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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

1:1000, group II $sPLA_2$ (H-74): sc-20105, Santa Cruz Biotechnology, Inc. USA), polyclonal rabbit anti-human $cPLA_2$ (dilution 1:1000, sc-454, Santa Cruz Biotechnology, Inc. USA) and polyclonal rabbit anti-human phosphorylated $cPLA_2$ antibody (Ser 505) (dilution 1:1000) (#2831, Cell Signalling Technology, Beverly, MA, USA). After washing with TBS-T, blots were incubated for 2 h at room temperature with the goat anti-rabbit IgG-HRP antibody (dilution 1:7000, sc-2004, Santa Cruz Biotechnology, CA, USA). The immunoreactive bands were visualized with ECL chemiluminescence detection kit (Amersham Biosciences) and their intensities were analyzed by using the ImageJ image processing program (U.S. National Institutes of Health, Bethesda, Maryland, USA). Equal protein loading was confirmed by using anti-β-actin mouse monoclonal IgG_1 antibody (C4) (dilution 1:500, sc-47778, Santa Cruz, CA, USA) and the goat anti-mouse IgG-HRP (dilution 1:7000, sc-2005, Santa Cruz, CA, USA) as a secondary antibody.

2.5. Intracellular protein expression of $sPLA_2$-IIA

The LPS-induced expression of $sPLA_2$-IIA was studied in A549 cells by indirect immunofluorescence followed by analysis by confocal microscopy. The cells were grown on cover slips and treated with the appropriate LPS concentration for 24 h. At the end of the incubation the cells were washed twice with PBS before being fixed with 4% paraformaldehyde in PBS. After two washings with PBS, the samples were briefly rinsed with an NH_4Cl solution in order to quench free paraformaldehyde. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 4 min at room temperature and incubated for 15 min with blocking buffer (10% FCS in PBS). Primary anti-$sPLA_2$-IIA antibodies were incubated with the cells for 45 min at room temperature in 10% fetal calf serum in PBS. After washing with PBS, the cells were incubated with donkey anti-rabbit IgG secondary antibodies (diluted in 10% FCS in PBS) conjugated with Alexa fluorophores, purchased from Invitrogen. Then, coverslips were mounted on glass slides using Mowiol containing 100 mg/mL diazabicyclo[2.2.2]octane (Sigma). Images were obtained on a Leica TCS SP5 II scanning confocal microscope, using the 63× HCX PL APO 1.3 NA objective and were subsequently processed with LAS AF and Adobe Photoshop software.

2.6. Total RNA isolation and qRT-PCR

For qRT-PCR experiments, cells were lysed directly on the Petri dish after the removal of the supernatant, with the addition of lysis buffer solution provided by the NucleoSpin RNA II kit (Macherey-Nagel, GmbH & Co. KG, Germany). Total RNA was isolated according to the manufacturer's recommended protocol. RNA integrity and purity was checked electrophoretically and verified with the criterion of an OD_{260}/OD_{280} absorption ratio > 1.7.

Real-time PCR was performed using the iScript One-Step RT-PCR kit with SYBR green (Bio-Rad Laboratories, Hercules, CA), using forward and reverse primers from Qiagen (USA) for *PLA2G2A*, *PLA2G4A* and *GAPDH* human genes, with the last used as a reference housekeeping gene.

Total RNA (100 ng) in a 25 μL total volume was first incubated at 50 °C for 10 min to synthesize cDNA, heated at 95 °C for 5 min to inactivate the reverse transcriptase, and then subjected to 35 thermal cycles (94 °C for 40 s, 60 °C for 40 s, and 72 °C for 1 min) of PCR amplification and 40 cycles from 55 °C to 95 °C (1 °C increase/cycle) for melting curve analysis using an MJ mini thermal cycler (Bio-Rad, Hercules, CA). Regarding A549 cells, three separate RNA isolations were analyzed in eight qRT-PCR runs, in duplicates for each experimental condition, whereas three separate RNA isolations analyzed in five qRT-PCR runs were performed for BAL AM, in duplicates for each experimental condition.

