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Dual role of lipoxin A_4 in pneumosepsis pathogenesis ☆

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ABSTRACT

Lipoxin A_4 (LXA_4) is an endogenous lipid mediator with potent anti-inflammatory actions but its role in infectious processes is not well understood. We investigated the involvement of LXA_4 and its receptor FPR2/ALX in the septic inflammatory dysregulation. Pneumosepsis was induced in mice by inoculation of *Klebsiella pneumoniae*. LXA_4 levels and FPR2/ALX expression in the infectious focus as well as the effects of treatment with receptor agonists (LXA_4 and BML-111) and antagonists (BOC-2 and WRW(4)) in early (1 h) and late (24 h) sepsis were studied. Sepsis induced an early increase in LXA_4, FPR2/ALX lung expression, local and systemic infection and inflammation, and mortality. Treatment with BOC-2 in early sepsis increased leukocyte migration to the focus, and reduced bacterial load and dissemination. Inhibition of 5- and 15-lipoxygenase in early sepsis also increased leukocyte migration. Early treatment with WRW(4) and BOC-2 improved survival. Treatment with authentic LXA_4 or BML-111 in early sepsis decreased cell migration and worsened the infection. In late sepsis, treatment with BOC-2 had no effect, but LXA_4 improved the survival rate by reducing the excessive inflammatory response, this effect being abolished by pretreatment with BOC-2. Thus, the anti-inflammatory and pro-resolution mediator LXA_4 and its receptor FPR2/ALX levels were increased in the early phase of sepsis, contributing to the septic inflammatory dysregulation. In addition, LXA_4 has a dual role in sepsis and that its beneficial or harmful effects are critically dependent on the time. Therefore, a proper interference with LXA_4 system may be a new therapeutic avenue to treat sepsis.

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1. Introduction

Sepsis is a disease with rising incidence and presenting a high mortality rate of about 50% [1]. One of the major causes of sepsis is pneumonia, responsible for almost half of all sources of infection [1,2].

Sepsis involves two distinct, non-sequential and non-exclusive phases known as the systemic inflammatory response and the compensatory anti-inflammatory response [3]. Survival in sepsis is influenced by the intensity of these phases as well as by the correct balance between them. When this balance is lost, the host is no longer able to limit the infection or to keep the inflammatory insult within limits. The result is the spreading of the infection and/or a dysregulation of the inflammatory response, affecting multiple organs and causing irreversible damage and death. The dysregulation of the inflammatory response in sepsis is associated with an exacerbated production of both pro- and anti-inflammatory mediators. The production/release of these mediators in inadequate amounts and/or timing compromises the ability of the host to mount or maintain a proper inflammatory response. Indeed, the release of anti-inflammatory mediators impairs the host response against infection [4].

Lipoxins, particularly lipoxin A_4 (LXA_4), are anti-inflammatory and pro-resolution mediators derived from the arachidonic acid biosynthesized by two main pathways, both mediated by lipoxygenases (LO). The first pathway involves lipoxygenation of arachidonic acid by 15-LO in epithelial cells and monocytes, or by 5-LO in neutrophils [5]. The second pathway involves interactions of platelets and leukocytes by transcellular conversion of the 5-LO epoxide product leukotriene to LXA_4 by the activity of 12-LO in platelets [6].

The actions of LXA_4 are mediated through its binding to FPR2/ALX membrane receptor or to the nuclear aryl hydrocarbon receptor (AhR) [7,8]. FPR2/ALX has been described as the main receptor responsible for the in vivo anti-inflammatory actions of LXA_4 [9,10]. The anti-inflammatory role of the FPR2/ALX receptor is well known,

Abbreviations: LXA_4, 5(S),6(R)-Lipoxin A_4; FPR2/ALX, formyl peptide receptor-2; AhR, aryl hydrocarbon receptor; LO, lipoxygenases; BOC-2, N-t-Boc-Phe-Leu-Phe-Leu-Phe; WRW(4), Trp-Arg-Trp-Trp-Trp-Trp-$CONH_2$; BML-111, 5(S),6(R),7-trihydroxyheptanoic acid methyl ester; BAL, bronchoalveolar lavage; CLP, cecal ligation and puncture.

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being LXA_4 and annexin-1 the most extensively studied agonists of this G-protein-coupled receptor [11].

LXA_4 exhibits a wide range of anti-inflammatory and pro-resolution effects, including the ability to reduce vascular permeability, to impair the entry of new neutrophils into sites of inflammation and to promote the non-phlogistic infiltration of monocytes into the site of injury [12–14]. LXA_4 and its analogs exhibit protective roles in several experimental inflammatory conditions, such as arthritis [15], cystic fibrosis [16], uveitis [17] and edema [18].

On the other hand, the role of LXA_4 in infectious processes is not well understood, since the benefits of anti-inflammatory mechanisms are difficult to evaluate during an active infection. For instance, in *Mycobacterium tuberculosis* infection high levels of LXA_4 were associated with increase in pathogen replication, being the inhibition of LXA_4 biosynthesis beneficial to the host [19]. In contrast, mice succumbed to *Toxoplasma gondii* infection in the absence of LXA_4 because of an exacerbated pro-inflammatory response despite the reduction in parasite number [20].

Therefore, we aimed to investigate the role of LXA_4 and the FPR2/ALX receptor in *Klebsiella pneumoniae*-induced sepsis and their involvement in the dysregulation of inflammatory process that occurs in this pathology.

2. Material and methods

2.1. Animals

Male Swiss mice (weighing 35–40 g) were housed in a temperature- and light-controlled room (23 ± 2 °C; 12 h light/dark cycle), with free access to water and food. The University Institutional Ethics Committee for Animal Care and Use (CEUA) approved all procedures.

