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Substance P-regulated leukotriene B4 production promotes acute pancreatitis-associated lung injury through neutrophil reverse migration

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ABSTRACT

Leukotriene B4 (LTB4) is a potent chemoattractant and inflammatory mediator involved in multiple inflammatory diseases. Substance P (SP) has been reported to promote production of LTB4 in itch-associated response *in vivo* and in some immune cells *in vitro*. Here, we investigated the role of LTB4 in acute pancreatitis (AP), AP-associated acute lung injury (ALI) and the related mechanisms of LTB4 production in AP. *In vivo*, murine AP model was induced by caerulein and lipopolysaccharide or L-arginine. The levels of LTB4 and its specific receptor BLT1 were markedly upregulated in both AP models. Blockade of BLT1 by LY293111 attenuated the severity of AP, decreased neutrophil reverse transendothelial cell migration (rTEM) into the circulation and alleviated the severity of ALI. *In vitro*, treatment of pancreatic acinar cells with SP increased LTB4 production. Furthermore, SP treatment increased phosphorylation of protein kinase C (PKC) α and mitogen activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), p-38 MAPK and c-Jun NH2-terminal kinase (JNK). Finally, blockade of neurokinin-1 receptor by CP96345 significantly attenuated the severity of AP and decreased the level of LTB4 when compared to AP group. In summary, these results show that SP regulates the production of LTB4 *via* PKCα/MAPK pathway, which further promotes AP-associated ALI through neutrophil rTEM.

1. Introduction

Acute pancreatitis (AP) is a common disorder with significant morbidity and mortality, with no specific treatment. Disease severity depends on whether the inflammatory response resolves or amplifies, leading to multi-organ failure [1,2]. In severe AP, acute lung injury (ALI) is the main contributing factor to early deaths as the consequence of the systemic inflammatory response [3,4]. Woodfin and colleagues demonstrated that neutrophils have the ability of reverse transendothelial cell migration (rTEM) back into the circulation, which is significantly associated with the dissemination of systemic inflammatory responses [5]. In our recent study we showed that rTEM neutrophils play an important role in AP-associated lung injury [6]. However, the mechanisms underlying neutrophil rTEM in AP need to be elucidated.

Leukotriene B4 (LTB4) is a potent chemoattractant for immune cells, including neutrophils and macrophages, which play a major role in inflammatory responses [7,8]. Two G protein-coupled receptors for LTB4 have been identified. BLT1 which has high-affinity is predominantly expressed on granulocytes and macrophages, while BLT2 with low-affinity is ubiquitously expressed but rarely studied [9]. Although accumulating evidence indicates that LTB4 is involved in the pathogenesis of AP [10–13], the exact role of LTB4-BLT1 system in AP has not been reported. Colom and colleagues have revealed that LTB4-driven neutrophil rTEM propagates a local sterile inflammatory response to become systemic in the cremaster ischemia-reperfusion (I-R) model [14]. Therefore, we hypothesized that LTB4 might regulate neutrophil rTEM in AP, which further promotes pancreatitis-associated lung injury.

Substance P (SP) is an 11-amino acid neuropeptide mainly involved in nociception and neurogenic inflammation. The biological functions of SP are primarily mediated through neurokinin-1 receptor (NK1R), which has the highest affinity for SP among NKRs [15–17]. Research increasingly suggests that SP/NK1R contributes to inflammation in AP

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and associated lung injury [18–21], but the molecular mechanism remains unclear. Moreover, SP/NK1R has been reported to activate PKC and downstream MAPK, such as ERK1/2, in macrophages and human astrocytoma cells [22,23]. Previous studies showed that activated ERK1/2 was involved in the production of LTB4 in neutrophils and mast cells [24,25]. In addition, pancreatic acinar cells (PACs) can produce LTB4 following bile acid or caerulein stimulation [13]. Based on these findings, we speculated that SP might regulate LTB4 production in AP *via* PKC/MAPK signaling pathway.

In the present study, we investigated the role of LTB4-BLT1 system in AP-associated ALI and the putative role of SP in LTB4 production. The correlation between SP and LTB4, two important and known inflammatory mediators, may provide helpful insights in understanding the mechanism of AP.

2. Materials and methods

2.1. Reagents

Caerulein was purchased from Anaspec (CA, USA). L-Arginine monohydrochloride, Lipopolysaccharide (LPS) and CP96345 were purchased from Sigma-Aldrich Chemical (MO, USA). LY293111 was purchased from Cayman Chemical (Ann Arbor, MI, USA). Substance P acetate salt was from Bachem (Torrance, CA, USA). Bovine Serum Albumin (BSA) was purchased from Roche (Basel, Switzerland). The antibody against BLT1 receptor was purchased from Cayman Chemical (Ann Arbor, MI, USA). Antibodies against phospho-PKCα, phospho-ERK1/2, phospho-p38 MAPK and phospho-SAPK/JNK were from Cell Signaling Technology (MA, USA). The antibody used to detect β-actin was purchased from Beyotime Biotechnology (Shanghai, China). The F4/80 antibody was from GeneTex (TX, USA) and the antibody against Ly6G was purchased from Abcam (MA, USA). PE-conjugated rat anti-mouse Ly6G and FITC-conjugated hamster anti-mouse ICAM-1 were purchased from BD Biosciences (NJ, USA). APC-conjugated mouse monoclonal anti-CXCR1 was from abcam (MA, USA).