Relative quantization of qPCR data from A549 RNA samples was carried out according to the method of Pfaffl [22], using *GAPDH* as an internal reference. Quantitative RT-PCR results were calculated as fold-increase in gene mRNA versus fold-increase in *GAPDH* mRNA. Data from qPCR from AM RNA samples were recorded and calculated with Bio-Rad CFX Manager software applying ΔΔCt method.

2.7. Statistics

Values were expressed as mean ± SD of three independent duplicate experiments. Comparisons were assessed by one-way ANOVA followed by post hoc comparisons with the Bonferroni method. The level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Effect of azithromycin on LPS-induced total PLA_2 activity in A549 cells

As depicted in (Fig. 1), total Ca^{2+}-dependent PLA_2 activity, measured fluorimetrically, was significantly increased in LPS-treated A549 cells in comparison with the untreated cells: control: [(4.10±0.98)nmolC12-NBD-FA/h/mg protein] and LPS: [(7.09± 1.03)nmolC12-NBD-FA/h/mg protein, P<0.05]. This increase was totally blocked in samples pre-treated with 5 or 20 μg/mL azithromycin, prior to the addition of 1 μg/mL LPS, resulting in PLA_2 values similar to the control: [5 μg/mL azithromycin + LPS: (5.15 ± 0.34) nmol C_{12}-NBD-FA/h/mg protein, 20 μg/mL azithromycin + LPS: (4.06 ± 0.63) nmol C_{12}-NBD-FA/h/mg protein]. Interestingly, azithromycin alone had no effect on total PLA_2 activity of the untreated-control cells. Additionally, reference experiments with standard porcine $sPLA_2$-IIA showed that the presence or absence of azithromycin did not affect the enzymic activity (Table 1). Moreover, the endogenous Ca^{2+}-independent PLA_2 activity levels measured in the presence of 10 mM EDTA (a chemical chelator) by the fluorimetric method in cell homogenates in the present study, were inconsiderable.

3.2. Effect of azithromycin on LPS-induced PLA_2 isoenzymes in A549 cells

Western blot experiments revealed that $cPLA_2$ and its activated form, $pcPLA_2$, were present at significant but invariable levels, both in untreated and LPS-treated cell homogenates (Fig. 2A and B). Azithromycin did not modify the $cPLA_2$ or $pcPLA_2$ levels under any of our experimental conditions.

Cell lysates were also examined for $sPLA_2$-IIA production: As shown in Fig. 3A and B, only the samples treated with LPS showed an induction of $sPLA_2$-IIA protein. Interestingly, azithromycin significantly reduced

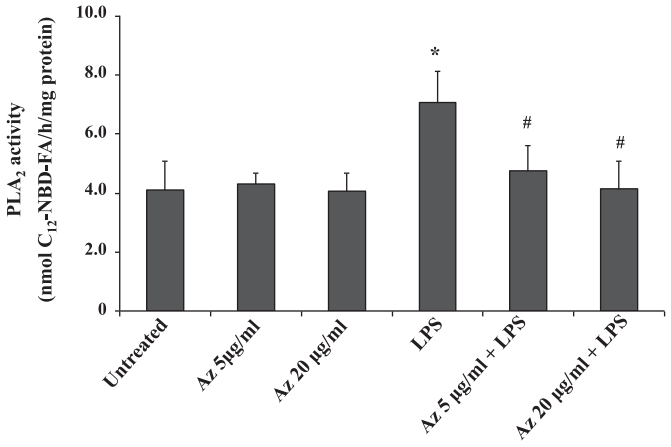

Fig. 1. Effect of azithromycin (Az) on the LPS-induced total PLA_2 activity in A549 cells. The levels of PLA_2 total activity were determined with a fluorimetric assay using C_{12}-NBD-PC as a substrate. Values represent mean ± SD of three independent experiments. $^{*}P < 0.05$: significant difference from control A549 cells; $^{\#}P < 0.05$: significant difference from LPS-treated A549 cells.

Table 1
Effect of azithromycin on standard $sPLA_2$-IIA activity.

