phospholipids and fatty acids when compared with that of corresponding control mitochondria. The decrease in levels of phosphatidylcholine and phosphatidylethanolamine with corresponding increases in their respective lysophospholipids, along with increases in levels of various free fatty acids, suggests increased activity of phospholipase A2. This is consistent with observations that have shown evidence of activation of this phospholipase in mitochondria in response to oxidative stress [48]. In addition, the increased levels of PA found are indicative of the activation of phospholipase D, a finding that is also supported by earlier work [49]. Thus, the current study suggests that activation of phospholipases occurs in villus enterocytes in response to indomethacin. This produces changes in the composition of lipids of the mitochondrial membranes in these cells. Changes in the

![Graph](image)

Fig. 4. Levels of MDA (A), conjugated dienes (B) and alpha-tocopherol (C) in mitochondria isolated from different enterocyte fractions from control and indomethacin-dosed rats. *P < 0.05 when compared with control. Each value represents mean ± SD of three separate experiments.

are also consistent with results published by Mancini et al. [46] who have demonstrated that enterocytic differentiation in Colo-205 cells (a human adenocarcinoma cell line derived from malignant ascites) predisposes such cells to apoptosis and that mitochondrial abnormalities precede the apoptotic changes. The relationship between differentiation and apoptosis in Colo-205 cells appears to mirror the changes in normal intestinal mucosa during the physiological process of terminal differentiation of enterocytes. The undifferentiated stem cells localized at the base of the intestinal crypts give rise to daughter cells that differentiate and migrate up the crypt to reach the mucosal surface, where they die by apoptosis and are sloughed from the surface. The findings by Mancini et al. therefore suggest a tight link between the enterocyte pathway of terminal differentiation and apoptosis. Thus, well differentiated
Fig. 5. Phospholipid composition of mitochondria from various enterocyte fractions from control and indomethacin-dosed rats. *P < 0.05 when compared with control. Each value represents mean ± SD of three separate experiments. PC: phosphatidylincholine, PE: phosphatidylethanolamine, LPC: lysophosphatidylincholine, LPE: lysophosphatidylethanolamine, PA: phosphatidic acid.

Phospholipid content are thought to destabilize membranes of organelles, either by simple depletion of phospholipid from the membranes, or through the accumulation of cholatecic products such as free fatty acids or lysophosphatides.

The accumulation of arachidonic acid within cells is known to induce apoptosis [50,51]. This mechanism may operational in the differentiated villus cells in the current study as the action of PLA2 in releasing arachidonic acid from the

Table 1
Free fatty acid composition of mitochondria from various enterocyte fractions from control and indomethacin-dosed rats

<table>
<thead>
<tr>
<th>Free fatty acids</th>
<th>Control (nmole/mg protein) (±SD)</th>
<th>Indomethacin (nmole/mg protein) (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Villus</td>
<td>Middle</td>
</tr>
<tr>
<td>Lauric acid</td>
<td>1.38 (0.15)</td>
<td>1.48 (0.30)</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>11.13 (0.85)</td>
<td>15.59 (0.98)</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>3.99 (0.24)</td>
<td>6.85 (0.84)</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>0.91 (0.13)</td>
<td>1.59 (0.08)</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>0.0016 (0.0004)</td>
<td>0.08 (0.0002)</td>
</tr>
</tbody>
</table>

Each value represents mean ± SD of three separate experiments.
*P < 0.05 when compared with control.
phospholipids and the inhibitory action of indomethacin on cyclooxygenase would cause intracellular accumulation of free arachidonic acid, thereby triggering apoptosis in these cells only.

Thus, these data suggest that the administration of indomethacin results in the generation of free radicals in these enterocytes, possibly as a result of the mitochondrial dysfunction produced and the infiltration of neutrophils into the mucosa. These oxidants appear to induce lipid peroxidation and activate phospholipases, which in turn alters the composition of various lipids in the mitochondrial membranes, thereby leading to further dysfunction.

It was also shown that arginine, a NO donor, has protective effects on villus cells against the deleterious effects of indomethacin. This is in agreement with studies, which have shown that NO-derivatives of NSAIDs are less ulcerogenic than the parent compounds [14,15]. NO is known to ameliorate vasoconstriction and improve organ blood flow in the small intestine during bacteremia [52], contribute to functional repair of the epithelial barrier following gut injury [53] and prevent intestinal mitochondrial damage that occurred during ischaemia reperfusion injury to the intestine [54]. In the current study, the specificity of the protection conferred by arginine through generation of NO is shown by abolition of the protective effect by prior treatment with L-NAME, an inhibitor of NO synthase. The activity of arginase was also found to be significantly higher in the indomethacin-treated villus cells when compared with control villus cells. This increase may lead to depletion of arginine in these cells, thereby depriving them of a source of NO. Such a change was not demonstrable in the middle and crypt cells, both of which did not show evidence of indomethacin-induced damage. These observations thus emphasize the importance of NO in mucosal defenses against indomethacin-induced injury in enterocytes.
Zinc is known to have anti-oxidant properties. It scavenges reactive oxygen metabolites and also acts as a membrane stabilizer. It stimulates tissue healing and repair in experimental ulcers directly through promoting cell proliferation, protein synthesis and growth factor production. It has also been shown to affect cytokine-activated transcription factors [55]. Several studies have reported that this element confers protection against gastric mucosal damage induced by NSAIDs [16–18]. The current study documents similar protection by zinc against indomethacin-induced changes in the villus cells of the small intestine. It has been shown that zinc stimulates the electron transport system and oxidative phosphorylation in hepatic mitochondria and thereby increases the ATP concentration in the cell [56]. It is possible that this mechanism may be operational in the current study also. The precise mechanism by which zinc offers protection against indomethacin-induced damage is, however, not completely clear and requires further study.

In conclusion, this study has shown for the first time that well differentiated villus tip cells of the intestine are more susceptible than the middle and crypt cells to the toxic effects of indomethacin. Impairment of cellular respiration and oxidant-induced damage are seen to occur in these cells. In response to the effects in the villus cells, crypt cell proliferation is enhanced to compensate for the damage. These deleterious effects of the drug may be mediated through the generation of oxygen free radicals and activation of phospholipases. Pre-treatment with arginine or zinc conferred protection on these cells against such damage.

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