Attenuation of intestinal ischemia/reperfusion injury with sodium nitroprusside: studies on mitochondrial function and lipid changes

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Abstract

Reactive oxygen species have been implicated in cellular injury during ischemia/reperfusion (I/R). Mitochondria are one of the main targets of oxygen free radicals and damage to this organelle leads to cell death. Reports suggest that nitric oxide (NO) may offer protection from damage during I/R. This study has looked at the functional changes and lipid alteration to mitochondria during intestinal I/R and the protection offered by NO. It was observed that I/R of the intestine is associated with functional alterations in the mitochondria as suggested by MTT reduction, respiratory control ratio and mitochondrial swelling. Mitochondrial lipid changes suggestive of activation of phospholipase A2 and phospholipase D were also seen after (I/R) mediated injury. These changes were prevented by the simultaneous presence of a NO donor in the lumen of the intestine. These studies have suggested that structural and functional alterations of mitochondria are prominent features of I/R injury to the intestine which can be ameliorated by NO. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Intestine; Ischemia-reperfusion; Nitric oxide; Mitochondria; Phospholipase

1. Introduction

A reduction in intestinal blood flow results in characteristic mucosal lesions. During reperfusion, intestinal mucosa shows significant cascade of destructive events [1,2]. Although reperfusion is essential in order to prevent anoxic cell death, it may be associated with additional severe cellular damage. It is well known that development of ischemia/reperfusion (I/R)-induced organ injury is closely associated with free radical production [3,4]. The efficiency of the endogenous antioxidant defense mechanism is important in protecting against reperfusion injury.

Oxygen free radicals are implicated in the pathogenesis of intestinal I/R injury [4-6]. The hypoxanthine-xanthine oxidase (XO) system appears to be an important source of reactive oxygen species (ROS) [7]. ROS react with nucleic acids, proteins, carbohydrates, and lipids to produce damage to these biological molecules. In addition to their direct tissue-damaging effect, ROS trigger the accumulation and activation of neutrophils [7,8]. Our earlier studies with XO-deficient animals suggest that during I/R injury to the intestine, the invading phagocytes, rather than XO, form an important source of oxygen radicals [9].
Mitochondrial respiration is the major source of ATP, which is indispensable for the maintenance of cellular integrity and the performance of cellular functions. Oxygen free radicals are also produced endogenously as a by-product by the mitochondrial electron transport systems and mitochondria have their own radical scavenging system to neutralize these radicals [10]. Alteration in the balance between radical production and scavenging ability is thought to lead to mitochondrial injury. Indeed, the energy producing system is impaired by free radicals in I/R-induced organ injury [11]. Recently, we have shown that mitochondria can withstand short periods of ischemia, whereas long duration ischemia or reperfusion results in irreversible damage to mitochondrial function [12]. Earlier we have shown that in vitro exposure of isolated intestinal mitochondria to ROS results in membrane phospholipid alteration, specifically a decrease in phosphatidylethanolamine (PE) and an increase in phosphatidic acid (PA) due to activation of a phospholipase D associated with intestinal mitochondria. These alterations in mitochondrial lipids may play a role in causing the functional alteration seen in oxidative stress [13].

The role that nitric oxide (NO) plays in cytotoxic events is unclear. Many reports suggest that NO mediates tissue injury during the I/R event [14–16]. Yet, other reports suggest that NO may have a protective effect during ischemia/reperfusion injury [17–19]. NO appears to be a regulator of regional blood flow during reperfusion following extremity ischemia. Decreased NO production may contribute to impaired regional blood flow and mortality [20]. NO donors have been shown to reduce the rise in reperfusion-induced intestinal mucosal permeability [21,22]. Recently, Terada et al. have shown that NO decreases lung injury following intestinal ischemia [23]. We have also recently shown that oxygen free radicals mediated intestinal mitochondrial phospholipase D (PLD) activation is attenuated by nitric oxide [24]. Little is known about intestinal mitochondrial lipid alterations due to oxidative stress, during I/R. Earlier reports indicate that reperfusion injury is associated with activation of mucosal phospholipase A2 (PLA2) [25] and accumulation of lysophospholipids could be involved in mucosal injury caused by small intestinal ischemia [26]. However, the role of PLD in I/R injury of the small intestine has not been investigated. The present study was carried out to clarify whether and to what extent phospholipases are involved in I/R injury of the small intestine in vivo, specifically looking at changes in mitochondria.