Sample	$sPLA_2$-IIA activity (nmol FA/h/mL sample)
Standard porcine $sPLA_2$	6.82 ± 0.38
Standard porcine $sPLA_2$ + 50 μg/mL azithromycin	6.74 ± 0.43

the LPS-induced $sPLA_2$-IIA signal, in a dose-dependent manner. Similarly, $sPLA_2$-IIA was secreted only upon treatment with LPS. Pre-treatment with 5 μg/mL azithromycin reduced the LPS-induced secretion of the enzyme, while with 20 μg/mL azithromycin, the $sPLA_2$-IIA signal was totally abolished (Fig. 3 A and C). The expression of $sPLA_2$ was also tested by confocal microscopy. Consistently with the western blotting data, LPS induced a robust expression of $sPLA_2$ (Fig. 4B), which was inhibited by azithromycin in a dose-dependent manner (Fig. 4C and D). In untreated cells we could not detect endogenous expression of $sPLA_2$ (Fig. 4A). A possible reason for the absence of endogenous $sPLA_2$ detection in untreated A549 cells is the low concentration of $sPLA_2$, below the detection limit of WB technique.

3.3. Effect of azithromycin on LPS-induced PLA_2-IIA production in human alveolar macrophages

3.3.1. Cytosolic PLA_2

Under our experimental conditions, $cPLA_2$ and its activated-phosphorylated form, $pcPLA_2$, were slightly expressed at the protein level, in both cells from control and ARDS patients. The protein band did not change significantly after cell treatment with azithromycin, LPS, or both (Fig. 5A).

3.3.2. Secretory PLA_2-IIA

Production and secretion of $sPLA_2$-IIA was induced by LPS in the alveolar macrophages from control, but not from ARDS patients (Fig. 5B). In particular, a low signal on western blotting was detected in the AM cell homogenate from control patients, but the majority of the protein was detected in the extracellular medium under treatment with LPS 1 μg/mL for 24 h. Azithromycin did not affect the production or the secretion of the LPS-induced $sPLA_2$-IIA (Fig. 5B), contrary to A549 cells. Even in higher concentration of azithromycin (40 μg/mL), no inhibitory effect was observed on the production or secretion of the $sPLA_2$-IIA protein (Fig. 5C).

