Effect of Neonatal Endotoxemia on the Pancreas of Adult Rats


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Bacterial endotoxin (lipopolysaccharide, LPS), is the component of the cellular wall of Gram negative bacteria. Endotoxemia (sepsis) could produce multiorgan failure and could be particularly danger in the early period of life. The effects of endotoxemia induced in the neonatal period of life on the pancreatic secretory function and on pancreatic defense of adult organism have not been investigated yet. To induce endotoxemia suckling rats (30g) have been injected intraperitoneally with LPS from E. coli (5, 10 or 15 mg/kg-day) during 5 consecutive days. Three months later in these animals (300g) the studies on pancreatic secretion and acute pancreatitis were carried out. In the adult rats, which have been subjected in infancy to endotoxemia, basal pancreatic secretion was unaffected, whereas amylase secretions stimulated by caerulein or by diversion of pancreatic-biliary juice to the exterior were significantly, and dose-dependently reduced as compared to the untreated control. In the rats pretreated with LPS in the suckling period of life caerulein-induced amylase release from isolated pancreatic acini was significantly decreased, and dose-dependent reduction of mRNA signal for CCK1 receptor on pancreatic acini have been observed. Caerulein infusion (25 µg/kg) produced caerulein induced pancreatitis (AP) in all animals tested, that was confirmed by histological examination. In the rats, which have been subjected in the neonatal period of life to LPS (10 or 15 mg/kg-day x 5 days) all manifestations of AP have been reduced. In these animals acute inflammatory changes of pancreatic tissue have been significantly diminished. Pancreatic weight and plasma lipase activity, have been markedly decreased in these animals as compared to the control rats, subjected in the infancy to saline injection instead of LPS. Caerulein-induced fall in an antioxidantive enzyme; SOD concentration was reversed and accompanied by significant reduction of lipid peroxidation products; MDA + 4 HNE in the pancreatic tissue. Conclusions: 1/ neonatal endotoxemia reduces gene expression for CCK1 receptor and could produce impairment of the exocrine pancreatic function at adult age; 2/ Prolonged exposition of suckling rats to bacterial endotoxin attenuated acute
pancreatitis induced in these animals at adult age and this effect could be related to the increased concentration of antioxidative enzyme SOD in the pancreatic tissue.

**Key words:** lipopolysaccharide (endotoxin, LPS), neonatal endotoxemia, pancreatic enzyme secretion, acute pancreatitis

**INTRODUCTION**

Lipopolysaccharide (LPS, endotoxin) is the constituent of outer leaflets of Gram negative bacteria, such as; *E. coli* or *S typhi* (1). LPS molecule constitutes of three elements; lipid A, the core and an antigen O. Lipid A represents the toxic part of LPS, the core consists of sugars linked to lipid A and to the antigen O, which is highly immunogenic and composed of 20-40 units, each unit represents 3 sugars (2).

Under normal conditions endotoxins are incorporated to the bacterial outer membrane, and not toxic, because they are covered with a polysaccharide capsule. Following cell damage LPS are released into the environment to activate immune cells (2, 3). These cells, such as macrophages, monocytes and polymorphonuclear leukocytes, produce and secrete the inflammatory mediators; cytokines, reactive oxygen species (ROS), nitric oxide (NO), etc (4-7). High concentrations of LPS leads to the septic shock (8, 9). In acute pancreatitis endotoxins are responsible for the multiorgan damage (10, 11).

When LPS are released into the blood, lipid A binds to the specific protein - LPS binding protein (LBP) (12). This is an acute phase protein, produced in the liver, and also in the kidney, heart and lungs in response to interleukin 1 (IL-1) and to interleukin 6 (IL-6) (13, 14). The constitutive blood level of LPB is low, but it increases during infection (15). LBP transfers LPS molecule to the cell, and this complex LPS-LBP binds to the CD14 receptor (16). CD14 is a membrane receptor lacking of the transmembrane domain and thus devoid of intracellular activity (17). The additional molecule involved in LPS-induced signal transduction is toll-like receptor 4 (TLR4), which initiates the signal pathway leading finally to the activation of NF-κB (18-20).