2.2. Animals

Male Balb/C mice aged 6 weeks were purchased from Shanghai SLAC Laboratory Animal Co Ltd. (Shanghai, China). The animals were acclimated for 1 week and kept in specific-pathogen-free conditions at a temperature of 22 °C and 12 h dark/light cycle. Mice had free access to water and standard rodent diet before experimentation. Thereafter, mice weighing 20-22 g were randomized into groups (n = 6 mice per group) to conduct the experiments. All animal experiments were approved by the Animal Ethics Committee of Shanghai Jiao Tong University School of Medicine (SYXK 2013-0050, Shanghai, China.).

2.3. Induction of experimental pancreatitis and treatments

Two AP models were used in this study: caerulein (CAE)-AP and L-arginine (L-Arg)-AP. Both models are noninvasive, rapidly induced and extensively used [26]. CAE-AP was induced by 10 intraperitoneal injections of caerulein (100 μg/kg) with a 1-hour interval between injections; LPS (5 mg/kg) was administered by intraperitoneal injection immediately after the last injection of caerulein; mice were sacrificed humanely at 12 h after the first caerulein injection. CAE-AP is highly reproducible and its severity can be easily controlled [27]. L-Arg-AP was induced by two intraperitoneal injections of L-Arg (4 g/kg, 8%, pH = 7.0) with a 1-hour interval between injections. The second L-Arg injection is defined as day 0. Mice were sacrificed humanely at day 3 after the induction of AP. L-Arg has toxic effects specific to the pancreas and L-Arg-AP is more severe than CAE-AP [28]. Normal control (NC) groups received only saline. LY293111 (LY, 5 mg/kg), a specific BLT1 antagonist, was administered intraperitoneally 0.5 h before the first injection of caerulein or L-Arg in LY groups. For NK1R blockade, the selective NK1R antagonist CP96345 (CP, 5 mg/kg) was injected intraperitoneally 0.5 h before the first L-Arg injection and equivalent CP was given every day for the following two days.

2.4. Measurements of serum amylase and lipase

Blood samples were collected and centrifuged at 3000 rpm for 15 min at 4 °C. The levels of serum amylase and lipase were measured by enzyme dynamics chemistry using commercial kits according to the manufacturer's protocols in a Roche/Hitachi modular analytics system (Roche, Switzerland).

2.5. Measurements of serum inflammatory cytokines

The serum levels of IL-6, IL-1β and TNF-α were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocols (Westang, China).

2.6. Measurements of LTB4 concentrations

LTB4 concentrations in the pancreatic tissue, serum and cell culture supernatants were determined by using LTB4 enzyme immunoassay kit according to the manufacturer's instructions (Cayman Chemical, USA).

2.7. Western blotting

Total protein isolated from PACs and pancreatic tissue were extracted as previously described [29]. Protein concentrations were detected using bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, China). Samples (40 μg per lane) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose membranes (Millipore, USA). Nonspecific binding was blocked with 5% BSA treatment. Next, the membranes were incubated with primary antibodies against BLT1 (1:200), p-PKCα (1:400), p-ERK1/2 (1:800), p-p38 MAPK (1:400), p-JNK (1:400) and β-actin (1:1000) overnight at 4 °C. The membranes were then washed with phosphate buffer solution containing 0.1% Tween (PBST). Samples were next probed with goat anti-rabbit or goat anti-mouse IR-Dye 800 or 700 CW labeled secondary antibodies for 1 h at 37 °C. Labeled proteins were visualized by Odyssey infra-red scanner (LI-COR, USA). Relative intensity was quantified *via* Odyssey scanning system and normalized to β-actin levels. The values of experimental groups were normalized to that of control groups and represented as fold changes compared to control values.

2.8. Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from pancreatic tissue using Trizol reagent (Invitrogen, CA, USA). RNA was reverse transcribed into cDNA with SuperScript II preamplification kit (Fermentas, MD, USA). Gene-specific, intron-spanning primers (Table 1) were synthesized by Shanghai Sangon Biotech Company (Shanghai, China). Real-time qRT-PCR was performed using KAPA SYBR Kits (Kapa Biosystems, Wilmington, USA) and ABI Prism 7900HT Sequence Detection System (Applied Biosystems, CA, USA). Relative expression levels of target genes and fold

Table 1
Sequences of mouse genes primers for qRT-PCR.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
BLT1	GGCTTCGTGGTCAAGCTACT	AGTCATGAAGCTGTCGGTGG
IL-6	CCGGAGAGGAGACTTCACAG	CATTTCCACGATTTCCCAGA
IL-1β	ATGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGATT
TNF-α	CCTCACACTCACAAACCACCA	ACAAGGTACAACCCATCGGC
GAPDH	GGTTGTCTCCTGCGACTTCA	TGGTCCAGGGTTTCTTACTCC