Lipopolysaccharide induced apoptosis of rat pancreatic acinar cells


Abstract
Background—Bacterial lipopolysaccharide (LPS) has been proposed to participate in the pathogenesis of pancreatic inflammatory disease.

Aims—This study investigated the role of endotoxaemia in the pathogenesis of pancreatic acinar cell injury.

Methods—Sixty eight male Sprague-Dawley rats were used in the study. Escherichia coli LPS (5 mg/kg) was injected into the peritoneal cavity of the rats. The concentration of pancreatic phospholipase A₂ (PLA₂) in plasma was measured and pancreatic tissue examined by histology, in situ detection of free DNA 3'-ends, and electrophoretic DNA analysis.

Results—The concentration of pancreatic PLA₂ increased in plasma and the catalytic activity of PLA₂ increased in pancreatic tissue after an LPS injection. Apoptosis in pancreatic acinar cells and fragmentation of DNA typical of apoptosis in pancreatic tissue was seen 24 hours after an LPS injection. Pancreatic acinar atrophy was seen 72 hours after the LPS injection.

Conclusions—These data show that LPS causes release of pancreatic PLA₂ into blood plasma, activation of PLA₂ in pancreatic tissue, and apoptosis of acinar cells.

Keywords: apoptosis, acinar cell, lipopolysaccharide, pancreas, phospholipase A₂.

Bacterial lipopolysaccharide (LPS, endotoxin) is an important component of the cell wall of Gram negative bacteria. LPS has been implicated in symptoms of septic shock such as vasodilatation, hypovolaemia, decreased arterial blood pressure, hypoxia, thrombocytopenia, and aggregation of platelets. In animal studies, loss of body weight, pulmonary oedema, neutrophil aggregation, hypoxia, and neutrophil activation and release from the bone marrow to blood have been reported. Effects of LPS are possibly mediated by the tumour necrosis factor (TNF) and interleukins.

Endotoxaemia has been seen in patients with severe acute pancreatitis. Increased endotoxin concentrations in blood and infection of pancreatic tissue have been found after the induction of experimental acute pancreatitis by sodium taurocholate. In these studies, the severity of pancreatitis was reduced by cecocystotomy and irrigation of colon or by antibiotic treatment. Moreover, infection of pancreatic tissue has been seen in caerulein induced acute pancreatitis. These results implicate bacterial infection of pancreatic tissue as a consequence of pancreatic disease. The role of endotoxaemia, however, in the induction of pancreatic disease is poorly understood.

Phospholipase A₂ (PLA₂) is an enzyme that cleaves a free fatty acid from the sn-2 position of phospholipids. PLA₂ is pivotal in the initiation of arachidonic acid metabolism and formation of lysophosphatidyl choline, which is the precursor of the platelet activating factor. Pancreatic phospholipase A₂ (group I PLA₂) is a digestive enzyme that is synthesized and secreted by pancreatic acinar cells into the duodenal lumen. Increased concentrations of group I PLA₂ are commonly found in serum in pancreatic diseases such as acute and chronic pancreatitis and pancreatic cancer. In experimental studies, increased concentrations of group I PLA₂ have been found in serum in both sodium taurocholate and caerulein induced acute pancreatitis.

The aim of this study was to investigate the role of endotoxaemia in the pathogenesis of pancreatic acinar cell injury. LPS was injected into the peritoneal cavity of rats and the rate of release of pancreatic PLA₂ into the circulation was measured as a marker of acinar cell injury. In addition, changes in pancreatic histology and DNA structure were studied to detect apoptosis.

Methods

ANIMALS AND EXPERIMENTAL PROTOCOL
Male Sprague-Dawley rats weighing 250 (30) g (mean (SD)) were kept under standard laboratory conditions and fed a commercial chow. Plasma samples from 20 animals were obtained for establishing a normal range for group I PLA₂ concentration in the rat. Another group of 48 rats was fasted for 24 hours, and 5 mg/kg Escherichia coli LPS (W E coli O55:B5, Difco Laboratories, Detroit, MI, USA) were injected into the peritoneal cavity of 42 rats. Six control animals (time 0) received saline only and were killed at 24 hours. Five ml blood were obtained at three minutes, 15 minutes, one, three, seven, 24, and 72 hours by cardiac puncture into heparinised tubes (six animals in each group). Plasma was separated, diluted with sterile saline, and stored at -20°C until the determination of group I PLA₂ concentration. A 0-50 gram sample of pancreatic tissue was homogenised in 2·5 ml of sodium phosphate buffer (0·05 mM, pH 7·6) and
centrifuged at 10,000 g for one hour. The supernatant was stored at −20°C for PLA₂ activity measurement. A sample of the splenic lobe of the pancreas was fixed in 10% formalin and embedded in paraffin wax. Consecutive sections were stained with haematoxylin and eosin or labelled for in situ visualisation of free DNA 3'-ends. A sample of pancreatic tissue was placed in liquid nitrogen and stored at −70°C for electrophoretic DNA analysis.