TLR4 receptors have been detected in the pancreas. They have been located in the ductal epithelium, vascular endothelium and in the islets (21, 22). Our recent study have shown that, TLR4 receptors are present on the normal pancreatic acinar cells isolated from the rat. Exposition of the animals to LPS resulted in the significant and dose-dependent increase of TLR4 on the pancreatic acinar cells.

Previous observations have revealed that chronic exposition of the rats to LPS (4 or 10 µg/kg during 5 days) resulted in mild pancreatic necrosis and in the reduction of pancreatic protein secretion (23, 24).
Acute pancreatitis is a serious disease of still unclear pathogenesis. Severe acute pancreatitis, which constitutes of 20-30% of cases, could be fatal (25). The mortality in acute pancreatitis results from the complications such as endotoxemia (sepsis), infected pancreatic necrosis and multiple organ damage (26-28). Endotoxemia in acute pancreatitis appears in the early phase of severe form of this disease (29). It has been reported recently, that the injury of intestinal mucosa and the increase of mucosal cells apoptosis leads to the increase of intestinal permeability (30). Translocation of Gram minus bacteria to the circulation and high concentration of LPS resulted in the systemic endotoxemia (31). Inhibition of caspases prevented the dysfunction of intestinal mucosa and attenuates endotoxemia (32).

Experimental studies in the rats have shown that pretreatment of these animals with low concentrations of LPS reduced tissue damage in acute pancreatitis and decreased pro-inflammatory cytokine production (33-35). However it is not known if endotoxemia, which take place in the early period of life, could affect pancreatic resistance to acute pancreatitis of the adult organism.

Endotoxemia is particularly danger for the young organisms, because it could produce tissue damage and sepsis with high mortality (36). It has been shown that endotoxemia, which take place in the early period of life often resulted in the

**Experimental protocol**

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<th>LPS (E.coli or S.typhi):</th>
<th>5, 10 or 15 mg/kg-day x 5 days</th>
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*Fig. 1.* The experimental protocol of the study on the effects of endotoxemia, induced in the suckling period of life, on the pancreatic secretory function and on pancreatic defense against acute pancreatitis in the rats.
decreased protein synthesis (37). In newborn rats LPS administration enhanced brain damage (38, 39). It is a question if endotoxemia in neonates could affect pancreatic function in adults? To answer this question we have investigated the influence of neonatal endotoxemia on the pancreatic enzyme secretion and on pancreatic defense of adult organism.

MATERIAL AND METHODS

The experimental protocol has been approved by the Jagiellonian University Ethical Committee for Animals Experimentation.

In our study we have employed the suckling rats (2 weeks old, weighing 30 - 40 g). Rat pups have been divided into two main groups (I and II). Rats from group I were injected with 0.5 ml of vehicle saline intraperitoneal (i.p), once a day, during 5 consecutive days (control group). Rats from group II were injected with LPS from *E. coli* during 5 consecutive days. LPS has been administered at various doses: 5, 10 or 15 mg/kg-day (Fig. 1). Each group of rats received separate dose of LPS, so rats obtained total doses of 25, 50 or 75 mg/kg. Three months later the same rats, as adults, have been used for the studies on pancreatic exocrine secretory functions and on the experiments on acute pancreatitis.