2. Materials and methods

Various lipid standards, HEPES, BSA, succinate (sodium salt), ADP, sodium nitroprusside (SNP), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), dimethyl sulfoxide (DMSO), pentobarbital, EDTA and EGTA were obtained from Sigma. Microtiter plates were supplied by Sterlin, Middlesex (UK). All other chemicals used were of analytical grade.

2.1. Development of ischemia/reperfusion

Development of I/R was done as described [12]. Briefly, overnight fasted rats were anesthetized with sodium pentobarbitone (50 mg/kg b.wt.). The small intestine was isolated after a midline abdominal incision and cannulated at both ends using silastic tubing. The luminal contents were removed by perfusion with normal saline and the superior mesenteric artery (SMA) was exposed retroperitoneally. To perform ischemia, the root of the SMA was occluded for different time periods namely, 30, 60 and 90 min with a clamp and released for 5 min for reperfusion. The isolated intestinal loop was returned to the abdominal cavity and the abdominal wall was closed to minimize dehydration of the gut segment during the course of the experiment. Body temperature was maintained at 37°C with an overhead lamp. After the corresponding time periods, the intestine was removed and enterocytes were isolated [27].

In a second group of animals, the same protocol was followed; however, the nitric oxide donor, sodium nitroprusside was dissolved in saline (1 mM final concentration). Saline at 37°C containing the solubilized NO donor was then perfused via a peristaltic pump (Vickers Medical Treonic IP4 pump, Basingstoke, UK) into the proximal jejunal lumen at a rate of 1 ml/min starting 5 min after saline wash. The test group was preconditioned with SNP solution for 15 min before being subjected to ische-
mia and this was continued throughout the experimental protocol.

Control rats were also anesthetized and the small intestine was isolated after midline abdominal incision. The intestine was cannulated at both ends using silastic tubing and the luminal contents were removed by perfusion with normal saline. Saline perfusion was then done as for the test group, except that the SMA was not clamped. Like the test group, the controls were also preconditioned for 15 min with SNP solution.

2.2. Preparation of mitochondria

Mitochondria were isolated from enterocytes as described by Masola and Evered [27]. Isolated mitochondrial fraction was suspended in EGTA free medium containing 250 mM sucrose and 5 mM HEPES, pH 7.4, and stored in ice at a protein concentration of 8–10 mg/ml. Protein was measured using BSA as standard [28]. Purity of the preparation was judged by the enrichment of the marker enzyme, succinate dehydrogenase [29].

2.3. MTT reduction by enterocyte mitochondria

Mitochondrial function was assessed by MTT reduction using a microplate reader as described with slight modification [12]. In a total volume of 150 μl in each well, mitochondrial suspension corresponding to 40–100 μg protein was taken, 6 μl of 1.25 mM MTT added and made up to the volume with 25 mM phosphate-buffered saline. MTT was dissolved in PBS and filtered to remove small amounts of insoluble residue in some batches of MTT. Plates were incubated at 37°C for 20 min followed by addition of 150 μl of DMSO and mixed thoroughly to dissolve the formazan. The plates were read on a multi-well scanning spectrophotometer (ELISA reader) using a test wavelength of 570 nm and reference wavelength of 630 nm. The amount of MTT formazan formed was calculated from the standard curve prepared using authentic MTT formazan (20–100 μM). Since succinate is a mitochondrial respiratory substrate, formation of MTT formazan in the presence and absence of 1 mM succinate (final concentration) was tested.

2.4. Measurement of oxygen uptake

Oxygen uptake was determined polarographically using a Clark-type electrode in 2 ml of respiratory medium (225 mM sucrose, 5 mM MgCl₂, 10 mM KH₂PO₄, 20 mM KCl, 10 mM Tris, and 5 mM HEPES pH 7.4), containing 5 mM succinate. One mg/ml of mitochondrial protein was used. Oxygen uptake was stimulated with 0.3 mM ADP. Respiratory control ratio was determined as described by Eastabrook [30].