BIOCHEMICAL AND MORPHOLOGICAL ANALYSIS

PLA₂ activity
The catalytic activity of PLA₂ in plasma and pancreatic homogenates was measured as described earlier, with slight modifications in the composition of the substrate. Briefly, unlabelled 1,2-dipalmitoylphosphatidylcholine (Sigma, St Louis, MO, USA) was mixed with 1-palmitoyl-2-[14C]-arachidonoylphosphatidylethanolamine (DuPont, Boston, MA, USA), in a ratio of 6 mM/325 μM (250 nCi), dissolved in a mixture of chloroform and methanol (2:1), dried under a flow of nitrogen, and redisolved in 10 ml 0·1 M glycine buffer (pH 8·1).

Protein content
The protein content of pancreatic homogenates was measured by the method of Lowry by using bovine serum albumin as a standard.

Time resolved fluoroimmunoassay (TR-FIA) for rat pancreatic PLA₂
PLA₂ was purified from rat pancreas and a polyclonal antibody was raised to this enzyme preparation in a rabbit as described earlier. Protein A purified anti-rat pancreatic PLA₂ antibody was labelled with an isothiocyanate derivative of a Europium chelate by using Eu-labelling kit (Wallac, Turku, Finland) according to the manufacturer’s instructions. TR-FIA was performed as described earlier. For comparison, the concentration of pancreatic PLA₂ in plasma was measured by an enzyme linked immunosorbent assay (ELISA) as described earlier.

DNA analysis
A 20 mg sample of pancreatic tissue was dispensed (Ultra-Turrax homogenisator, IKA, Staufen, Germany) in ice cold buffer consisting of 0·1 M NaCl, 0·01 M EDTA, 0·3 M TRIS-HCl (pH 8·0), and 0·2 M sucrose. DNA was extracted as described earlier. After isolation and spectrophotometric quantification, 500 ng of each sample was labelled at 3' -ends with [32P]dideoxy-ATP (ddATP, 3000 Ci/mmol, Amersham, Buckinghamshire, England) with terminal transferase (25 U/sample, Boehringer, Mannheim, Germany). Half of the labelled samples were separated in 2% agarose gels (6·5 V/cm) for 2·5 hours. The gel was dried without heating for two hours in a gel dryer, and exposed to Konica MG Super Rapid x ray film (Konica, Tokyo, Japan) at −70°C.

In situ detection of apoptotic cells
In situ detection of apoptotic cells in paraffin wax sections was performed as described earlier, with slight modifications. Briefly, deparaffinised sections were treated with 10 μg/ml of proteinase K (Boehringer, Mannheim, Germany) at 37°C in 2 mM CaCl₂ and 20 mM TRIS-HCl, pH 7·4, for 30 minutes. DNA 3' -end labelling was performed after 10 minutes incubation with terminal transferase buffer (Promega, Madison, WI, USA). The labelling mixture contained fresh terminal transferase buffer, 5 μM non-radioactive digoxigenin-dideoxy-UTP (dig-ddUTP, Boehringer, Mannheim, Germany), 45 μM ddATP (Pharmacia, Uppsala, Sweden), and 0·34 U/μl terminal transferase (Promega, Madison, WI, USA). The reaction was allowed to continue for one hour at 37°C in a humidified chamber. After washing, the slides were incubated with blocking buffer containing 2% (w/v) blocking reagent and 0·05% (w/v) sodium azide (Boehringer, Mannheim, Germany) for 30 minutes. Antidigoxigenin antibody, conjugated to alkaline phosphatase (1:3000, Boehringer, Mannheim, Germany), in 2% (w/v) blocking buffer was added and incubated for two hours in a humidified chamber. The slides were treated with alkaline phosphatase buffer (0·1 M NaCl, 0·05 M MgCl₂, and 0·1 M TRIS-HCl, pH 9·5) for 10

Figure 1: The concentration of pancreatic PLA₂ in rat plasma after LPS administration. Significant increase is seen three hours after LPS injection: *p<0·05, n=6 in the control group (time 0) and in each LPS treated group, values are mean (SEM).
Lipopolysaccharide induced apoptosis of rat pancreatic acinar cells

Figure 2: PLA2 activity in pancreas after LPS administration. Significant increase is seen one, three, seven, and 24 hours after LPS injection: *p<0.05, values are mean (SEM).

Results

Time resolved fluoroimmunoassay
The linear measuring range for rat pancreatic PLA2 by the TR-FIA was 1.5–324 μg/l. The coefficient of inter assay variation ranged from 1-66% to 4-11%. The sensitivity of the assay was determined to be 0.31 μg/l. This concentration corresponds to the mean fluorescence of zero standard (10 replicates) + 3 SD.