![Pancreatic amylase outputs in response to caerulein in the control adult rats and in the adult animals, which have been subjected to exposition to LPS from *E. coli* (25, 50 or 75 mg/kg) in the suckling period of life. Basal = unstimulated amylase outputs. Results are means ± SEM from 4 separate experiments, each performed on 8-10 rats. Asterisk indicates significant (p<0.05) decrease below the value obtained from control rats, without previous pretreatment with LPS.](image-url)
RESULTS AND DISCUSSION

Effect of neonatal endotoxemia on the pancreatic enzyme secretion in adults

The effect of neonatal endotoxemia on the pancreatic exocrine secretion in the adult organism has not been previously investigated. In the study on the mature rats Vaccaro et al. have demonstrated that 7-days treatment of these rats with LPS resulted in the subsequent decrease of pancreatic secretory function (40). Above effect has been related to the inflammation of the pancreas produced by LPS, because prolonged administration of high doses of endotoxin to the rats resulted in the acute inflammatory changes in the pancreatic tissue in these animals (23, 40).

It has been reported that LPS is able to affect directly pancreatic cells, to induce apoptosis in AR42J cells exposed to endotoxin and to increase mRNA signal for pancreatitis associated protein (PAP), and for pro-inflammatory cytokines in these (24). Chronic treatment of the adult rats with LPS resulted in the pancreatic tissue damage (41).

Fig. 3. Pancreatic amylase outputs evoked by diversion of pancreatic juice to the exterior (DPJ) in the control adult rats and in the adult animals, which have been subjected to exposition to LPS from E. coli (25, 50 or 75 mg/kg) in the suckling period of life. Basal = unstimulated amylase outputs. Results are means ± SEM from 4 separate experiments, each performed on 8-10 rats. Asterisk indicates significant (p<0.05) decrease below the value obtained from control rats without previous pretreatment with LPS.
In our study, histological assessment of pancreatic tissue taken from adult rats, which have been subjected to endotoxin pretreatment in the suckling period of life, have not revealed any manifestations of pancreatic inflammation. In our study on the adult rats, which have been subjected to LPS in the early period of life, amylase blood level was not significantly different from the enzyme blood concentration observed in the control rats, not pretreated with LPS. Also in the adult rats, pretreated in infancy with LPS, unstimulated (basal) secretion of amylase was not affected by neonatal endotoxemia (Fig. 2). However, enzyme secretion induced by caerulein (1 µg/kg) was significantly diminished by pretreatment of the rats with LPS at total doses of 50 or 75 mg/kg in the suckling period of life (Fig. 2). The lowest response to caerulein was observed in the rats, which have been pretreated with the highest used dose of LPS (75 mg/kg). Also enzyme secretion stimulated by diversion of pancreatic juice to the exterior (DPJ) was reduced in the animals, which have been subjected to neonatal endotoxemia, comparing to the untreated controls (Fig. 3).

Fig. 4. Amylase release from isolated pancreatic acini in response to caerulein (10⁻¹⁰ M). The acini were isolated from the pancreata of rats pretreated in the suckling period of life with LPS from *E. coli* (50 or 75 mg/kg). Control - animals pretreated with 0.9% NaCl instead of LPS. Results are means ± SEM from 3 separate experiments, each performed in duplicate. Asterisk indicates significant (P<0.05) decrease below the value obtained from control rats without previous pretreatment with LPS.
Fig. 5. mRNA signal for CCK1 receptor measured by RT-PCR in the pancreatic acini isolated from the control rats (lane 1) or from the animals pretreated in the suckling period of life with LPS from \textit{E. coli} at dose of 75 mg/kg (lane 2), at dose of 50 mg/kg (lane 3) or at dose of 25 mg/kg (lane 4). Control - animals pretreated with 0.9% NaCl instead of LPS.

Fig. 6. Histological picture of the pancreatic tissue taken from the adult rats, with caerulein-induced pancreatitis, which have been subjected in the suckling period of life to i.p injections of physiological saline (control), or treated with LPS from \textit{E.coli} at total dose of 75 mg/kg.
Fig. 7. Plasma lipase activity measured in the adult rats with caerulein-induced pancreatitis, which have been pretreated with LPS from *E. coli* (25, 50 or 75 mg/kg) in the suckling period of life. Results are means ± SEM from 6 separate experiments, each performed on 8-10 rats. Asterisk indicates significant (P<0.05) decrease below the value obtained from rats subjected to caerulein-induced pancreatitis without previous pretreatment with LPS.

