Increased phospholipase A\textsubscript{2} and decreased lysophospholipase activity in the small intestinal mucosa after ischaemia and revascularisation

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SUMMARY The influence of ischaemia and revascularisation on lipid peroxidation and phospholipid metabolism in the rat small intestinal mucosa was investigated. Two hours of total ischaemia followed by five minutes of revascularisation caused not only accumulation of malondialdehyde in the mucosa, but also increased activity of phospholipase A\textsubscript{2}, decreased activity of lysophospholipase, and increased ratio between lysophosphatidylcholine and phosphatidylcholine. Pretreatment with the phospholipase A\textsubscript{2} inhibitor, quinacrine, prevented the increases in mucosal phospholipase A\textsubscript{2} activity and lysophosphatidylcholine/phosphatidylcholine ratio after ischaemia and morphological examinations revealed that the mucosa was then also protected against ischaemic injury. These findings point to the possibility that activation of phospholipase A\textsubscript{2} and accumulation of lysophosphoglycercides could be involved in mediating the mucosal injury caused by small intestinal ischaemia.

The mechanisms underlying mucosal injury caused by small intestinal ischaemia have not been fully elucidated. Intraluminal proteolytic enzymes like pancreatic trypsin have been claimed to be of importance, although ischaemic mucosal damage occurs also in animals who have had their pancreatic ducts ligated five to six days before the ischaemia. It has also been suggested that the countercurrent exchange of oxygen in the intestinal villi could aggravate the mucosal hypoxia and initiate necrosis in the epithelial cells near the villous tips. More recently, oxygen-derived free radicals produced in the tissue during a postocclusive hyperaemia have been put forward. Such free radicals can increase lipid peroxidation and damage cellular membranes but a precise molecular mechanism by which they might injure the intestinal mucosa during ischaemia has not been substantiated.

We recently found that the intestinal permeability increase after ischaemia was potentiated by lysophosphatidylcholine (lysoPC), a potentially membrane damaging surfactant occurring naturally in the gut but also known to induce mucosal injury at high concentrations. The ischaemic intestine was thus affected by lysoPC concentrations that had no effect on the non-ischaemic intestine, and it was therefore suggested that increased amounts of lysoPC was being formed in the ischaemic gut mucosa, perhaps by the action of phospholipase A\textsubscript{2} on phosphatidylcholine (PC). This possibility would be consistent with the recent findings that lysoPC and other lysophosphoglycerides accumulate in the ischaemic heart and are important mediators of sequelae after myocardial ischaemia.

In order to further examine this possibility, we have now investigated the influence of ischaemia and revascularisation on phospholipase A\textsubscript{2} activity, lysophospholipase activity, and lysoPC/PC ratio in the small intestinal mucosa. In addition, we have examined the effect of a phospholipase A\textsubscript{2} inhibitor, quinacrine, on the accumulation of lysoPC and on the mucosal damage after ischaemia and revascularisation.

Methods

ANIMALS Female Sprague/Dawley rats (225–250 g body weight) were anaesthetised by an intraperitoneal injection of ketamine (Ketalar\textsuperscript{®}, Parke Davis Co Inc, USA, 50 mg/kg body weight) and xylazin.
Experimental Design

An experimental model described in detail previously was used to create ischaemia and revascularisation. A thin thread (5-0) was placed under the mesenteric vessels supporting the tied loop. The thread was heightened about 2 cm, which lifted the vessels up and caused the blood flow to the tied loop to stop. After two hours, the animal was killed and the tied loop excised. In another group of animals, the vessels were brought down again and the intestinal segment revascularised for five minutes before the animal was killed. In yet another group (the non-ischaemic controls), the vessels were left untreated for two hours and five minutes.

The blood flow in the tied loop before, during, and after the ischaemia was recorded using laser Doppler flowmetry. As described previously, lifting the vessels 2 cm caused total ischaemia in the bowel wall whereas bringing the vessels down again reestablished the blood flow to starting values.

To study the influence of a phospholipase A2 inhibitor, rats were given intravenous injections of quinacrine (10 mg/kg body weight) dissolved in 0.5 ml 150 mM NaCl. After 30 minutes, the animals were prepared as described above. Controls were given 0.5 ml 150 mM NaCl only. The animals were then subjected to two hours ischaemia and five minutes revascularisation as described above.

