ORIGINAL ARTICLE

The protective effects of Lipoxin A4 during the early phase of severe acute pancreatitis in rats

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Abstract

Objective. Our aim was to investigate the protective effects of a Lipoxin A4 analogue (LXA4) in the early phase of acute pancreatitis in rats. Materials and methods. Severe acute pancreatitis (SAP) was induced by injection of 5% sodium taurocholate into the pancreatic duct. Rats with SAP were treated with LXA4 (0.1 mg/kg), 10 min after the 5% sodium taurocholate injection, after which LXA4 was administrated every 8 hours, three times (LXA4 group). The sham group was only given the vehicle after operation. Plasma amylase activity, serum levels of interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α) were measured at 4, 12, and 24 h after induction of SAP. The pancreatic index and histopathologic observations were evaluated and the expression of intercellular adhesion molecule-1 (ICAM-1) and NF-κB p65 in the pancreas, and the expression of ICAM-1 in the lungs were detected by immunohistochemistry. Results. LXA4 treated rats had lower serum levels of TNF-α, IL-1, and IL-6 at all time points measured (p < 0.05), but significantly differed in plasma amylase activity only at 24 h as compared with the SAP group. The pancreatic index and the scores of pancreatitic histopathologic evaluations were lower in the LXA4 group as compared to the SAP group. Immunohistochemistry showed that LXA4 attenuated the expression of ICAM-1 in the lungs as well as in the pancreas (p < 0.05). Conclusions. We demonstrate that LXA4 has protective effects in experimental SAP, which may be achieved by inhibiting the NF-κB signalling pathway, thereby reducing the production of proinflammatory cytokines.

Key Words: Acute pancreatitis, lipoxins A4, LXA4, systemic inflammatory response syndrome, SIRS

Introduction

Since the excessive leukocyte activation theory was proposed [1], the importance of inflammatory cells and the inflammatory cascades in severe acute pancreatitis (SAP) has been emphasized. The main adverse link during the early phase of SAP is the systemic inflammatory response syndrome (SIRS), and this is the main driving factor for the development of the acute respiratory distress syndrome (ARDS), the dominant component of multiple organ dysfunction syndrome (MODS) and the main cause of death [2]. A systemic inflammatory response syndrome can increase both endothelial and epithelial barrier permeability [3], resulting in compromised oxygenation and lung gas exchange. About three-fifths of all deaths in acute pancreatitis in the first week has been reported to be associated with acute lung injury [4]. Therefore, one of the strategies when treating early SAP is to focus on minimizing or avoiding excessive

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SIRS. Most clinical trials targeting the blockade of specific inflammatory mediators have not been successful [5,6]. The major methods of potential clinical intervention suggested have been antioxidants, protein C inhibitors, and hemofiltration [7,8,9].

Lipoxins, produced during the late phase of inflammation, represent powerful anti-inflammatory lipids [10]. In similarity with prostaglandins and leukotrienes, lipoxins are important arachidonic acid metabolites which play a crucial role in the progress of inflammation. However, lipoxins have different biological effects as compared to leukotrienes [11]. LXA₄ represents a key member among lipoxins [12], and in the late phase of inflammation it prevents further inflammatory response by influencing neutrophil apoptosis [13], inhibiting neutrophil chemotaxis and respiration [14], and stimulating the uptake and clearance of apoptotic polymorphonuclear neutrophils [15].

LXA₄ can reduce the excretion of proinflammatory cytokines and modulate excessive neutrophil stimulation, factors also involved in the development of both local and systemic complications of SAP. Therefore, our hypothesis was that LXA₄ analogues may have anti-inflammatory effects during the early phase of SAP, though LXA₄ itself may play a role not at least during later phases of inflammation. The aim of the study was thus to investigate whether an LXA₄ analogue could attenuate the severity of acute pancreatitis, the systemic inflammatory response, and the associated lung injury in experimental acute pancreatitis in the rat.