Fig. 8. Plasma interleukin 1β (IL-1β) concentration in the adult rats with caerulein-induced pancreatitis, which have been pretreated with various doses of LPS from *E. coli* (25, 50 or 75 mg/kg) in the suckling period of life. Results are means ± SEM from 6 separate experiments, each performed on 8-10 rats. Asterisk indicates significant (P<0.05) decrease below the value obtained from rats subjected to caerulein-induced pancreatitis without previous pretreatment with LPS.
For the *in vitro* experiments, pancreatic acini, obtained from rats pretreated in the suckling period of life with LPS have been used. These acini showed decreased responsiveness to caerulein. Amylase release from pancreatic acini isolated from the rats subjected to LPS (at total dose of 50 or 75 mg/kg) was significantly lower than those observed in the control acini originating from untreated with endotoxin young rats (*Fig. 4*).

What about the CCK receptors? We have observed that the signal for CCK1 was significantly and dose dependently reduced in the pancreatic acini obtained from rats injected with endotoxin in the early period of life (*Fig 5*). It is very likely that this reduction of signal for CCK1 receptor could be responsible, at least in part, for the some kind of down-regulation of this receptor and decreased secretory response of pancreatic acini to caerulein.

Our observations could be summarized as follows: exposition of suckling rats to LPS resulted in the impairment of pancreatic enzyme secretion in the adults. This effect could be related, at least in part, to the changes of CCK1 receptor on pancreatic acini.

**Effect of neonatal endotoxemia on the course of acute experimental pancreatitis in adults**

In this part of the study rat pups were injected with various doses of LPS during 5 consecutive days, in the same way as in the secretory studies. After 3 days...
Fig. 10. The amount of lipid peroxidation products; malondialdehyde and 4-hydroxynonenal (MDA+4 HNE) in the pancreatic tissue taken from adult rats with caerulein-induced pancreatitis, which have been pretreated with various doses of LPS from *E. coli* (25, 50 or 75 mg/kg) in the suckling period of life. Results are means ± SEM from 6 separate experiments, each performed on 8-10 rats. Asterisk indicates significant (P<0.05) decrease below the value obtained from rats subjected to caerulein-induced pancreatitis, without previous pretreatment with LPS.

Fig. 11. Concentration of superoxide dismutase (SOD) in the pancreatic tissue taken from rats with caerulein-induced pancreatitis pretreated with from LPS *E. coli* (25, 50 or 75 mg/kg) in the suckling period of life. Results are means ± SEM from 6 separate experiments, each performed on 8-10 rats. Asterisk indicates significant (P<0.05) decrease below the value obtained from rats subjected to caerulein-induced pancreatitis, without previous pretreatment with LPS.
months animals were subjected to caerulein-induced pancreatitis (AP). In the groups of rats which have been treated, in the early period of life, with LPS at total doses of 50 or 75 mg/kg pancreatic inflammatory changes were significantly less pronounced, as was shown by macroscopic observation supported by histological assessment (Fig. 6). Lipase blood level is the indicator of the severity of acute pancreatitis. In these groups of rats plasma lipase levels, were significantly diminished, as compared with that measured in the control rats with acute pancreatitis untreated with LPS in neonatal period of life (Fig. 7).

Attenuation of acute pancreatitis in the rats pretreated in the suckling period of life with LPS at total doses of 50 or 75 mg/kg was expressed by significant decrease of an pro-inflammatory IL-1β plasma concentration, as compared to the control rats with acute pancreatitis without LPS pretreatment (Fig. 8). In spite of above reduction of pro-inflammatory IL-1β, exposition of the infant rats to LPS significantly increased production of an anti-inflammatory IL-10 in the course of acute pancreatitis (Fig. 9).