Sample Preparation

The excised segment was opened and the mucosa washed with cold saline and scraped off with a curette. Special precaution was taken to remove only the superficial layers of the mucosa. The mucosal cells were suspended in 150 mM NaCl, weighed, and disintegrated in a Dounce homogeniser by five strokes with a Teflon pestle. The homogenised cells were then analysed as described below.

Chemical Analysis

Malondialdehyde (MDA) was determined using the thiobarbituric acid (TBA) reaction. The reaction mixture contained 0.2 ml 8.1% sodium dodecyl sulphate (SDS), 1.5 ml 20% acetic acid pH 3.5, 1.5 ml 0.8% TBA, and 0.2 ml homogenised cells (2-3 mg protein). The mixture was made up to 4.0 ml with distilled water and heated at 95°C for 60 minutes. After cooling with tap water, 1 ml distilled water was added and the red pigment produced was extracted with 5.0 ml n-butanol:pyridine (15:1, v/v) and assayed fluorometrically. Results were expressed as nmol MDA/mg protein.

Phospholipase A2 (PLA2, EC 3.1.1.4) was determined as described previously. The reaction mixture contained, in a final volume of 450 ml radiolabelled E. coli (5000 cpm), 70 mM buffer of suitable pH (pH 9.8 glycine-NaOH, pH 7.2 Tris-maleate, and pH 5.5 citrate), 2 mM CaCl2 (this was included when assaying PLA2 at pH 9.8 and 7.2 but excluded at pH 5.5). 0.01% (wt/vol) bovine serum albumin (BSA), and mucosal cells corresponding to 2-8 μg protein. The mixture was incubated at 37°C and the reaction stopped by adding 3 ml 0.15 M NaCl with 1% BSA. The suspension was then passed through a 0.45 μm Millipore filter (Millipore SFHA 025 BO, Millipore AB, V Frölunda, Sweden) and the radioactivity in the filtrate was assayed and expressed as percentage of total radioactivity.

LysoPhospholipase (EC 3.1.1.5) was determined using lysoPC as substrate. The reaction mixture contained, in a total volume of 500 μl 1 mM lysoPC (Sigma type 1), 15,000 cpm radiolabelled lysoPC (1-α-cycl-13C-palmitoyl-sn-glycero-3-phosphorylcholine, 52.3 mCi/mmol; NEN Chemicals, GBH, Dreieich, Germany), 400 μl Tris-maleate, pH 7.3, and 0-20 μg homogenised mucosal cells. The mixture was incubated at 37°C and the reaction stopped by the addition of 1 ml cold 20% trichloro-acetic acid. To the reaction products were added 0.5 ml 16% Triton X-100 and 5 ml hexane. One millilitre of the top layer (the hexane phase) was then counted for radioactivity and the total radioactivity in the hexane phase expressed as percentage of total radioactivity.

LysoPhosphatidylcholine (lysoPC) and phosphatidylcholine (PC) were determined essentially as described by Chen and Kou. Lipids were extracted according to Folch et al., the extracts evaporated to dryness under vacuum, and the residues dissolved in 250 μl CHCl3:CH3OH (2:1, v/v). The extracted phospholipids were then subjected to high performance liquid chromatography (HPLC). The HPLC equipment was an LKB 2152 controller, a LKB 2150 pump, a Rheodyne 7125 injector with a 20 μl loop, a LDC spectromonitor III, and a Hewlett Packard 3393 A integrator. The column was an Apex Silica 5 μ (250×4.5 mm, Jones Chromatography Ltd, Colnbrook, UK) and the mobile phase CH3CN:CH3OH:H3PO4 (100:3:1). The column was equilibrated overnight with mobile phase (0.05 ml/min) before analysis. A flow program was used to speed up elution of the phospholipids and to narrow and point the chromatographic peaks; this program (0 min: 1 ml/min, 18

(Rompun®, Bayer Ag, Leverkusen, West Germany, 5 mg/kg body weight). Laparotomy was carried out and the most distal 10 cm of the ileum was ligated at both ends. In order to minimise evaporation from the tissue, the small intestine was covered with sterile gauze pads soaked with saline at 37°C. In addition, 5 ml Ringerdex solution was given subcutaneously to prevent dehydration of the animals.
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Min: 1.5 ml/min, 20 min: 2 ml/min, 30 min: 1 ml/min) was started at the time of sample injection. The phospholipids were monitored by UV detection at 203 nm and the peaks identified by comparison with known standards: phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin (P7769, P4513, P6013, L5379, and S7004 from Sigma Chemical Company, St Louis, Mo, USA). A typical chromatogram is depicted in Figure 1. The amounts of lysoPC and PC in the extracted samples were calculated by the integrator as Us, corresponding to area under curve. These data were then used to calculate the ratio between lysoPC and PC.

