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Effect of azithromycin on the LPS-induced production and secretion of phospholipase A_2 in lung cells

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ABSTRACT

Azithromycin is a member of macrolides, utilized in the treatment of infections. Independently, these antibiotics also possess anti-inflammatory and immunomodulatory properties. Phospholipase A_2 isotypes, which are implicated in the pathophysiology of inflammatory lung disorders, are produced by alveolar macrophages and other lung cells during inflammatory response and can promote lung injury by destructing lung surfactant. The aim of the study was to investigate whether in lung cells azithromycin can inhibit secretory and cytosolic phospholipases A_2, ($sPLA_2$) and ($cPLA_2$), respectively, which are induced by an inflammatory trigger. In this respect, we studied the lipopolysaccharide (LPS)-mediated production or secretion of $sPLA_2$ and $cPLA_2$ from A549 cells, a cancer bronchial epithelial cell line, and alveolar macrophages, isolated from bronchoalveolar lavage fluid of ARDS and control patients without cardiopulmonary disease or sepsis. Pre-treatment of cells with azithromycin caused a dose-dependent decrease in the LPS-induced $sPLA_2$-IIA levels in A549 cells. This inhibition was rather due to reduced *PLA2G2A* mRNA expression and secretion of $sPLA_2$-IIA protein levels, as observed by western blotting and indirect immunofluorescence by confocal microscopy, respectively, than to the inhibition of the enzymic activity per se. On the contrary, azithromycin had no effect on the LPS-induced production or secretion of $sPLA_2$-IIA from alveolar macrophages. The levels of LPS-induced c-PLA_2 were not significantly affected by azithromycin in either cell type. We conclude that azithromycin exerts anti-inflammatory properties on lung epithelial cells through the inhibition of both the expression and secretion of LPS-induced $sPLA_2$-IIA, while it does not affect alveolar macrophages.

1. Introduction

Macrolides belong to an old class of antibiotics containing 14-, 15-, or 16-membered lactone ring linked by glycosidic bonds with sugars [for review, see 1]. They modulate the virulence activity of both gram positive and negative bacteria by inhibiting protein synthesis through reversible binding to the 50S subunit of bacterial ribosomes [2,3]. High concentrations of macrolides can be accumulated in the lysosomal compartments of phagocytes, because of their weak alkaline character, from where they are released to the sites of inflammation. Macrolides are now being considered for treatment of acute and chronic lung inflammatory diseases. In particular, azithromycin suppresses quorum sensing, that regulates bacterial density [4–6].

Apart from their antimicrobial activity, there is accumulating evidence that macrolides can also act as immunomodulators. This was initially recognized in patients with diffuse panbronchiolitis, who presented improved survival after treatment with erythromycin [5,7]. Macrolides act on a wide range of cells including bronchial epithelial cells, alveolar macrophages, monocytes, eosinophils and neutrophils [6,8]. They regulate leukocyte function and production of inflammatory mediators, control mucus hyper-secretion and resolution of inflammation, and modulate host defense mechanisms [9]. It has been proposed that macrolides exert their anti-inflammatory properties by inhibiting nuclear factor κB and activating protein-1, suppressing the secretion of pro-inflammatory cytokines and chemokines [7–10].

Abbreviations: AMs, alveolar macrophages; ARDS, Acute Respiratory Distress Syndrome; BAL, bronchoalveolar lavage; C_{12}-NBD-FA, 12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoic acid; C_{12}-NBD-PC, 1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-*sn*-glycero-3-phosphocholine; $cPLA_2$, cytosolic phospholipase A_2; FCS, fetal calf serum; LPS, lipopolysaccharide; PaO_2/FiO_2, partial pressure of arterial oxygen/inspired fraction of oxygen; PBS, phosphate-buffered solution; PVDF, polyvinylidine difluoride; $sPLA_2$, secretory phospholipase A_2; TBS, Tris-buffered saline; TLR, Toll-like receptor

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Endotoxin induces inflammatory response in various cells [11], leading to the production of pro-inflammatory mediators as well as phospholipases A_2 (PLA_2) [12]. This group of enzymes catalyzes the hydrolysis of the *sn*-2 ester bond of membrane phospholipids, producing lyso-phospholipids and free fatty acids, such as arachidonic acid, the precursor of eicosanoids. Members of the PLA_2 superfamily are implicated in lung inflammatory disorders [for review, see ref. 13], while especially secretory, $sPLA_2$ isotypes are considered as markers of inflammation [14]. These enzymes play important role in the initiation and amplification of the inflammatory reaction. They induce degranulation of mast cells and eosinophils and activate exocytosis in macrophages. Furthermore, $sPLA_2$s promote cytokine and chemokine production from macrophages, neutrophils, eosinophils, monocytes, and endothelial cells. These effects are mediated upon binding of $sPLA_2$s to specific receptors expressed on effector cells and are not related to their enzymic activity [15].

Cytosolic PLA_2, a Ca^{2+}-dependent enzyme, is involved in cell signaling processes. It produces arachidonic acid and its metabolites, eicosanoids, which constitute a group of bioactive signaling molecules [16].

So far, the information on the effect of azithromycin on suppressing the expression of pro-inflammatory agents is not fully understood.

The aim of this study was to investigate whether macrolides and especially azithromycin affects $cPLA_2$ levels or the production and secretion of $sPLA_2$-IIA from LPS-activated human alveolar macrophages and lung epithelial cells.

2. Materials and methods

A549 cell line, representing a model of type II alveolar cells, was purchased from American Type Culture Collection (ATCC, Manassas, VA); the cell culture media and its supplements were from Gibco (BRL, Grand Island, NY) and the fluorescent lipid reagents C_{12}-NBD-PC and C_{12}-NBD-FA were from Avanti Polar Lipids (Alabaster, AL, USA). Standard porcine $sPLA_2$ type IIA [E.C. 3.1.1.4] was obtained from Sigma Chemical Company (St Louis, MO, USA). Azithromycin, intravenous solution, was obtained from Pfizer Hellas AE.

