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A synthetic analog of 15-epi-lipoxin A_4 inhibits human monocyte apoptosis: Involvement of ERK-2 and PI3-kinase

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ABSTRACT

Human monocytes play a central function in several steps of the immune response and the process involved in regulating their survival are critical to population control. Lipoxins are lipid mediators and members of the eicosanoid family that exhibit selective stimulatory but nonphlogistic activities in mononuclear cells. In this study, we investigated the effects of 15-epi-16-(*para*-fluoro)phenoxy-LXA_4 (ATL-1), a synthetic analog of 15-epi-lipoxin A_4, in human monocytes survival and apoptosis. ATL-1 concentration-dependently increased monocyte survival, as a consequence of cell apoptosis reduction by the analog. Treatment of these cells with PD98059 or LY294002 blocked ATL-1 effects, indicating the involvement of ERK-2 and PI3-K, both pathways associated with cell survival. Confirming the activation of these pathways, we demonstrated an increase in ERK-2 nuclear translocation and Akt phosphorylation. Furthermore, we showed that ATL-1 inhibits Bax translocation to the mitochondria. These results confirm a cytoprotective effect of lipoxins in monocytes and might contribute to the elucidation of the mechanisms associated with the resolution phase of the inflammatory process in different pathophysiological events.

1. Introduction

Human monocytes play a central function in the initiation, development and outcome of the immune response by differentiating into macrophages and dendritic cells and by releasing cell-signaling molecules, including cytokines. Mononuclear cells are known to play a key role in the inflammatory process of various pathophysiological events such as cancer, angiogenesis, atherosclerosis, rheumatoid arthritis or sepsis [1]. In the absence of an inflammatory stimulus, more monocyte precursors develop from the bone marrow than are needed to replace normal tissue macrophage numbers [2]. However, during inflammatory responses, a dramatic up-regulation of monocyte survival and differentiation may be required. Furthermore, as inflammation wanes, the number of monocytes in the inflammatory sites decreases, a clue for a physiologic death program in monocytes [2].

The processes involved in regulating monocyte removal and survival are critical to population control. Monocytes are produced in the bone marrow and can circulate in the blood stream for 24–48 h before undergoing spontaneous apoptosis, a form of programmed cell death [2]. Apoptosis is a physiological self-destruction mechanism regulated by an endogenous program which leads to the removal of the dying cell by macrophages in a way that prevents the onset of inflammatory reactions [3,4]. Following exposure of cells to stimuli that trigger programmed cell death, cytochrome c is rapidly released from mitochondria into the cytoplasm where it activates proteolytic molecules known as caspases that are crucial for the execution of apoptosis. In apoptotic cells, Bax translocation to the mitochondria induces the release of cytochrome c, activation of caspase-3, membrane blebbing, nuclear condensation, and internucleosomal DNA fragmentation, typical features of apoptosis [5]. The apoptotic process has been well described by its important function in different diseases including atherosclerosis, cancer and Alzheimer's disease [1].

Mitogen-activated protein kinases (MAPKs) are a family of 38–45 kDa kinases whose activity is modulated by phosphorylation leading to the transcription of genes that govern cellular processes such as cell growth, differentiation and migration. Furthermore, MAPKs are linked to the regulation of cell death in various different cell types [6,7]. Three subgroups of the MAPK family enzymes have been cloned: c-jun NH2-terminal kinase (JNK) and p38 MAP-kinase, mainly triggered by stress and inflammation, and extracellular signal-regulated protein kinase (ERK), frequently activated by mitogens [8]. ERK activity is, therefore, regarded to be important for cell growth and the inhibition of cell death [9].

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Recently, a Ser/Thr kinase, Akt, was shown to play an important role in cell survival in various cells. Akt activation is mediated by phosphoinositide 3-kinase (PI3-kinase) [10]. The PI3-K/Akt signaling pathway promotes prosurvival and anti-apoptotic activities, activating DNA transcription factors, such as NF-kB, and proapoptotic proteins [11,12]. Among the apoptotic proteins, Bad and caspases, especially caspase-3, have been shown to regulate cell survival in several cells [5,13–15]. Furthermore, Akt activation is able to induce Bcl-2 activation, a well-known anti-apoptotic protein [16]. Although ERK and Akt pathways seem to play crucial roles in cell survival, the functional discrimination of the two molecules has not yet been achieved, especially in human monocytes.

Lipoxins (LX) are members of the eicosanoid family of bioactive lipid mediators with trihydroxytetraene structures that represent a distinct class of arachidonic acid metabolites. LX are generated during cell–cell interactions under a variety of conditions, such as infection and inflammation and can be produced by one of at least three biosynthetic routes working independently or in concert, in particular biological settings [17]. The original pathways identified for LX formation were via lipoxygenase–lipoxygenase interactions. More recently, a novel pathway for LX synthesis was identified, in which aspirin, well-known to inhibit prostaglandin and thromboxane biosynthesis, has a unique ability to trigger, via a mechanism involving cyclooxygenase-2 inhibition, formation of 15-epimers of LXA_4 or aspirin-triggered LX (ATL), both *in vitro* and *in vivo* [18]. LX and ATL are rapidly biosynthesized in response to specific stimuli, act locally and are quickly enzymatically inactivated by different pathways in human monocytes [19].