2.5. Mitochondrial swelling

Mitochondrial swelling was determined by the decrease in absorbance at 540 nm as described [31].

2.6. Histologic studies

Full-thickness samples of the small intestinal tissue were fixed in 10% formalin, embedded in paraplast and 3–4-μm sections cut. The sections were stained with hematoxylin and eosin and observed under a light microscope.

2.7. Ultrastructural studies

Full-thickness samples of the small intestine were fixed in 2–5% glutaraldehyde, post-fixed in osmium tetroxide, and embedded in araldite. Sections of 1 μm were cut, stained with Toluidine blue and used to select appropriate area for ultrastructural study. Ultrathin sections were cut on a Leica Ultracut UCT ultramicrotome with a diamond knife (Diatome, Switzerland), stained with uranyl acetate and lead citrate, and examined with a Phillips EM201C electron microscope (Eindhoven, The Netherlands).

2.8. Lipid analysis

Total lipids were extracted by Bligh and Dyer’s method [32]. Neutral lipids were separated on silica gel G plates using the solvent system hexane-diethyl ether–acetic acid (80:20:1, v/v). Spots were identified by iodine exposure, scraped and eluted. Cholesterol [33], diglycerides and triglycerides [34] were estimated as described. Free fatty acids were methylated and
quantitated by gas chromatography after separation on a 5% EGSS-X column. Individual phospholipids were separated on silica gel H plates using the solvent system chloroform–methanol–acetic acid–water (25:15:4:2 v/v) [35] and quantitated by phosphate estimation after acid hydrolysis [36]. For PA quantitation, extracted lipids were spotted on silica gel G plates impregnated with 0.5 M oxalic acid and separated using the solvent system chloroform–methanol–Con. HCl (85:134:0.5 v/v) [37]. PA spot corresponding to standard was identified by iodine exposure, scraped, eluted and quantitated by phosphate estimation after acid digestion. Our earlier studies using intestinal mitochondrial PLD have shown that this enzyme does not catalyze transphosphatidylation in the presence of alcohol [13,38], like the recently reported yeast PLD [39,40].

2.9. Statistical analysis

Data are expressed as mean ± S.D. from three separate experiments. Statistical analysis was performed with Student’s t-test to compare the changes.

3. Results

3.1. Effect of nitric oxide donor on morphological changes triggered by ischemia/reperfusion

Control mucosa with and without nitric oxide perfusion showed normal crypts and villi (Fig. 1A1, B1). Blood vessels were also normal. With ischemia, there was detachment of the villus epithelium leaving denuded villi (Fig. 1A2) whereas luminal perfusion with 1 mM nitric oxide donor, SNP, showed greater preservation of villus epithelium (Fig. 1B2). The lamina propria showed marked vascular dilatation, congestion and hemorrhage both with and without nitric oxide perfusion. Submucosal vessels showed dilatation and congestion. In ischemia followed by reperfusion, villi appeared collapsed, shortened and broadened and there was further detachment of villus epithelium (Fig. 1A3) whereas luminal perfusion of SNP showed preservation of villus epithelium (Fig. 1B3). I/R showed focal necrosis in crypt bases that was not seen in the tissue exposed to nitric oxide donor.

3.2. Effect of nitric oxide donor on ultrastructural changes during ischemia/reperfusion

Control mucosa with and without nitric oxide perfusion showed normal crypts and villi. There were no remarkable changes seen in mitochondria (Fig. 2a). With ischemia, the villus epithelium showed detachment. There was peripheral condensation of nuclear chromatin and the mitochondria were markedly dilated with lucency of the matrix and disruption of cristae. Endoplasmic reticulum was also dilated. Crypts in the mucosa showed focal dilatation of mitochondria with matrix lucency and disrupted cristae as described in the villi (Fig. 2b). In ischemia followed by short duration of reperfusion, villus epithelial cells showed changes similar to those with ischemia alone. The crypt cells also showed focal mitochondrial dilatation. Luminal perfusion with nitric oxide donor, SNP, prevented the remarkable changes that were seen in the ischemic mitochondria. The microvilli and intercellular junctions were relatively well preserved. Crypts of tissue exposed to nitric oxide did not show mitochondrial dilatation or