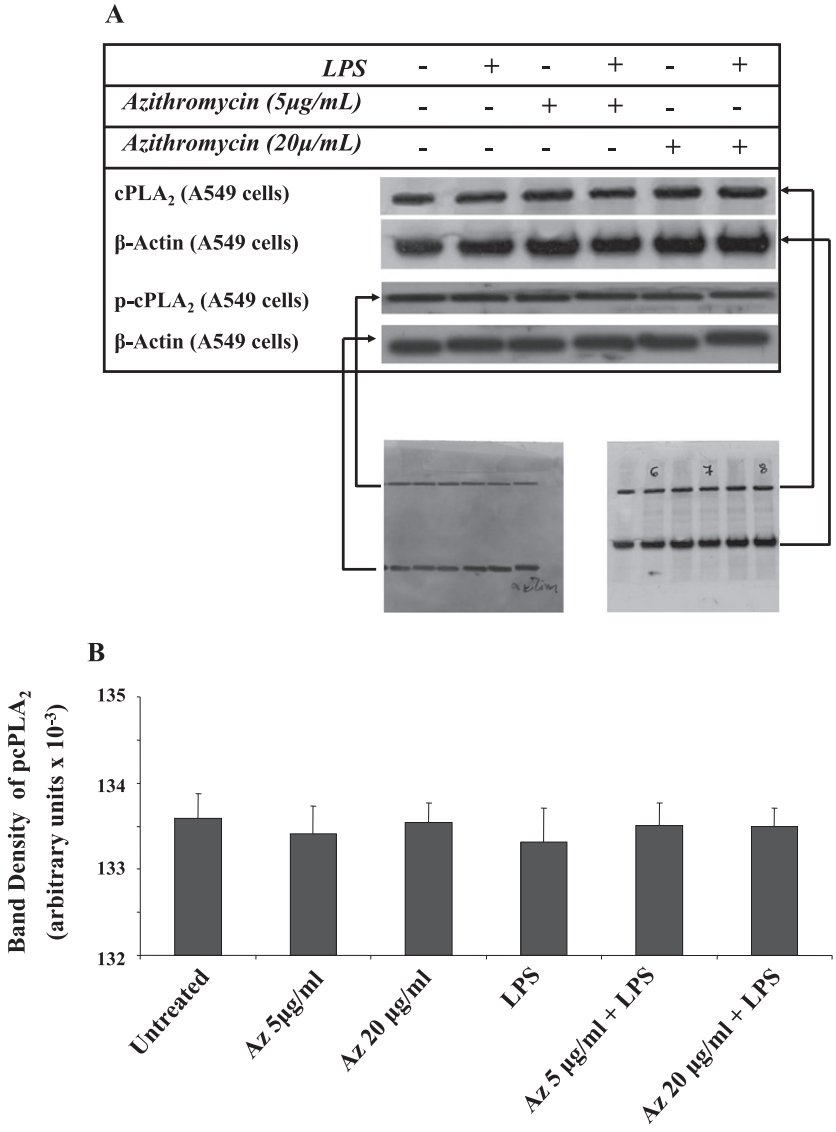

Fig. 2. Effect of LPS and azithromycin (Az) on $cPLA_2$ and p-$cPLA_2$ production in A549 cells. (A) Incubation with LPS took place in the presence (5 and 20 μg/mL) or absence of azithromycin. Cell homogenates were analyzed by western blotting using anti-$cPLA_2$ and $pcPLA_2$ antibodies. Antibodies against β-actin were used as control of protein loading. (B) Bar graphs represent intensities of $pcPLA_2$ intensities analyzed by the ImageJ image processing program. Values represent mean ± SD of three independent experiments.