Activation of immune response and production of cytokines is essential for the intensity of pancreatic necrosis and systemic complications in acute pancreatitis (42-45). Aggravation of inflammatory process is often associated with increased production of pro-inflammatory interleukins such as tumor necrosis factor α (TNF α), interleukin 1 β (IL-1β), interleukin 6 (IL-6), or interleukin 8 (IL-8) (23, 97).
Anti-inflammatory cytokines, such as interleukin 4 (IL-4), or interleukin 10 (IL-10) alleviated the severity of this disease (42, 50).

In our study it was observed that exposition of the rats to endotoxin in the infancy, reduced concentration of pro-inflammatory IL-1β plasma concentration together with increased blood level of an anti-inflammatory IL-10 in these animals subjected to acute pancreatitis at adult age. This indicates that neonatal endotoxemia affects the ability of the immune cells to produce the cytokines and increases the resistance of the organism to pancreatic inflammation.

Acute pancreatitis is characterized by the dramatic increase of reactive oxygen species (ROS) in the pancreatic tissue (52). ROS are the toxic compounds responsible for membrane cell damage (53). These substances are critically implicated in the development of septic shock and pancreatitis-associated multiple organ dysfunction syndrome (MODS) (54-57). In acute pancreatitis high amounts of ROS are generated in the neutrophils infiltrating the pancreas and in the pancreatic acinar cells (58). The level of lipid peroxidation products; malondialdehyde and 4-hydroxynonenal (MDA + 4 HNE) is commonly used as an indicator of ROS formation in acute pancreatitis (59). Pancreatic inflammation caused sudden increase of MDA + 4 HNE (42-44). Above rise of lipid peroxidation products was significantly lower in the pancreatic tissue taken from the rats subjected in the suckling period to endotoxemia, than in the rats with acute pancreatitis without LPS pretreatment (Fig. 10). This indicates that in the animals pretreated with endotoxins formation of ROS was reduced.

Under normal conditions nonenzymatic or enzymatic antioxidants (such as superoxide dysmutase; SOD) protect the tissue against the noxious effects of ROS (58). Impairment of the scavenging system and increased production of ROS promoted leukocyte activation, cytokine production, and caused dysfunction of pancreatic microcirculation (54-57). Concentration of SOD, an antioxidative enzyme, in pancreatic tissue shows negative correlation with lipid peroxidation products in the gland of rats with acute pancreatitis (42, 60-62).

Looking for the mechanism of LPS-induced protection of the pancreas the level of SOD, in the pancreatic tissue have been measured. It was observed in our study, that pancreatic SOD increased in the rats with AP, which have been treated in the early period of life with LPS (at total doses of 50 or 75 mg/kg), comparing to the untreated control (Fig. 11). This is consistent with the observation that pancreatic level of lipid peroxidation products; such as MDA + 4 HNO, was markedly diminished in the these rats (Fig. 10).

Among the other defense mechanisms activated by neonatal endotoxemia the increased production of heat shock protein (HSP) in the pancreatic acini could be taken into consideration. HSP has been reported to protect the pancreatic tissue against the damage and to limit the severity of acute pancreatitis (63). Several mechanisms, which have been shown to protect the pancreas against the damage act via activation of HSP's (64, 65). We have observed that in the pancreatic acini
obtained from the rats pretreated in the early period of life with LPS the level of HSP60 protein was significantly increased (66) (Fig. 12).

CONCLUSIONS

Above results of the study on the effects of neonatal endotoxemia on the pancreas of adult rats could be summarized as follows:

- Endotoxemia in the early period of life resulted in the impairment of pancreatic enzyme secretion.
- Endotoxemia in the neonatal period of life improved pancreatic defense against inflammation though the activation of SOD, stimulation of HSP60 and modulation of cytokine production.

Conflict of interest statement: None declared.

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