2.1. Patients

Ten consecutive mechanically ventilated patients were employed in this study, 6 with early, moderate to severe ARDS (3 with primary and 3 with secondary) and 4 control patients. Standard criteria for ARDS diagnosis were based the Berlin Definition of ARDS [17].

The first 48 h from the initiation of ARDS are considered as early stage of the syndrome. The causes of primary ARDS were pneumonia and aspiration of gastric content. The risk factors for secondary ARDS were severe sepsis due to catheter-related infections and abdominal sepsis. The control group included intubated and mechanically-ventilated patients with neuromuscular diseases who developed ventilatory failure. The inclusion criteria for control subjects were as follows: no evidence of cardiopulmonary disease, $PaO_2/FiO_2 > 400$ mm Hg, without evidence of systemic inflammation. All patients underwent diagnostic bronchoalveolar lavage (BAL) according to ref. [14]. The protocol was approved by the review board for human studies of the University Hospital of Ioannina, while the patients or the next of kin gave a written informed consent to the study.

2.2. Cell cultivation and treatment conditions

Human alveolar macrophages were isolated from BAL fluid of control and ARDS patients. BAL fluid was filtered for mucus removal and centrifuged at 500 *g* for 10 min at 4 °C for BAL. The sediment, representing the isolated cells, was washed twice with phosphate-buffered solution (PBS), pH 7.4, and was finally re-suspended in RPMI-1640 culture medium. Cell count and viability were measured after cell staining with Trypan Blue exclusion dye. Alveolar macrophage (AM) population was purified by a negative-isolation protocol, using Dynabeads (Dynal LTD, UK) [18].

AMs (1×10^6 cells/well) were cultured in a growth medium consisting of RPMI-1640 without phenol red (to avoid interference with the fluorimetric assays), supplemented with 10% FBS, 2 mM L-glutamine, Ca^{2+}/Mg^{2+} and antibiotic cocktail, at 37 °C in a 5% CO_2 incubator. The cells were allowed to adhere for 3 h and then, the supernatants were collected, while adherent cells were washed twice with 37 °C PBS. Cells were made quiescent by incubation for 24 h in medium without FBS prior to the addition of LPS and azithromycin.

Human A549 cells ($6–8 \times 10^5$ cells/well) were cultured in Ham's F12 K medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotic cocktail, at 37 °C in 5% CO_2 complete medium until they reached ~80% confluence. Subconfluent cells were made quiescent for 24 h by incubating in serum-free Ham's F12K.

AM and A549 cells were treated with 1 μg/mL LPS (Sigma, Saint Louis, USA) for 24 h. In separate experiments, the cells were pre-treated with azithromycin (5 μg/mL, 20 μg/mL and 40 μg/mL for qRT-PCR experiments) for 1 h before the addition of LPS. Reference samples included untreated cells (control) and cells treated only with azithromycin.

After the incubation period, the cell supernatants were collected and centrifuged at 800 *g* for 10 min at 4 °C, the sediment was discarded and the 800g centrifuged supernatants were aliquoted and kept at −80 °C until analysis. Adherent cells were harvested by scrapping in ice-cold PBS, washed and were finally re-suspended into 1 mL PBS. Homogenization was assessed by sonication and aliquots were stored at −80 °C for less than 2 weeks for PLA_2 determination. Total protein was determined by the method of Bradford [19]. For western blot experiments, cells were lysed directly on the Petri dish after the addition of 50 μL electrophoresis sample buffer [25 mM Tris–HCl, pH 6.8, 4% (w/v) SDS, 10% glycerol, 10% (v/v) β-mercaptoethanol, 0.006%, w/v, bromophenol blue]. The lysates were collected and boiled for 5 min, aliquoted and stored at −80 °C until use. All the cell samples from ARDS and control patients were analyzed each one separately.

2.3. Fluorimetric determination of total PLA_2 activity

Total Ca^{2+}-dependent PLA_2 activity in cell homogenates and cell supernatants was measured by a fluorimetric assay developed in our laboratory [20,21]. The incubation mixture contained 240 μL of 10 mM Tris–HCl buffered solution, 2 mM Ca^{2+}, pH 7.4, and 5 μM C_{12}-NBD-PC (1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-*sn*-glycero-3-phosphocholine) as a fluorescent substrate. The reaction started with the addition of the source of the enzyme containing 5–15 μg of total protein. Incubation took place for 4 h. Excitation and emission wavelengths were adjusted to 475 and 535 nm respectively. The enzymic activities were calculated from the slope of the response curve by using C_{12}-NBD-FA as an internal standard.

To investigate whether azithromycin blocked the activity of the enzyme per se, the above assay was performed using standard $sPLA_2$ preparations dissolved in normal saline (0.8×10^{-3} IU) and the cells were pre-incubated for 30 min with a final concentration of 50 μg/mL azithromycin.

2.4. Analysis of PLA_2 isoenzymes by western blotting

Secretory, $sPLA_2$ type IIA, cytosolic $cPLA_2$ and its activated-phosphorylated form, $pcPLA_2$, were analyzed by western blotting: Samples from whole-cell protein extracts or cell supernatants were subjected to 15% SDS-PAGE, followed by immunoblotting, onto a polyvinylidine difluoride (PVDF) membrane. Nonspecific binding sites were blocked by incubation with 5% skim milk in TBS containing 0.1% Tween-20 (TBS-T), for 4 h at 4 °C. The membranes were then incubated overnight with polyclonal rabbit anti-human $sPLA_2$ group IIA (dilution