For the comparison of the new TR-FIA with the ELISA, both based on the use of the same polyclonal rabbit anti-rat pancreatic PLA2 antibody, plasma samples from 16 rats were measured by both methods. There was a good correlation between the TR-FIA and ELISA methods. Regression analysis yielded:

\[ y = 1.19x - 1.06, r = 0.97 \]

PLA2 in plasma and pancreas
In intact control rats, the concentration of pancreatic PLA2 in plasma was 60–0 (6-1) μg/l. The PLA2 concentration increased to 86–0 (6-8) μg/l at three hours after LPS administration (p=0.029), remained increased at seven hours, and decreased at 24 hours (Fig 1).

There was no correlation between PLA2 activity and the concentration of pancreatic PLA2 in plasma after LPS administration (\( y = 0.0213x + 64.4, r = 0.06 \)).

The PLA2 activity of pancreatic tissue increased at one hour after LPS administration and remained increased at 72 hours (Fig 2).

DNA analysis
Ladder-like fragmentation of pancreatic DNA typical of apoptosis was seen in pancreatic tissue homogenate 24 hours after LPS administration. DNA of animals treated with saline or seven or 72 hours after LPS administration were unfragmented (Fig 3).

Histological examination
No histological changes were seen during the first seven hours after LPS injection; only occasional apoptotic cells were seen in the pancreas (Figs 4a and b). Several apoptotic acinar cells with pale or eosinophilic cytoplasm and with or without fragmented nuclear material were seen 24 hours after LPS administration in the haematoxylin and eosin stained section in all animals examined (Fig 5a). Most of these cells were positive in the in situ detection of free 3'-ends of DNA performed in adjacent sections (Fig 5b). In control sections (0, 7, 24, or 72 hours after LPS injection), no colour reaction was noted when terminal transferase, dig-ddUTP or antibody was omitted from the reaction mixture.

Occasional apoptotic cells and autophagic vacuoles were seen in pancreatic acini three days after LPS administration. Moreover, the size of acini and the amount of cytoplasmic material in the apical zone of acini decreased because of atrophy of the exocrine pancreas (Fig 6). No inflammatory cell infiltration was seen in the pancreas during the experiment.
Figure 4: In situ labelling of free 3'-ends of DNA and histology of pancreas. (a) Rat pancreatic sample seven hours after LPS injection. Occasional apoptotic acinar cells (arrowheads) appear in the acini. Haematoxylin and eosin (×410). (b) Consecutive section of the same pancreatic sample as seen in (a). Strong positive immunostaining is seen in the cells undergoing apoptosis. Free DNA 3'-labelling with terminal transferase and antidigoxigenin antibody (×410).

Figure 5: (a) Rat pancreatic tissue 24 hours after LPS injection. There are several apoptotic cells in the acini (arrows). Some of the apoptotic cells show fragmented nuclear material and eosinophilic cytoplasm while some have lost their cellular structure completely (asterisks). Haematoxylin and eosin (×410). (b) In situ labelling of free 3'-ends of DNA in a consecutive section of the same pancreatic sample as seen in (a). There is a large number of apoptotic acinar cells showing DNA fragmentation as shown by positive immunoreaction in the affected cells. Intensive staining is also seen in some cells that do not contain fragmented nuclear material (asterisks, ×410).

A statistically significant increase in the number of acinar cells undergoing apoptotic DNA fragmentation was seen 24 hours after LPS injection (from 2.46 (0.16) in the saline treated controls to 53.6 (15.0), p<0.0001.

Figure 6: Rat pancreas 72 hours after LPS injection. The acini are atrophic, and there are occasional autophagic vacuoles (arrows). Haematoxylin and eosin (×410).

Figure 7: Time related changes in the number of apoptotic cells in the pancreas during LPS exposure. The number of apoptotic cells is considerably increased at 24 hours after LPS administration (**p<0.001). The values show the number of apoptotic cells per mm² of tissue section area, mean (SEM). Control level (time 0) shows the number of apoptotic acinar cells in rats treated with saline and killed at 24 hours.

Fig 7). The islet cells showed no apoptosis during the experiment.

Discussion

In this study, we show pronounced apoptosis of rat pancreatic acinar cells 24 hours after an intraperitoneal injection of LPS. Three days after the LPS injection, there was pronounced atrophy in the gland. Apoptotic fragmentation of DNA was confirmed by gel electrophoresis, which showed DNA fragments of approximately 200 base pairs. The finding that only acinar cells but not islet cells of the pancreas underwent apoptosis shows that LPS has a selective capability to induce cell injury in different cell types of the pancreas.