Table 1

<table>
<thead>
<tr>
<th>Neutral lipids</th>
<th>Control</th>
<th>I30</th>
<th>I60</th>
<th>I90</th>
<th>I30R</th>
<th>I60R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free fatty acids</td>
<td>111.90 ± 7.50</td>
<td>116.20 ± 3.90</td>
<td>120.36 ± 6.46</td>
<td>154.00 ± 8.2a</td>
<td>132.64 ± 7.2a</td>
<td>148.20 ± 7.3a</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>86.89 ± 4.89</td>
<td>84.87 ± 5.90</td>
<td>87.14 ± 5.20</td>
<td>85.95 ± 5.10</td>
<td>85.79 ± 7.12</td>
<td>87.42 ± 7.60</td>
</tr>
<tr>
<td>Cholesteryl ester</td>
<td>30.74 ± 3.00</td>
<td>31.70 ± 2.70</td>
<td>32.27 ± 1.92</td>
<td>28.10 ± 2.50</td>
<td>30.37 ± 3.40</td>
<td>30.62 ± 1.37</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>86.96 ± 5.00</td>
<td>86.03 ± 3.97</td>
<td>88.78 ± 6.36</td>
<td>90.35 ± 5.34</td>
<td>86.41 ± 5.85</td>
<td>86.43 ± 6.29</td>
</tr>
<tr>
<td>Diglycerides</td>
<td>54.43 ± 2.73</td>
<td>57.48 ± 2.20</td>
<td>58.18 ± 4.50</td>
<td>57.85 ± 3.48</td>
<td>58.92 ± 3.20</td>
<td>60.70 ± 2.88</td>
</tr>
</tbody>
</table>

Each value (nmol/mg protein) represents mean ± S.D. of three separate estimations. I30, 30-min ischemia alone; I60, 60-min ischemia alone; I90, 90-min ischemia alone; I30R, 30-min ischemia followed by 5-min reperfusion; I60R, 60-min ischemia followed by 5-min reperfusion.

*P < 0.05 as compared to control mitochondria.
Fig. 1. Light microscopic appearance of intestinal mucosa after ischemia and I/R and the effect of NO on these alterations. 
(A1) Small intestine of control rat with normal crypts and villi. 
(A2) Small intestine of rat subjected to 90-min ischemia showing mildly shortened, broadened villi and extensive denudation of the villus epithelium. 
(A3) Small intestine subjected to 60-min ischemia and 5-min reperfusion showing further shortening and broadening of villi and loss of villus epithelium. 
(B1) Small intestine of control rat perfused with nitric oxide. 
(B2) Small intestine subjected to 90-min ischemia with simultaneous luminal perfusion of nitric oxide donor. 
(B3) Small intestine subjected to 60-min ischemia and 5-min reperfusion along with luminal perfusion of nitric oxide donor showing partial shortening and broadening of villi and disruption of villus epithelium. Experimental details are described in the text.
epithelial necrosis (Fig. 2c). However they did show prominent intercellular edema.

3.3. Effect of nitric oxide donor on mitochondrial function as assessed by MTT reduction

Mitochondrial function can be assessed by the reduction of the tetrazolium salt MTT to its formazan, which gives an index of cell viability and mitochondrial function. Our earlier work with ischemia of 30, 60, and 90 min and ischemia followed by 5-min reperfusion suggested that, 30-and 60-min ischemia alone produce reversible change in MTT reduction, whereas 90-min ischemia alone or 30- or 60-min ischemia followed by 5-min reperfusion results in irreversible damage to mitochondria as assessed by

Fig. 2. Electron microscopic appearance of epithelial cells after ischemia and the effect of NO. (a) Epithelial cells of control rat with normal mitochondria (arrow). (b) Epithelial cells of rat subjected to 90-min ischemia with markedly dilated mitochondria (arrow), showing decreased matrix density and peripherally situated cristae. (c) Epithelial cells of rat exposed to nitric oxide and 90-min ischemia with normal mitochondria (arrow). Experimental details are described in the text.