Protein was measured by the method of Lowry et al.20

Morphological Examinations
To study morphological changes after ischaemia and revascularisation, the animals were perfused via the heart;21 22 with 300 ml 2% glutaraldehyde in 0.1 M Na-cacodylate-HCl with 0.1 M sucrose and 2% polyvinylpyrrolidone (PVP, molecular weight 44000 dalton). The perfusion was conducted during 10 minutes at a constant hydrostatic pressure of 110 mm Hg. The pH of the fixture was 7.2, its temperature 37°C and its total osmolality about 560 mOsm (vehicle osmolality about 300 mOsm). Five 2 mm thick segments were sampled from each loop and immersed in the above fixative for another three days at 4°C. The specimens were then dehydrated, embedded in paraffin, cut, and stained with either haematoxylin and cosin, haematoxylin-van Gieson, or Alcian blue – PAS.

The morphological changes in the villous tips were quantitatively assessed by examining five intestinal circumferences per animal in sections stained with haematoxylin-van Gieson. Tips were classified into four categories according to their morphological appearance: (i) normal morphology with regular columnar epithelium, (ii) slight changes with slightly

**Fig. 1** Reversed phase HPLC separation of phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), lysophosphatidylcholine (lysoPC), and sphingomyelin (SM). One μg of each phospholipid was injected. Details concerning the chromatographic conditions are given in Methods. AUFS=absorbance units full scale.

**Fig. 2** Influence of ischaemia and revascularisation on MDA content in the small intestinal mucosa. The intestine was either left untreated for two hours ( ), or subjected to two hours ischaemia ( ) or two hours ischaemia with five minutes revascularisation ( ). Mean of four to five determinations, vertical bar indicates one SD. *** = p<0.001 v untreated.
increased desquamation of epithelium, (iii) moderate changes with loss of columnar characteristics, heaping up of cells and partial desquamation, karyolysis and karyorrhexis, and (iv) severe changes with patchy desquamation of epithelium leaving denuded areas of basal membrane. A mean of 36 villi (range 22–65) were examined in each circumferential section.

STATISTICAL ANALYSIS
Significance of differences were calculated using Student’s t test.

Results

MDA CONTENT
Figure 2 shows the accumulation of MDA in the intestinal mucosa after ischaemia for two hours and revascularisation for five minutes. The difference in MDA content between control rats and those rendered ischaemic for two hours without revascularisation was not statistically significant (p>0.05). On the other hand, ischaemia for two hours followed by revascularisation for five minutes caused a statistically significant increase in MDA accumulation (p<0.001).

PHOSPHOLIPASE A2 ACTIVITY
Figure 3 shows the effect of ischaemia and revascularisation on PLA2 activity at different pH values in the small intestinal mucosa. Ischaemia for two hours alone caused small increases over control values. On the other hand, ischaemia for two hours followed by revascularisation for five minutes caused more pronounced, statistically significant increases in the activity of PLA2 at different pH values (p<0.01).

LYSOPHOSPHOLIPASE ACTIVITY
The effect of two hours ischaemia on mucosal lysophospholipase activity is illustrated in Figure 4. It thus appeared that the lysophospholipase activity was lower in the mucosa subjected to ischaemia than in the untreated mucosa. As shown in Figure 5, the lysophospholipase activity was further decreased after revascularisation for five minutes (p<0.001 v control).

LYSOPC/PC RATIO
The lysoPC/PC ratio in the intestinal mucosa after different treatments is given in Table 1. Thus, the ratio in the mucosa subjected to ischaemia and revascularisation was significantly higher than the ratio in non-ischaemic control mucosa (p<0.001).