LXA_4 and ATL exert anti-inflammatory effects through signals generated by binding to a high-affinity, G-protein-coupled receptor, denoted ALX [20]. The ALX receptor is conserved in mammalian species and constitutively expressed in various cellular types, thus being ideally localized to play key roles in modulating cell–cell interactions and cell-mediated immune responses, such as inhibition of polymorphonuclear cells (PMN) chemotaxis, adhesion, and transmigration across endothelial and epithelial cells [21]. Eosinophil-driven allergic reactions are also blocked by LXA_4 and its stable analogs [22,23]. Furthermore, LX and synthetic analogs inhibit superoxide production and cytokine release in PMN, as well as leukocyte trafficking [24,25].

In contrast to the down-regulation of PMN, LX exhibit selective stimulatory but nonphlogistic activities, i.e., namely without pro-inflammatory consequences *in vivo*, in the monocyte, as potent activation of cell migration and adherence to laminin [26,27]. It has been described that LX significantly enhance phagocytosis of apoptotic PMN by monocyte-derived macrophages [28]. These actions of lipoxins and their temporal separation from prostaglandins and leukotrienes in experimental inflammation indicate that they play unique roles in inflammation as mediators of resolution [29,30]. Although a few studies show evidence pointing to signaling pathways induced by lipoxins and ATL, the current understanding of the LXA_4 receptor's signal transduction pathways in monocytes still remains incomplete [31–34].

In this study, we investigated the effects of 15-epi-16-(*para*-fluoro)phenoxy-LXA_4 (ATL-1), a synthetic analog of 15-epi-lipoxin A_4, in human monocytes survival and apoptosis, as well as the signaling pathways involved in these processes. Here, we report that ATL-1 concentration-dependently increased human monocyte survival, as a consequence of cell apoptosis reduction by the analog. Treatment of these cells with PD98059 or LY294002 blocked ATL-1 effects, indicating the involvement of ERK-2 and PI3-K, both pathways associated with cell survival. We also demonstrated an increase in ERK-2 nuclear translocation and Akt phosphorylation, confirming that ATL-1-promoted apoptosis inhibition depends on the activation of ERK-2 and PI3-K pathways. Furthermore, we showed that ATL-1 inhibited Bax translocation to the mitochondria, an important step on apoptosis induction. These results confirm a cytoprotective effect of lipoxins in monocytes and might contribute to the elucidation of the mechanisms associated with the resolution phase of the inflammatory process.

2. Material and methods

2.1. Reagents

Ficoll-Hypaque and Percoll were purchased from Amersham Biosciences. PD98059 and LY294002 were obtained from Calbiochem. Boc-2 peptide was obtained from Phoenix Pharmaceuticals Inc. LTD_4 was purchased from Cayman Chemical. Abs were purchased from Santa Cruz Biotechnology and streptavidin from Caltag Laboratories. All other reagents and chemicals were purchased from Sigma–Aldrich. LXA_4 was a generous gift from Dr. Charles N. Serhan, Harvard Medical School; ATL-1, the stable 15-epi-LXA_4 analog, was a generous gift from Dr. John F. Parkinson, Bayer Healthcare Pharmaceuticals.

2.2. Isolation of human monocytes

Isolated PBMC were obtained from EDTA (0.5%)-treated venous blood of healthy volunteers by Ficoll-Hypaque density gradient centrifugation. The PBMC monolayer was collected, washed twice with Hank's buffered saline solution (HBSS) without Ca^{2+} and Mg^{2+}, suspended in RPMI 1640 medium without serum (incomplete medium) and incubated for 60 min at 37 °C to allow the monocytes to attach to culture dishes. Lymphocytes and platelets were removed, and isolated monocytes (98% purity), estimated to be >96% viable by trypan blue exclusion, were ressuspended overnight in complete medium (10% FBS) before treatment.

2.3. Measurement of monocyte survival

A modified MTT assay was used to quantify the effect of ATL-1 on monocyte survival. The method measures mitochondrial function as described previously [35]. Isolated human monocytes were plated on 96-well plates (1.5×10^5 cells/well) and maintained overnight in complete medium. Cells were then changed to serum-free medium in the absence or presence of various concentrations of ATL-1 (1–100 nM). Cells incubated in complete medium (10% FBS) were used as a positive control. Cells incubated in medium without serum were used as a negative control. After 48 h, the medium was aspirated from the wells and MTT reagent (40 μg/well) was added to each well. The cells were then incubated for 4 h at 37 °C followed by incubation with 100 μL of isopropyl alcohol. Survival was expressed as % of live cells above negative control. The optical density at 570 nm was read with an enzyme-linked immunosorbent assay (ELISA) reader (Model 550, BioRad).

2.4. Assessment of monocyte apoptosis

2.4.1. Fluorescence-activated cell sorting (FACS)

To measure the phosphatidylserine exposure on apoptotic cell surface, a flow cytometric assay using annexin V binding (annexin V-FLUOS; Roche Molecular Biochemicals, Mannheim, Germany) was performed. A working solution of annexin V-FITC was made from stock annexin V-FITC (0.1 μg/mL) diluted 1/3000 in HBSS supplemented with 2.5 mM $CaCl_2$. Monocytes (4×10^6 mL^{-1}) were incubated in the absence or presence of LY294002, PD98059, Boc-2 peptide or Montelukast prior to the treatment with ATL-1. In some experiments, cells were treated with LXA_4 or LTD_4. Cells (20 μL) were then added to 200 μL of a working solution of annexin V before being assessed on a FACSCalibur flow cytometer (BD Biosciences,