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MTT reduction [12]. Hence MTT reduction was assessed with and without succinate using isolated enterocyte mitochondria from animals subjected to intestinal ischemia for 90 min, and 60-min ischemia with 5-min reperfusion. As can be seen, compared to control, in 90-min ischemia alone or 60-min ischemia followed by 5-min reperfusion, the ability of the mitochondria to reduce MTT is significantly decreased (Fig. 3A), whereas luminal perfusion of rats with 1 mM nitric oxide donor, sodium nitroprusside, enhanced the mitochondrial MTT reduction significantly in both ischemia or ischemia followed by reperfusion (Fig. 3B). This was also true in the presence of the respiratory substrate, succinate.

### 3.4. Mitochondrial respiratory function

Changes in the respiratory control ratio (RCR) are shown in Fig. 4. Both ischemia and I/R affected mitochondrial respiratory function as compared to control. Respiratory control ratio markedly decreased during ischemia in all groups or ischemia followed reperfusion (Fig. 4A). Administration of nitric oxide donor significantly enhanced the recovery of the RCR (Fig. 4B).

![Fig. 3. MTT reduction by isolated enterocyte mitochondria after graded ischemia and I/R and the effect of nitric oxide. I, control; II, 90-min ischemia; and III, 60-min ischemia followed by 5-min reperfusion. (A) MTT reduction by mitochondria in the presence and absence of succinate. (B) Effect of NO treatment on MTT reduction. Experimental details are described in the text. Data represent mean ± S.D. of three separate experiments. *P < 0.05 versus that of mitochondria from control.](image1)

![Fig. 4. Respiratory control ratio (RCR) of isolated enterocyte mitochondria after graded ischemia and I/R (A) and the effect of nitric oxide (B). Con, control; I30, I60 and I90, ischemia alone for 30, 60 and 90 min; I30R and I60R, ischemia of 30 and 60 min followed by 5-min reperfusion. Experimental details are described in the text. Data represent mean ± S.D. of three separate experiments. *P < 0.05 versus that of control mitochondria.](image2)
3.5. Effect of nitric oxide donor on mitochondrial swelling

A significant decrease in absorbance at 540 nm was observed after ischemia in all groups (Fig. 5A), indicating that the mitochondria have undergone the permeability transition, whereas this decrease was not seen in ischemia or I/R with luminal perfusion of nitric oxide donor. This indicated that the mitochondrial permeability transition is prevented by the presence of nitric oxide (Fig. 5B).

3.6. Effect of nitric oxide on mitochondrial lipid changes after ischemia/reperfusion

Table 1 summarizes the neutral lipid changes after ischemia or ischemia followed by 5-min reperfusion as compared to control. No significant differences were found in the neutral lipids except free fatty acids. Ischemia of increasing duration caused a progressive increase in free fatty acids, with ischemia of 90 min or ischemia followed by 5-min reperfusion causing significant increase as compared to control. Interestingly, when simultaneous luminal perfusion with nitric oxide donor, SNP showed a complete prevention of free fatty acid release in either long-term ischemia (90 min) or ischemia (60 min) with short-term reperfusion (5 min) (Table 2).

Phospholipid composition was also analyzed following ischemia or ischemia with short-term reperfusion. As can be seen in Table 3, ischemia of all groups showed a significant decrease in major phospholipids namely, phosphatidylcholine and phosphatidylethanolamine. Similar changes were observed in I/R but degradation was much more prominent than

Table 2
Neutral lipid composition of intestinal mitochondria in control, ischemia and I/R in NO-treated animals

<table>
<thead>
<tr>
<th>Neutral lipids</th>
<th>Control</th>
<th>I₉₀</th>
<th>I₉₀R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free fatty acids</td>
<td>114.50 ± 10.50</td>
<td>120.00 ± 8.40*</td>
<td>118.70 ± 7.00*</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>84.00 ± 5.00</td>
<td>82.90 ± 4.00</td>
<td>85.70 ± 6.00</td>
</tr>
<tr>
<td>Cholesteryl ester</td>
<td>32.70 ± 4.90</td>
<td>30.70 ± 3.10</td>
<td>34.00 ± 2.70</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>86.00 ± 8.00</td>
<td>84.70 ± 6.20</td>
<td>88.10 ± 4.90</td>
</tr>
<tr>
<td>Diglycerides</td>
<td>52.37 ± 3.00</td>
<td>55.70 ± 4.00</td>
<td>58.70 ± 4.30</td>
</tr>
</tbody>
</table>

Each value (nmol/mg protein) represents mean ± S.D. of three separate estimations. I₉₀, 90-min ischemia alone; I₉₀R, 60-min ischemia followed by 5-min reperfusion. Lumen was perfused with 1 mM SNP during 15 min prior to and during the active experimental duration.