Materials and methods

Materials

The following materials were purchased from the sources as follows: sodium taurocholate (Sigma Chemical Company, St Louis, MO, USA); pentobarbital (Abbott Laboratories, North Chicago, IL, USA); 5(S),6(R)-lipoxin A4 methyl ester (Cayman Chemical Company, Ann Arbor, MI, USA); rat ELISA kit for detecting interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α) (Rapidbio Company, USA); NF-κB-P65 antibody, intercellular adhesion molecule-1 (ICAM-1) antibody and immunohistochemical test kit (Santa Cruz Biotechnology, Inc., USA); and serum amylase activity test kit (Nanjing Jian Cheng Biotechnology Research Institute, Nanjing, China).

Animals

A total of 72 healthy male Sprague-Dawley (SD) rats (weighing 190 ± 20 g, 6–8 weeks old) were supplied by the Laboratory Animal Center of Wenzhou Medical College (Wenzhou, China). The rats were maintained under specific pathogen free (SPF) conditions. The temperature was kept at 20–22°C, a 12 h light-dark cycle was maintained, and all rats were fed with a standard rat chow and water except for a day of fasting before the operation. The protocol for the animal experiment was approved by the Institutional Animal Committee of Wenzhou Medical College. All animals received care in accordance with ‘Guide for the Care and Use of Laboratory Animals’.

Experimental design

Animals were randomly divided into three experimental groups: the SAP group that consisted of 24 rats with induction of SAP by 5% sodium taurocholate injection into the pancreatic duct and the administration of an equal amount of physiological saline via the dorsal penile vein; the LXA₄ group containing 24 rats treated with LXA₄ (0.1 mg/kg) via the dorsal penile vein, administered 10 min after the 5% sodium taurocholate injection, after which LXA₄ was administered every 8 hours, three times; and the sham group of 24 rats subjected to a surgical procedure without induction of SAP and administration of an equal amount of physiological saline via the dorsal penile vein.

Induction of severe acute pancreatitis

The rat model of SAP was established according to Aho et al [16]. Briefly, the rats were operated under aseptic condition, using 3% sodium pentobarbital (0.2 ml/100 g) anesthesia. The pancreas was exteriorized through a midline abdominal incision, the proximal bile duct was clamped at the level of the distal bile duct, and the distal pancreatic duct was cannulated using a 19G polyethylene catheter through the duodenal wall. SAP was induced by intraductal injection of 5% sodium taurocholate (0.1 ml/100 g) through a microinjection pump at a speed of 0.1 ml/min. In the sham group, the sodium taurocholate injection was omitted, but the surgical procedure was identical to the other groups, including bile duct cannulation.

Administration of LXA₄ and saline

Administration of 0.1 mg/kg of LXA₄ via the dorsal penile vein was performed 10 min after the induction of SAP and the same dose was repeated every 8 hours, three times. Administration of an equal volume of
physiological saline via the dorsal penile vein was performed after the induction of SAP and this was repeated every 8 hours in both the SAP and sham groups. The administration of LXA₄ and saline was carried out under aseptic conditions.

**Specimen collection**

Eight rats from each group were randomly submitted to a midline laparotomy incision under anesthesia after 4, 12, and 24 h following the induction of SAP. Blood, as well as pancreatic and pulmonary tissue, was collected at these time points for further analysis.

**Amylase and cytokines estimation**

Plasma amylase activity was determined by means of iodine-amylum colorimetry and expressed in units per deciliter. Plasma levels of IL-1, IL-6, and TNF-α were determined by means of ELISA using kits provided by the manufacturing company and expressed in picograms per milliliter.

**Histopathologic analysis**

The pancreatic index refers to the percentage of the pancreatic wet weight as compared to the body weight [17]. This ratio (pancreatic weight g/body weight g × 1,000) was utilized to evaluate the degree of pancreatic edema. Specimens of pancreas and lungs were harvested and fixed in 10% formaldehyde solution, embedded in paraffin, sectioned, and stained immuno-histochemically. For immunohistochemistry, sections were incubated with anti-ICAM-1 antibody (the primary antibody, dilution 1:50; Santa Cruz Biotechnology, INC. USA) overnight at 4°C. Sections were then washed and incubated in horseradish peroxidase-conjugated secondary antibodies for 30 min at room temperature. Finally, the slides were treated with chromogen 3, 3-diaminobenzidine for 10 min, rinsed, counterstained with hematoxylin, dehydrated in Histoclear, and coverslipped. Negative controls were obtained by replacing the primary antibody with PBS. All incubations and washings were performed with PBS (3 × 5 min) and carried out at room temperature. The sections were then examined under a light microscope. The result was obtained by the SPOT and IPP image acquisition systems (the pictures were 40 × 10 field of view) and processed by professional image analysis software (Image-Pro Plus 6.0 version). The relative intensity of ICAM-1 was reflected by integrated optical density (IOD) [20].