On one hand, some acinar cells with fragmented nuclear material and eosinophilic cytoplasm did not stain with DNA 3'-in situ hybridisation. On the other hand, there were a number of cells that had lost their cellular structure completely, and stained intensively. This finding shows that the condensation of nuclear material and DNA fragmentation are parallel phenomena, but DNA is fragmented later.

A number of agents, such as ethionine,23 triethylenetetramine tetrahydrochloride,24 1-cyano-2-hydroxy-3-butene,25 and zinc26 have been reported to induce apoptosis of pancreatic acinar cells. An interesting finding is that an intragastric infusion of ethanol together with long-term high fat diet induces apoptosis of pancreatic acinar cells and pancreatic atrophy in rats.27

The proposed mechanism of apoptosis is an initial milde plasma membrane damage with consequent influx of calcium ions28 and thereby the activation of endonucleases29 and proteases,30 which leads to apoptotic cell...
Lipopolysaccharide induced apoptosis of rat pancreatic acinar cells

dead. It has been assumed that, in endothelial cells, TNF induces both arachidonic acid release and apoptosis, but PL-A2 activity is not involved in the induction of apoptosis.31 On the contrary, activation of PL-A2 has been reported to trigger apoptosis in phototoxinsensitised mouse lymphoma cells.32

Endotoxaemia has been implicated as a prognostic factor in patients with acute hemorrhagic pancreatitis.5,6 However, the mechanisms by which bacterial endotoxins might take part in the initiation of pancreatic disease are largely unknown. Moreover, no evidence has been presented for the ability of LPS to induce apoptosis in pancreatic acinar cells. LPS prevents apoptosis of peripheral blood monocytes33 and delays apoptosis of neutrophils.34 On the contrary, apoptosis is induced by LPS in malignant CD5+ B-cells35 and in mouse thymus cells in vivo.36 A feasible pathway for the effects of LPS is the initial activation and release of TNF into circulation during the first phase of endotoxaemia, followed by activation of PL-A2.4 TNF is able to induce both PL-A2 activity41 and apoptosis.42 Baffy and coworkers proposed that PL-A2 activity, but not arachidonic acid release, participates in TNF mediated cytotoxicity.42 Thus, PL-A2 activation may exert cytotoxicity by non-specific hydrolysis of membrane phospholipids.

The average concentration of pancreatic PL-A2 in the serum of healthy humans is 5-8 μg/l,43 and, at the early stages of acute pancreatitis, the concentration increases up to sevenfold.12 In experimental sodium taurocholate-induced acute pancreatitis in rats, the increase of pancreatic PL-A2 in serum is 40-fold at 60 minutes after the induction.13 In our study, there was 60 μg/l of pancreatic PL-A2 in intact rat plasma and in the plasma of saline treated control animals. The concentration increased significantly at three hours after LPS administration. These results suggest that acinar cells release pancreatic PL-A2 into blood plasma during the early stages of endotoxaemia possibly caused by cell injury leading to apoptosis. The decreased concentration of pancreatic PL-A2 in plasma at 24 hours after LPS administration suggests that the capability of pancreatic acinar cells to release PL-A2 diminishes after apoptotic acinar cell death.

In this model of endotoxaemia, intraperitoneally injected LPS may reach pancreatic acinar cells both directly from the peritoneal cavity or after absorption via the bloodstream. After intravenous administration of LPS, apoptosis can be seen in pancreatic acinar cells at 24 hours, but the number of apoptotic cells seems to be smaller than after intraperitoneal LPS administration (unpublished data). However, the time related changes in the pancreas in both models seem to be comparable (unpublished data). An intraperitoneal injection of a high dose of LPS represents the situation in which high amounts of endotoxin have an effect on the pancreas both directly and via the bloodstream, as may be the case in clinical endotoxaemia resulting from abdominal diseases. Direct and indirect effects of LPS could not be distinguished in this study. Therefore, further experiments with intravenous injection of LPS and administration of LPS to isolated pancreatic acinar cell preparation are warranted.

In summary, experimental endotoxemia causes profound changes in rat exocrine pancreas. Pancreatic PL-A2 is released into blood plasma during the first few hours before the appearance of histologic changes in acinar cells. This finding supports the notion that an increased concentration of pancreatic PL-A2 in plasma is an early marker of injury of pancreatic acinar cells. Apoptosis of pancreatic acinar cells is a somewhat later phenomenon. Finally, at day three after LPS administration, pancreatic acinar atrophy appears throughout the gland. The activation of PL-A2 that takes place simultaneously with apoptosis in pancreatic tissue may have a role in triggering the development of apoptosis through cytokine activation.

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Lipopolysaccharide induced apoptosis of rat pancreatic acinar cells.

V J Laine, K M Nyman, H J Peuravuori, K Henriksen, M Parvinen and T J Nevalainen

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