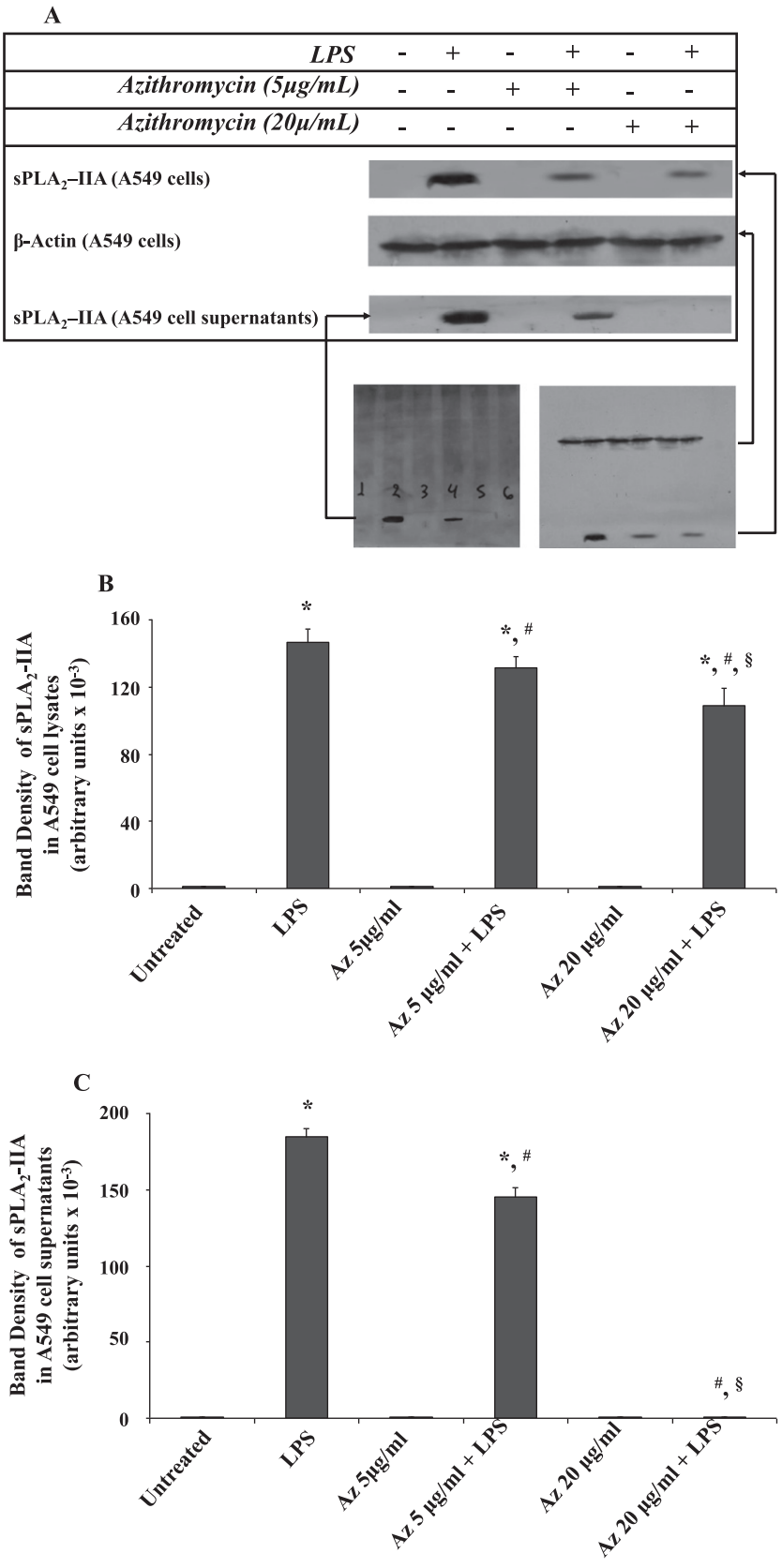

Fig. 3. Effect of LPS and azithromycin (Az) on sPLA$_2$-IIA induction in A549 cells: (A) The gels are representative of one cell preparation under different treatment conditions. Untreated cells were used as a reference for each experiment. Incubation with LPS took place in the presence (5 and 20 μg/mL) or absence of azithromycin. Cell homogenates were analyzed by western blotting using anti-sPLA$_2$-IIA antibodies. Antibodies against β-actin were used as control of protein loading. (B) Bar graphs represent intensities of sPLA$_2$-IIA in A549 cell lysates analyzed by the ImageJ image processing program. Values represent mean ± SD of three independent experiments. (C) Bar graphs represent intensities of sPLA$_2$-IIA in A549 supernatants analyzed by the ImageJ image processing program. Values represent mean ± SD of three independent experiments. $^{*}P < 0.05$: significant difference from control; $^{\#}P < 0.05$: significant difference from samples treated with LPS; $^{\S}P < 0.05$: significant difference from samples treated with LPS + azithromycin 5 μg/mL.