The detection of NF-κB P65 in pancreatic tissue

NF-κB P65 was detected by immunohistochemical staining and the process was the same as ICAM-1 immunostain. Cytoplasm or nuclear positive staining was identified according to the location of brown dyeing. Ultimately, the positive rate was calculated.

**Statistical analysis**

The data were analyzed by SPSS software (11.5 version). Statistical significance of differences among multiple groups was determined by one-way ANOVA (when the variance was even) or rank-sum test (when the variance was uneven). Multiple comparisons were determined by SNK-q test (when the variance was even) or Nemenyi test (when the variance was uneven). For enumeration of data, statistical significance for differences was determined by chi-square test. For all analyses, statistical significance was defined as p < 0.05.

**Results**

Histopathologic examination of the pancreas and plasma amylase activity

The induction of SAP was successful in all animals. Two rats in the SAP group and one in the LXA₄ group...
Serum levels of proinflammatory cytokines

Serum IL-1 and -6 reached the highest levels at 12 h after induction of SAP (compared with levels at 4 and 24 h). Serum TNF-α levels were highest at 4 h and then declined in the SAP group. The LXA₄ group had the same pattern of changes in proinflammatory cytokines as the SAP group, but levels of IL-1, IL-6, and TNF-α were significantly lower as compared with the SAP group (p < 0.05; Figure 1).

Pancreatic index and immunohistochemical staining

The pancreatic index is described in Table I. In the LXA₄ group, the pancreatic weight/body weight ratio significantly decreased as compared with the SAP group (p < 0.01). Immunohistochemical staining demonstrated that no ICAM-1 and NK-κB P65 positive dyeing on the surface of pancreatic vascular endothelial cells and acinar cells could be detected in the sham group. Pancreatic vascular endothelial cells were positively staining brown during the whole study period (from 4 to 24 h in the SAP and LXA₄ groups). Semi-quantitative analyses were performed by IOD evaluation of ICAM-1 positive cells. In the SAP group, the IOD value of ICAM-1 positive cells gradually increased, and reached the highest levels at 24 h. The IOD results indicate that the LXA₄ group had a decreased ICAM-1 expression as compared to the SAP group at every time point studied (p < 0.01; Figure 2).

In the pancreas, 81.8% (18/22) of the SAP cases had NK-κB P65 positive cells, while this was only found in 56.5% (13/23) in the LXA₄ group (p > 0.05). In both the SAP and LXA₄ groups, the highest positive ratio of NF-κB P65 staining was at 24 h, but the LXA₄ group had a lower ratio of NF-κB P65 nuclear positive staining cells as compared to the SAP group (59.1 vs. 4.3%; p < 0.01; Table II).

Pulmonary injury assessment

The lung injury was evaluated by the method described by Su et al [19]. In the sham group, the alveolar structure was clear, without alveolar isolation, outstretched mesenchymal blood vessels, and infiltration of neutrophils. In the SAP and LXA₄ groups, at 4 h, partial alveolar walls collapsed; mesenchymal telangiectasis, hyperemia, and infiltration of neutrophils were observed. At 12 h, there were distinct mesenchymal telangiectasis and hyperemia, infiltration of neutrophils, leakage of red blood cells into the alveolar cavity, and at 24 h, more serious histopathologic changes appeared, including aggravating hemorrhage and pneumonedema (Figure 3). According to the assessment of lung injury, the LXA₄ group had alleviated histopathologic changes and lower scores as compared to the SAP group (Table III).

The expression of ICAM-1 in the lungs in the sham group was negative. In the SAP and LXA₄ groups,
expression of ICAM-1 was observed at all three time points evaluated, but only at 24 h, the IOD of the LXA4 group was significantly lower as compared to the SAP group (Figure 2; $p < 0.05$).