*P < 0.05 compared to ischemia and I/R.
ischemia alone. Simultaneously, progressive increase in lysophosphatidylcholine was seen in all these groups. There was no significant change in lysophosphatidylethanolamine in either ischemia or ischemia followed by reperfusion. These changes in phospholipids were prevented by the luminal perfusion of NO donor (Table 4). Our earlier work has shown that intestinal mitochondria have a PLD activity, which can be stimulated by oxygen free radicals, divalent metal ions and polyamines [13,38,41]. The increased PE degradation observed in ischemia and I/R suggests that possibly PE degradation is brought about by PLD activity. This was further confirmed by PA estimation, which showed a significant increase in PA formation in ischemia and I/R (Fig. 6A). This decrease in PE and increase in PA formation were completely prevented by the luminal perfusion of NO donor (Fig. 6B). There was no change in the level of other phospholipids namely, phosphatidylserine, phosphatidylinositol, sphingomyelin, and cardiolipin (Tables 3 and 4).

4. Discussion

Intestinal I/R results in epithelial cell damage and is primarily due to generation of oxygen free radicals. Xanthine oxidase and infiltrated phagocytes have been suggested to be important sources of oxygen free radicals during I/R injury [2]. Nitric oxide, produced by the NO synthase enzyme is an important regulator of intestinal blood flow and mucosal barrier function [42–44]. $\text{N}^\text{G}$-nitro-$l$-arginine methyl ester (L-NAME) was ineffective in preventing these changes in PLD activity possibly because NO synthase is not involved in this process.

Table 3

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Control</th>
<th>$I_{30}$</th>
<th>$I_{60}$</th>
<th>$I_{90}$</th>
<th>$I_{30}R$</th>
<th>$I_{60}R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>86.67 ± 3.67</td>
<td>81.45 ± 3.20</td>
<td>79.65 ± 2.58</td>
<td>62.29 ± 3.63 $^a$</td>
<td>66.90 ± 3.60 $^a$</td>
<td>59.25 ± 3.10 $^a$</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>83.93 ± 3.50</td>
<td>75.94 ± 3.35 $^a$</td>
<td>69.00 ± 4.00 $^a$</td>
<td>54.70 ± 3.10 $^a$</td>
<td>53.17 ± 4.00 $^a$</td>
<td>43.30 ± 3.70 $^a$</td>
</tr>
<tr>
<td>LysoPC</td>
<td>8.85 ± 0.74</td>
<td>9.85 ± 0.50</td>
<td>11.55 ± 1.00</td>
<td>38.70 ± 2.30 $^a$</td>
<td>28.60 ± 2.10 $^a$</td>
<td>38.23 ± 2.90 $^a$</td>
</tr>
<tr>
<td>LysoPE</td>
<td>11.26 ± 0.65</td>
<td>12.20 ± 1.40</td>
<td>12.58 ± 1.00</td>
<td>12.86 ± 1.10</td>
<td>11.80 ± 0.80</td>
<td>13.60 ± 1.40</td>
</tr>
<tr>
<td>PI and PS</td>
<td>22.46 ± 1.56</td>
<td>21.19 ± 1.60</td>
<td>22.75 ± 1.22</td>
<td>22.12 ± 1.90</td>
<td>22.10 ± 1.90</td>
<td>23.20 ± 2.00</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>15.35 ± 0.73</td>
<td>15.56 ± 1.68</td>
<td>14.77 ± 1.08</td>
<td>14.71 ± 1.85</td>
<td>15.00 ± 1.20</td>
<td>15.15 ± 1.56</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>25.82 ± 1.88</td>
<td>24.88 ± 2.23</td>
<td>24.98 ± 2.21</td>
<td>23.34 ± 2.59</td>
<td>25.15 ± 1.23</td>
<td>25.20 ± 1.70</td>
</tr>
</tbody>
</table>

Each value (nmol/mg protein) represents mean ± S.D. of three separate estimations. The abbreviations are the same as given in Table 1.