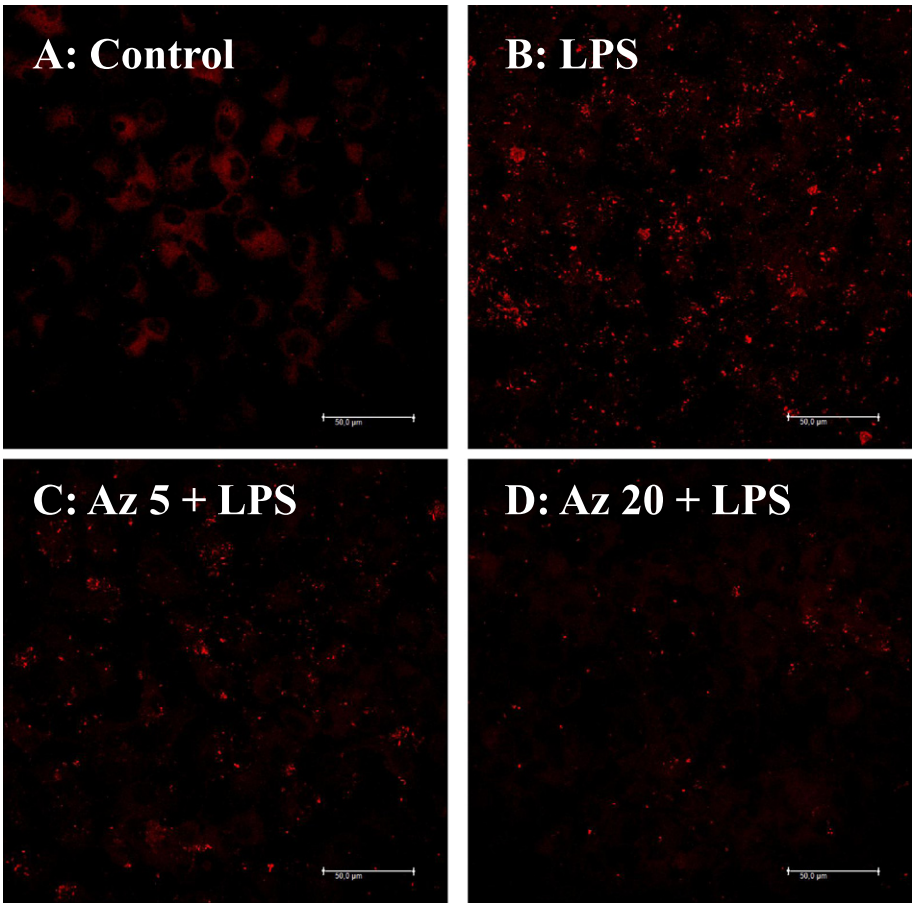

Fig. 4. Effect of azithromycin (Az) on LPS-induced expression of $sPLA_2$ in A549 cells: $sPLA_2$-IIA was detected by immunofluorescence microscopy after incubation with appropriate secondary antibodies labeled with Alexa 594. Samples were analyzed using a Leica TCS SP5 II scanning confocal microscope, using the Leica 63× HCX PL APO 1.3 NA objective. Images were acquired using the Leica Software and files were subsequently processed in LAS AF and Adobe Photoshop. (A) Control A549 cells without activation with LPS; (B) A549 cells activated with 1 μg/mL LPS; (C) A549 cells treated with 5 μg azithromycin/mL, 30 min prior to activation with 1 μg LPS; (D) A549 cells treated with 20 μg azithromycin/mL, 30 min prior to activation with 1 μg LPS. The bar represents 50 μm.

Although a significant amount of $sPLA_2$-IIA protein was detected in the extracellular medium of AM from ARDS patients, the enzyme was not detected within the cells. It is noteworthy that $sPLA_2$-IIA was detected only in the BAL fluid from ARDS patients, contrary to the control patients (Fig. 6).

3.3.3. PLA2G2A and PLA2G4A mRNA expression

The qRT-PCR data revealed that *PLA2G2A* mRNA is slightly expressed in untreated A549 cells (cycle threshold 29 from 100 ng RNA sample). This expression presented a 2.9 ± 1.4-fold increase after LPS treatment ($P < 0.05$). Azithromycin 20 μg/mL alone did not affect the expression levels of *PLA2G2A* mRNA (0.8 ± 0.3). However, the LPS-induced increase of *PLA2G2A* mRNA expression was inhibited by pre-incubation of the A549 cells with azithromycin 20 μg/mL (1.6 ± 1.6, $P < 0.05$) (Fig. 7A). The genes' expression was normalized to the housekeeping gene of *GAPDH*. Regarding samples treated with 5 μg/mL azithromycin (±LPS), the signal was very low.

We did not detect any *PLA2G2A* mRNA expression neither in control nor in ARDS RNA samples from AMs.

On the contrary, *PLA2G4A* mRNA expression was measured in AM RNA samples from both control and ARDS patients: The expression levels were not altered significantly after treatment of AMs from control patients with LPS (1.2 ± 0.5), 40 μg/mL azithromycin alone (0.9 ± 0.4) or LPS + azithromycin (40 μg/mL) (0.8 ± 0.4) as compared to the untreated cells ($P > 0.5$) (Fig. 7B). Regarding ARDS AM, similar pattern of *PLA2G4A* expression was observed before or after the incubation of the cells with LPS (0.9 ± 0.9), azithromycin 40 μg/mL (0.8 ± 0.6) or LPS + azithromycin 40 μg/mL (0.7 ± 0.8) as compared to control.

4. Discussion

In this study, we demonstrated that pre-incubation of A549 cells, a model of alveolar epithelial cells, with azithromycin, suppressed the LPS-induced total PLA_2 activity, secretory PLA_2-IIA protein levels as well as the LPS-induced *PLA2G2A* mRNA expression. In parallel, azithromycin could not inhibit the activity of standard $sPLA_2$-IIA in vitro. Thus, the reduction of total PLA_2 activity in azithromycin pre-treated cells could be due to suppressed endogenous expression of $sPLA_2$-IIA, as it was shown by confocal microscopy and qRT-PCR data.