**Discussion**

The effect of lipoxin treatment in SAP has never been studied previously. For the first time we report the use of LXA4 in order to modulate the inflammatory response in experimental acute pancreatitis induced by 5% sodium taurocholate. In the pancreatitis model, LXA4 ameliorated the pathological changes in both the pancreas and the lungs, including the expression of ICAM-1 and the NF-κB P65 activation rate in pancreatic acinar cells, and also the pancreatic index was attenuated by the LXA4. Furthermore, a systemic protective effect provided by LXA4 was noted, evidenced by a decline in levels of serum proinflammatory cytokines and a decrease in lung pathology evaluation scores.

Acute pancreatitis is characterized by a severe inflammatory process of the pancreas and may frequently be associated with a systemic inflammatory response. About 10–20% of acute pancreatitis cases has been classified as severe, still associated with a high mortality rate [21]. In our study, the explosive
increase of serum levels of proinflammatory cytokines and the run-up plasma amylase activity, together with pathological features demonstrating the development of SAP and SIRS have been shown [22]. During the 24 h observation period in this setting, to be defined as a comparably early phase [23], 3 rats (3/48) died. Early mortality has mainly been attributed to SIRS and organ dysfunction [2]. Inflammatory cells and mediators in SIRS play a significant role in the progress of the early phase of SAP and cause systemic complications such as acute lung injury [24]. A novel treatment strategy should focus on anti-inflammatory interventions in order to improve outcome, at least during the early phase of the disease. In the present study, the stable LX analogue LXA4 proved to be beneficial in experimental acute pancreatitis, modulating SIRS and MODS.

LXA4 is involved in the resolution of inflammation. Moreover, systemic administration LXA4 reduced pathological changes in the pancreas and the pancreatic index, suggesting a suppression of local pancreatic inflammation, decreasing the degree of pancreatic edema, pancreatic damage, and leukocyte infiltration. LXA4 could not reverse the local inflammatory process during the earliest phase of SAP and overall changes were gradually aggravated and most pronounced at 24 h, well in line with the co-localization hypothesis, where activated digestive enzymes result in cell injury and necrosis, and an increasingly evident inflammatory response [25]. During this process LXA4 seemingly act positively, not at least later on during the course modulating both the local and systemic inflammatory response by, for example, modulating the excretion of inflammatory mediators, activation of NF-κB, and leukocyte stimulation, thereby attenuating damage of the pancreas and also remote organs (e.g., the lungs).

The levels of IL-1 and -6 have been identified as predictors of severity during the early phase of acute pancreatitis [26,27]. Cytokines released from the

Table II. The immunohistochemistry result of expression of NF-κB P65 in pancreas.

<table>
<thead>
<tr>
<th>Time point (h)</th>
<th>Sham group</th>
<th>SAP group</th>
<th>LXA4 group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (-) (+)</td>
<td>(-) (+)</td>
<td>(-) (+)</td>
</tr>
<tr>
<td></td>
<td>4 24 8 0</td>
<td>4 4 2</td>
<td>5 3</td>
</tr>
<tr>
<td></td>
<td>12 24 8 0</td>
<td>0 8 5</td>
<td>4 4</td>
</tr>
<tr>
<td></td>
<td>24 21 8 0</td>
<td>0 6 6</td>
<td>1 6</td>
</tr>
</tbody>
</table>

SAP = severe acute pancreatitis.
LXA4 = SAP + administration of Lipoxin A4.
(-) = Negative.
(+) = Positive.
pancreas exacerbate the local inflammatory progression. Previous studies have demonstrated that LXA4 can inhibit monocytes and neutrophils from secreting IL-1 and TNF-α [28]. TNF-α represents an initial factor, mostly secreted by monocytes and acinar cells, inducing the expression of IL-1, -6, and -8, exaggerating the cascade response [3]. IL-1 and TNF-α both act locally to aggravate the pancreatitis process and also systemically activate cytokines and leukocytes. Once the local inflammation has formed an uncontrolled positive feedback loop, the result is an excessive release of inflammatory mediators and leukocyte activation, eventually resulting in SIRS and organ dysfunction [3]. LXA4 efficiently and early on inhibited cytokine release, and the lower cytokine levels paralleled and attenuated the local inflammatory response and decreased acinar cell injury.