INFLUENCE OF QUINACRINE
Table 1 also shows the influence on quinacrine on the lysoPC/PC ratio after ischaemia and revascularisation. Apparently, the lysoPC/PC ratio in animals pretreated with quinacrine before the ischaemia was not significantly different from the lysoPC/PC...
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Fig. 5  Influence of ischaemia and revascularisation on lysophospholipase activity in the small intestinal mucosa. The intestine was either left untreated for two hours (□) or subjected to two hours ischaemia (■) or two hours ischaemia with five minutes revascularisation (■). The lysophospholipase activity in mucosal cells corresponding to 2 μg protein was then determined. Mean of four to five determinations, vertical bar indicates one SD. ** = p < 0.01 and *** = p < 0.001 v untreated.

ratio in non-ischaemic control animals (p > 0.05). Analogously, the phospholipase A₂ activity in mucosa from quinacrine – treated ischaemic animals was not different from that in non-ischaemic controls (p > 0.05, Fig. 6).

The influence of quinacrine on the morphological changes caused by ischaemia and revascularisation in the intestinal mucosa is shown in Table 2. In the mucosa not subjected to ischaemia, the surface

Table 1  Lysophosphatidylcholine/PC ratio in the intestinal mucosa after different treatments. Rats were pretreated with saline or quinacrine, and the intestine either subjected to two hours ischaemia (I) and five minutes revascularisation (R) or left untreated for that time (non-ischaemic control). Values are means ± standard errors of three to five rats in each group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lysophosphatidylcholine/PC</th>
<th>Significance of difference†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-ischaemic control</td>
<td>0.6±0.3</td>
<td></td>
</tr>
<tr>
<td>Saline + I + R</td>
<td>3.1±0.9</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Quinacrine + I + R</td>
<td>1.2±0.6</td>
<td>p &gt; 0.05</td>
</tr>
</tbody>
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*per cent; † versus non-ischaemic control.

Fig. 6  Phospholipase A₂ activity in the intestinal mucosa after different treatments. □ = non-ischaemic controls—that is, rats given saline and not subjected to ischaemia, ■ = ischaemic controls—that is, rats given saline and subjected to two hours ischaemia and five minutes revascularisation, ○ = rats given quinacrine and subjected to two hours ischaemia and five minutes revascularisation. The phospholipase A₂ activity was assayed at pH 7.2. Mean of four to five determinations, vertical bars indicate one SD.

Table 2  Influence of quinacrine on morphological changes in the small intestinal mucosa after two hours ischaemia (I) and five minutes revascularisation (R). The table gives the percentage of villous tips showing different degrees of morphological changes after various treatments. Slight changes mean a slight increase in the number of desquamating cells, moderate changes include loss of columnar characteristics, vacuolisation, and heaping up of epithelium as well as karyorrhexis, karyolysis, and partial desquamation, whereas severe changes represent total desquamation of epithelium leaving some areas of the basal membrane denuded. Values are means ± standard errors; number of animals are given within parenthesis. Five circumferences per animal were counted and a mean of 36 villi (range 22–63) were examined in each intestinal circumference.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>None</th>
<th>Slight</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-ischaemic control</td>
<td>44±5</td>
<td>56±5</td>
<td>1±1</td>
<td>1±1</td>
</tr>
<tr>
<td>Saline + I + R (6)</td>
<td>6±3</td>
<td>16±6</td>
<td>40±8</td>
<td>39±15</td>
</tr>
<tr>
<td>Quinacrine + I + R (6)</td>
<td>32±8</td>
<td>41±2</td>
<td>20±8*</td>
<td>8±2*</td>
</tr>
</tbody>
</table>

*p < 0.01 v saline + I + R (moderate plus severe)
epithelium was either columnar or slightly changed — that is, it showed a slight increase in the number of desquamated cells (Fig. 7a). Moderate epithelial changes with heaping up and vacuolisation of cells, karyorrhexis, and karyolysis (Fig. 7b) were rarely seen. On the other hand, two hours ischaemia and five minutes revascularisation caused regular changes of this moderate kind. This treatment also caused severe changes, including total desquamation of the epithelium, leaving the basal membrane denuded (Fig. 7c). Detachment of the epithelium of the villous tips without desquamation was observed occasionally, and oedema and extravasation of red blood cells in the lamina propria was seen in specimens showing the most severe epithelial damage. It appeared, however, that pretreatment with quinacrine reduced this epithelial damage. Indeed, when the degree of epithelial damage was quantitatively assessed (Table 2), it was found to be significantly reduced by pretreatment with quinacrine. Thus, quinacrine treatment resulted in significantly less villous tips with moderately and severely damaged epithelium (p<0.01) and consequently significantly more normal or only slightly changed tips.