Primary cultures of alveolar macrophages (AMs) responded in a different way: LPS induced the production and secretion of $sPLA_2$-IIA in cells from patients without cardiopulmonary disease, contrary to cells from patients with ARDS, where $sPLA_2$-IIA protein was observed only in the extracellular medium. Azithromycin did not suppress the LPS-induced $sPLA_2$-IIA protein expression, even at the high concentration of 40 μg/mL, or the total PLA_2 activity in the AM of any group of patients. Under all our experimental conditions, the protein levels of $cPLA_2$ and its activated form, $pcPLA_2$, were low, and were not modified after treatment with LPS or azithromycin.

Lung injury is a consequence of diffuse alveolar damage. Alveolar cells produce a variety of inflammatory cytokines that recruit neutrophils to the lung parenchyma. Activation of these cells causes an excessive release of cytokines, reactive oxygen species, and proteases,

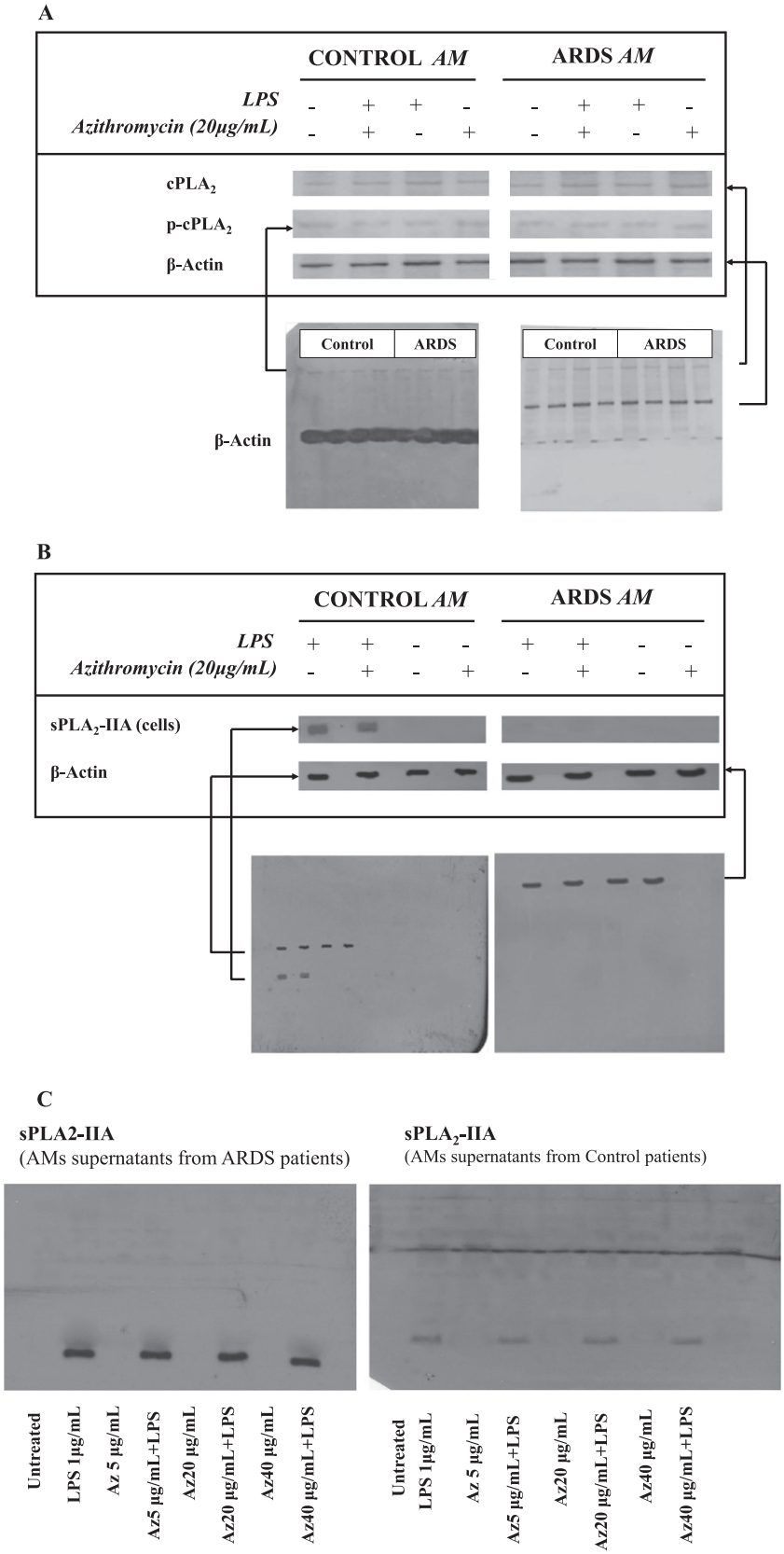

Fig. 5. Effect of LPS and azithromycin (Az) on the production of $cPLA_2$, as well as the production and secretion of $sPLA_2$-IIA, from alveolar macrophages. Cells were isolated from four control and six ARDS patients. Incubation with LPS took place in the presence (5 and 20 μg/mL) or absence of azithromycin. Analysis took place by western blotting using 5 μg protein loading and appropriate antibodies for $cPLA_2$, $pcPLA_2$ and $sPLA_2$-IIA. Antibodies against β-actin were used as control of protein loading. (A) Levels of $cPLA_2$ and its activated form, $pcPLA_2$. (B) Intracellular levels of $sPLA_2$-IIA. (C) Levels of $sPLA_2$-IIA in cell supernatants. Gel images are representative of one AM preparation from control and one from ARDS patient under different treatment conditions.