NF-κB, activating the local inflammation, seems to be a key mediator influenced by LXA4 [29]. It is a nuclear transcription factor that plays an essential role in the inflammatory process of acute pancreatitis. Activated NF-κB can integrate with the corresponding sequences in the gene promoters of a variety of inflammatory cytokines and thus influence gene transcription [30]. Being a central proinflammatory signalling regulator, the activation of NF-κB may initiate a proinflammatory ‘cytokine storm’ [31]. As a key step in the positive feedback loop reaction, NF-κB activation, reduced by LXA4, significantly influences cytokine generation. Cytokines are essential in the pathogenesis of SAP and the systemic complications associated with SAP, but a decrease in this response could be seen following the administration of LXA4. Inhibition of the cytokine production has also been proposed as an intervention in order to decrease the severity of acute pancreatitis, as inflammatory cytokines aggravate the pancreatitis process [32,33]. The simultaneous decline in cytokines indicates that LXA4 in a broad fashion can decrease levels of pro-inflammatory cytokines related to the NF-κB pathway. Therefore, we believe that LXA4 possesses beneficial effects,

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Table III. The scores of pathological grading of lung injury.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Sham group</th>
<th>SAP group</th>
<th>LXA4 group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 h</td>
<td>3.08 ± 0.05</td>
<td>4.51 ± 0.14</td>
<td>4.36 ± 0.08</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>12 h</td>
<td>3.04 ± 0.04</td>
<td>8.71 ± 0.12</td>
<td>7.75 ± 0.12</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>24 h</td>
<td>3.08 ± 0.05</td>
<td>12.83 ± 0.12</td>
<td>11.04 ± 0.10</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

SAP = severe acute pancreatitis.
LXA4 = SAP + administration of Lipoxin A4.
P value = the statistical value of SAP group vs. LXA4 group.
modulating the SIRS process in SAP. Moreover, inhibiting the activation of NF-κB also significantly reduces the expression of ICAM-1, an important factor otherwise promoting pancreatic injury together with proinflammatory mediators and activated inflammatory cells [26]. Thus, LXA₄ seemingly decreases neutrophil flux across the endothelial barrier in the pancreas in acute pancreatitis.

High levels of proinflammatory mediators and activated inflammatory cells can cause remote organ injury. Previous studies have shown that inflammatory mediators (TNF-α, IL-1, -6, and -8) and adhesion molecules (ICAM-1, VCAM-1, E-selectin) and NOS are important cytokines involved in SIRS [32,34]. The most frequently affected organ in SAP driven SIRS are the lungs. ARDS and acute lung injury (ALI) represent primary clinical manifestations [35]. Inflammatory mediators can also activate peripheral, otherwise unstimulated, inflammatory cells, trigger the expression of adhesion molecules such as ICAM-1 in distant organs, and promote the adherence of leukocytes to the vascular endothelium, for example, in the pulmonary microcirculation. LXA₄ inhibits the chemotaxis, adhesion, and exudation of leukocytes by reducing the expression of chemotactic factors, inflammatory cytokines, and production of adhesion molecules and reactive oxygen species (ROS) [36,37]. The pathological changes of ALI in SAP demonstrated a significant reduction in the infiltration of PMNs following LXA₄ treatment in both the pancreas and lungs, attributed to the characters of LXA₄, promoting clearance of senescent neutrophils, blocking adhesion and respiratory burst activity. LXA₄ can also inhibit the function of mononuclear phagocytes and the production of ROS [14], and moreover, promote phagocytosis by mononuclear phagocytes to apoptotic neutrophil granulocytes [15]. We thus suggest that LXA₄ may ameliorate ALI by reducing cytokine generation, decreasing ICAM-1 expression, and inhibiting neutrophil activation.

Most studies have shown that high levels of LXA₄ can reduce the inflammatory response [38,39]. In the present study, we verified that LXA₄ possesses powerful anti-inflammatory properties during the course of acute pancreatitis. Our findings moreover suggest that LXA₄, with effects visible early on, but mainly with most pronounced manifestations of effectiveness later on during the process of inflammation [24], possesses a potential future intervention in SAP. These observations might contribute to reduce both the local and systemic inflammatory response and potentially ameliorate remote organ dysfunction in SAP.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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