$^a$ $P < 0.05$ as compared to control mitochondria.
ter (l-NAME) is an inhibitor of nitric oxide synthesis and has been used to inhibit the effect of NO. Studies of ischemia reperfusion in the feline small intestine have shown that I/R induced mucosal and microvascular permeability increases were dramatically augmented by NAME infusion and this effect was reversed by infusion of l-arginine [21]. Other studies also have indicated a beneficial effect of NO in I/R injury [45,46], whereas there are few reports that indicated an exacerbation of I/R injury upon treatment with NO [47]. This study has looked into the structural and functional alterations in enterocyte mitochondria during I/R injury and the effect of NO donor, sodium nitroprusside on these alterations. We used SNP as a NO donor at a concentration of 1 mM, and a flow rate of 1 ml/min and this would result in an available NO concentration of 10 nM. Reaction with oxygen would result in a decrease of NO concentration over time, and hence, the final NO level available to enterocytes would be compatible with physiological levels [46].

The intestinal villi are extremely susceptible to ischemic damage and their necrosis is one of the earliest histological changes that occur during intestinal ischemia [48]. Our earlier histological studies have indicated morphological changes in the gut due to ischemia and reperfusion and the damage was found to be more severe after reperfusion. These results suggested that mucosal damage occurs during I/R and free radicals generated by the infiltrated neutrophils may play a role in this damaging process [9,49]. Intestinal alkaline phosphatase activity, which is localized in the villus cells [50], was significantly decreased during I/R [12]. Sisley et al. [51] suggested that a loss in activity of intestinal alkaline phosphatase occurs during reperfusion and this may be a specific marker for reperfusion injury. In this study, we have observed that nitric oxide protected the mucosa from the ischemia and ischemia followed by reperfusion damage. Epithelial detachment and villus collapse were less severe in the mucosa exposed to nitric oxide donor than in the mucosa subjected to I/R. I/R showed focal necrosis in crypt bases that was not seen in the tissue exposed to nitric oxide donor.

A prominent feature of cell damage caused by oxygen free radicals is structural and functional damage to mitochondria. It was seen that long duration ischemia alone or I/R had a damaging effect on mitochondrial function as assessed by MTT reduction and the respiratory control ratio. We have earlier shown that short duration of ischemia alone (30 or 60 min) results in reversible damage to mitochondria as seen by MTT reduction in presence of succinate, whereas long-duration (90 min) ischemia alone or 30- or 60-min ischemia followed by 5-min reperfusion results in irreversible damage [12]. Hence, in this study, MTT reduction was carried out only for 90-min ischemia alone or 60-min ischemia followed by 5-min reperfusion and this was done with and without NO donor. The presence of NO donor completely reversed the effect of I/R damage as seen by measurement of respiratory control ratio and mitochondrial permeability transition. These parameters, which are indicative of functional damage to mitochondria, are reversed by NO, indicating a beneficial role for this compound in I/R injury.

Functional alterations may be induced by structural changes in the mitochondria and this was checked by analyzing the lipid composition of the mitochondria that are essential components of the mitochondrial function.

<table>
<thead>
<tr>
<th>Phospholipids</th>
<th>Control</th>
<th>I90</th>
<th>I60R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>84.94 ± 6.03</td>
<td>81.60 ± 6.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>82.20 ± 7.00&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Phosphatidylethanolamine</td>
<td>80.78 ± 5.97</td>
<td>79.20 ± 4.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79.25 ± 5.06&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>LysoPC</td>
<td>8.62 ± 1.00</td>
<td>11.58 ± 1.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.04 ± 1.58&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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<td>LysoPE</td>
<td>10.81 ± 0.66</td>
<td>12.19 ± 1.18</td>
<td>12.80 ± 1.62</td>
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<td>PI and PS</td>
<td>21.23 ± 1.26</td>
<td>21.52 ± 1.60</td>
<td>21.04 ± 1.95</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>16.04 ± 1.90</td>
<td>15.01 ± 1.93</td>
<td>15.03 ± 0.76</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>25.23 ± 1.78</td>
<td>23.58 ± 1.19</td>
<td>23.84 ± 2.03</td>
</tr>
</tbody>
</table>