2.2. Bacterial inoculum and pneumosepsis induction

The bacterium used was *K. pneumoniae* — ATCC 700603 (American Type Culture Collection, Rockville, MD). Pneumosepsis was induced as previously described [21]. Briefly, bacteria were made pathogenic by 10 passages in C57-Bl6 mice. Before each experiment, individual aliquots were washed twice with sterile Dulbecco's PBS (in mM 137 NaCl, 2.7 KCl, 1.5 KH_2PO_4, and 8.1 $NaHPO_4$; pH 7.4), suspended in Brain Heart Infusion broth and incubated for 18 h at 37 °C. The broth was then centrifuged and the resultant pellet was washed twice with sterile PBS. Bacterial concentration was determined by light absorbance at 600 nm and compared to a standard curve. Mice were anesthetized with tribromoethanol and ketamine (375/25 mg/kg, i.p.) and placed in supine position. Under aseptic conditions, a 5 mm vertical incision was made in the frontal neck, the trachea was identified and 0.05 mL of either sterile PBS (sham-inoculated) or bacterial suspension (pneumosepsis) was injected into the trachea. Based on previous experiments, the number of viable bacteria inoculated was 4×10^8 CFU. The skin was closed with surgical suture. Animals received 30 mL/kg of sterile warm PBS subcutaneously. Until anesthesia recovery, animals were maintained at 37 °C in their cages, and afterward they were housed in a temperature- and light-controlled room as described above.

2.3. Flow cytometry

To recover intrapulmonary inflammatory cells, 1 mL of sterile PBS was instilled into the lungs through the trachea, and bronchoalveolar lavage (BAL) was aspirated back. This procedure was repeated 3 times. BAL samples were evaluated only when the volume recovery was greater than 80%. Briefly, BAL cells were obtained 6 and 24 h after bacterial inoculation and incubated with rabbit anti-mouse FPR2 (clone M-73; Santa Cruz Biotechnology, CA, USA) and various combinations of fluorochrome-conjugated rat anti-mouse mAbs specific for CD11b-APC (integrin αM chain, Mac-1α chain; clone M1/70), CD11c-PE-Cy7 (integrin αX; clone HL3), Gr-1-PE (Ly-6G and Ly-6C; clone RB6-8C5) (all from BD Pharmingen, NJ, USA), and F4/80 eFluor® 450 (panmacrophage marker; clone BM8, eBioscience) for 1 h in FACS buffer containing 0.5 mg/mL anti-mouse FcγIII/II receptor (clone 2.4G2; BD Pharmingen). Samples were washed and the final staining was made with Alexa Fluor 488-conjugated anti-rabbit IgG (Invitrogen, UK). Data were collected using a FACSCanto II with FACSDiva software and data were analyzed with Flowjo software (Tree Star, Ashland, OR, USA). Depending on the number of cells available, 30,000 to 50,000 events per sample were analyzed.

2.4. Immunofluorescence microscopy

Six and 24 h after infection, animals were anesthetized as above and perfused through the heart with saline (0.9% NaCl) followed by 4% paraformaldehyde fixative solution. The trachea was exposed and cannulated; lungs were inflated with the same fixative solution, removed from the animal and placed in fixative for 24 h. Then, lungs were inflated with 25% (v/v) optimal cutting temperature freezing medium (OCT; Tissue Tek®) in 20% sucrose in PBS, and embedded for frozen sectioning. Sections (5 μm thick) were obtained using a cryostat (Leica, Germany), mounted on gelatin-coated slides and blocked with 5% fetal bovine serum in PBS for 1 h. Then, slices were incubated with rabbit polyclonal anti-FPR2 Ab (1:100, Santa Cruz Biotechnology, CA, USA) overnight at 4 °C. Subsequently, slices were washed three times with PBS and incubated with Alexa Fluor 488-conjugated anti-rabbit IgG (1:1000, Invitrogen, UK) for 1 h at room temperature and washed three times with PBS. Coverslips were mounted on the slides using Gel Mount™ aqueous mounting medium (Sigma-Aldrich, St Louis, MO, USA), and examined by fluorescence microscopy (Olympus BX41, Olympus, Tokyo, Japan). Images were captured using Q-capture Pro 5.1 (Q-imaging). Values were expressed as arbitrary fluorescence intensity relative to control conditions and were quantified using ImageJ software.

2.5. Leukocyte counts

Total cell numbers were determined with a hemocytometer and differential counts were determined on Cytospin smears stained with rapid Panoptic stain (Laborclin, Pinhais, PR, Brazil). The main cell population in septic animals was polymorphonuclear cells, but we have also observed a substantial number of immature myeloid cells with a ring-like nucleus. Therefore, cell counts were divided in polymorphonuclear neutrophils, mononuclear cells and immature myeloid cells.

2.6. Quantification of bacterial CFUs

Hearts and spleens were aseptically harvested, placed in 200 μL of sterile PBS, and homogenized. BAL was also obtained under sterile conditions and was diluted serially in sterile PBS. Ten microliters of each dilution or tissue homogenate was aseptically plated and cultured on Mueller-Hinton agar dishes at 37 °C. After 24 h, CFU were counted and results were expressed as log CFU.

2.7. Determination of LXA_4, cytokine, lactate and creatinine levels

Blood and BAL samples were obtained, centrifuged at 1200 *g* for 10 min at 4 °C, the plasma or supernatant was saved. The measurement of LXA_4 was done after extraction and assay according to the manufacturer's recommendations using a commercially available enzyme-linked immunosorbent assay kit (Oxford Biomedical Research, Michigan, USA). Plasma cytokines TNF-alpha and IL-1beta were measured according to the manufacturer's recommendations using commercially available enzyme-linked immunosorbent assay kits (PeproTech Inc., New Jersey, USA). Plasma levels of lactate and creatinine were

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