**Discussion**

This investigation shows an accumulation of MDA, an increased activity of PLA₂, and a decreased activity of lysophospholipase in the small intestinal mucosa after ischaemia and revascularisation. The MDA accumulation was not unexpected, as ischaemia and, in particular, revascularisation after ischaemia, are known to initiate the formation of oxygen derived free radicals with increased lipid peroxidation and products thereof as a plausible consequence. Rather, the accumulation of MDA may be looked upon as a strong indication that under the experimental conditions used in the present investigation; free radicals were formed in the intestinal mucosa and were allowed to exert toxic effects on fatty acid residues in membrane lipids.

The reason why PLA₂ activity was increased is less clear. One possibility is that cellular influx of calcium was augmented, and that the ensuing increase in cytosolic calcium caused a PLA₂ activation. It has previously been shown that myocardial ischaemia results in increased cellular calcium content and it is well known that calcium activates membrane bound PLA₂. Alternatively, the treatment could have inactivated an endogenous PLA₂ inhibitor; such inhibitors have been found in a number of cells and tissues. Other possibilities include chemical conversion or configurational changes of the PLA₂ molecule so as to increase its catalytic activity, as well as release of latent enzyme from membranes or membrane-bounded organelles.

The mechanism by which lysophospholipase activity was decreased is also unclear. The enzyme has optimal activity at pH 7 and since ischaemia is accompanied by a decrease in cellular pH this could have caused a decrease in activity. On the other hand, lysophospholipase has been shown to be inhibited by lysoPC concentrations above 100 μM and it is therefore possible that the decreased activity was caused by increased lysoPC concentrations formed in the mucosa. This way, the decreased lysophospholipase activity could be connected to the increased PLA₂ activity via increased lysoPC formation. This possibility is consistent with the finding that the lysoPC/PC ratio was increased in the mucosa after ischaemia and revascularisation (Table 1).

A further connection between the MDA formation and the alterations in phospholipid metabolism is worth considering. It has previously been shown that increased phospholipase activity is detected during the peroxidic decomposition of mitochondrial and erythrocyte membrane lipids, and that the removal of peroxidation products originating in membranes is PLA₂-dependent. Sevanian et al. thus showed that PLA₂ may serve to eliminate membrane peroxides, which in turn decompose to yield MDA. Moreover, increased lysoPC formation was evident following lipid peroxidation in PLA₂-containing liposomes and was inhibited by PLA₂ inhibitors. Thus, lipid peroxidation induced PLA₂ activity, as indicated in the present study, agrees well with previous observations.

It can be assumed that this PLA₂ activation produces a membrane sparing effect by catalysing the elimination of peroxidised lipid that otherwise would facilitate the propagation of lipid peroxidation. At the same time, the action of PLA₂ could be the first step in a membrane repair process where the lysophosphoglycerides formed are utilised for synthesis of new membrane diacylphosphoglycerides. The decreased activity of lysophospholipase could then be one way by which lysophosphoglycerides are maintained and kept available for diacylphosphoglyceride synthesis rather than being degraded via the action of lysophospholipase. On the other hand, lysophosphoglycerides are potentially toxic molecules that can interact with and, at higher concentrations, damage cellular membranes.
lyosphosphoglycerides, if exceeding a critical level, might therefore contribute to the mucosal injury after small intestinal ischaemia. This would explain why low concentrations of lysoPC (1 mM) increased the permeability of the ischaemic intestine but had no influence on the normal.

The present investigation also showed that the intestinal mucosa can be protected against ischaemic injury by quinacrine, a phospholipase A2 inhibitor (Table 2). The ischaemic injury in our experimental system was primarily confined to the epithelium. Epithelial lifting at the villous tips or down the sides of the villi was seen only occasionally, though this has been described as a typical morphological finding during intestinal hypoperfusion or vascular obstruction. This might be explained by different degrees and durations of ischaemia, and lifting of the epithelium might well have appeared during earlier stages of the ischaemia. Notably, the morphological changes after ischaemia and revascularisation were similar to those obtained after exposure of the epithelium to lysoPC. It also appeared that quinacrine not only protected the mucosa against ischaemic damage but also prevented ischaemia induced increases in PLA2 activity (Fig. 6) and lyso PC/PC ratio (Table 1). This lends further support to the possibility that activation of phospholipase A2 and accumulation of lysophosphoglycerides could be involved in mediating the mucosal injury caused by small intestinal ischaemia. This possibility is now being further investigated, as is the possibility to use different phospholipase A2 inhibitors for protecting the intestinal mucosa against ischaemic damage.

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