Each value (nmol/mg protein) represents mean ± S.D. of three separate estimations. The abbreviations are same as given in Table 2. <sup>a</sup>P < 0.05 as compared to ischemia and I/R.
drial membrane. During the course of hypoxia, or ischemic tissue injury, membrane phospholipid breakdown occurs, leading to the accumulation of various cytotoxic products. In this study, during ischemia or I/R, lipid alterations in the mitochondria were seen. The prominent alterations include decrease in PC and PE and an increase in lysoPC and free fatty acids. Phospholipids comprise the major structural framework of cell membranes and fatty acids and lysophospholipids once generated can exert toxic effects [52–54]. These phospholipid changes are attributed to PLA2-induced membrane damage. Earlier reports have shown that I/R injury is associated with activation of mucosal PLA2 [55]. Both membrane associated and cytosolic PLA2 have been shown to be present in the mucosal cells [56]. Similarly, calcium-dependent and -independent PLA2 have been reported [57,58]. It has been shown that the translocation of cytosolic PLA2 to membranes occurs during O2 deprivation potentially enhancing their ability to evoke membrane damage [57]. The increase in free fatty acids and lysophospholipids was completely prevented by the luminal perfusion with NO donor.

In addition to PLA2 products, an increase in PA was seen during I/R injury in the enterocyte mitochondria. This PA is generated by the activation of PLD and we have shown earlier that intestinal mitochondria have an active PLD, which can be stimulated by oxygen free radicals or calcium [13,38]. It was also shown that this PLD specifically utilizes PE as substrate when stimulated by free radicals or calcium [13,38]. The changes seen in this study clearly showed that concomitant with the decrease in PE, an increase in PA was seen, suggesting that the PLD is likely to be activated by free radicals and/or calcium. Increase in PA in the mitochondrial membrane may induce functional alterations in the mitochondria. PA is known to facilitate calcium transport and increase in PA in mitochondrial membrane alters the calcium homeostasis. It has been reported that alteration in calcium homeostasis plays an important role in I/R injury [25]. This alteration in PE and PA levels was completely prevented by the simultaneous presence of NO donor in the lumen. This supports our earlier observation that enterocyte mitochondrial PLD is activated by oxygen free radicals and calcium, and inhibited by NO [24]. There is very little information on the mitochondrial damage during I/R injury and this study has clearly shown that concomitant with the lipid alterations in the mitochondria, functional alterations are also seen. Both the structural and functional alteration to intestinal mitochondria brought about during I/R is completely prevented by NO. Since, in this study, we observed an increase in PLD activity with 30-min ischemia it is probable that this is an early event in ischemic damage that is prevented by nitric oxide. To our knowledge, this is the first report showing mitochondrial PLD inhibition by nitric oxide under in vivo oxidative stress, namely I/R.

NO may bring about protection to the tissue through various mechanisms. Superoxide is known to be generated during I/R, and nitric oxide has the capacity to inhibit reactive oxygen metabolites, including superoxide anion by rapidly reacting with superoxide and abolishing its biological activity [45]. Nitric oxide can also suppress xanthine oxidase activity [59]. The antioxidant action of NO was also shown in vivo where the antioxidant capacity of plasma was doubled by addition of NO donors [60]. In our experiments, perfusion with NO did not cause any damage to mitochondrial structure and function and hence it is unlikely that peroxynitrite may have a role in this process. NO is also known to affect the activity of neutrophil NADPH oxidase which is responsible for generation of O2·− by infiltrated neutrophils, and NO may increase the blood flow and hence offer protection.

In conclusion, this study has shown that long duration of ischemia or ischemia followed by reperfusion potentially damages enterocyte mitochondrial structure and function. The data also strongly support the contention that nitric oxide plays a protective role in the intestine during periods of ischemia and I/R, and its decreased level may exacerbate tissue injury. This may be brought about by alterations in mitochondrial lipid composition through altered PLD activity and this enzyme may have a crucial role in enterocyte mitochondrial damage during I/R injury.

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