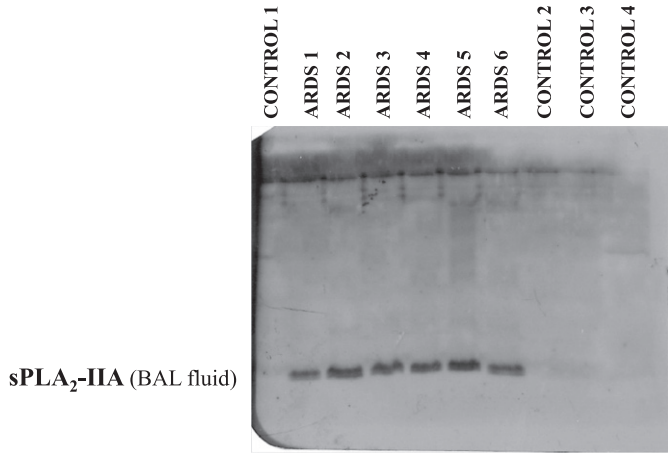

Fig. 6. Detection of $sPLA_2$-IIA in BAL fluid: The levels of $sPLA_2$-IIA were analyzed by western blotting using anti-$sPLA_2$-IIA antibody in BAL fluids from six ARDS and four control patient samples.

including PLA_2, which disturb the permeability of the alveolar-capillary membrane and can lead to the development of ARDS. Walkey and Wiener investigated the association between administration of macrolide antibiotics and clinical outcomes in patients with ARDS [23]. They found that patients who received early treatment with macrolides showed significant reduction in mortality. In our study, we used LPS for the activation of epithelial cells. LPS induces an excessive production of pro-inflammatory substances, including various PLA_2 isoforms [24–28]. Secretory PLA_2s are known to play a role in the pathogenesis of many inflammatory diseases: Increased levels of $sPLA_2$ have been detected in the plasma and biological fluids of patients with ARDS, acute pancreatitis, rheumatoid arthritis, pneumonia, etc. [14,29]. $sPLA_2$s cause the liberation of free fatty acids, such as arachidonic acid (AA) and lyso-phospholipids [30] from membrane phospholipids, while they can destruct lung surfactant and promote the development of lung injury [13].

The fact that the LPS-induced $sPLA_2$-IIA production and release were suppressed after pre-treatment of A549 cells with azithromycin reveals its anti-inflammatory properties on alveolar epithelial cells. Our findings are in agreement with the observations of Walkey and Wiener

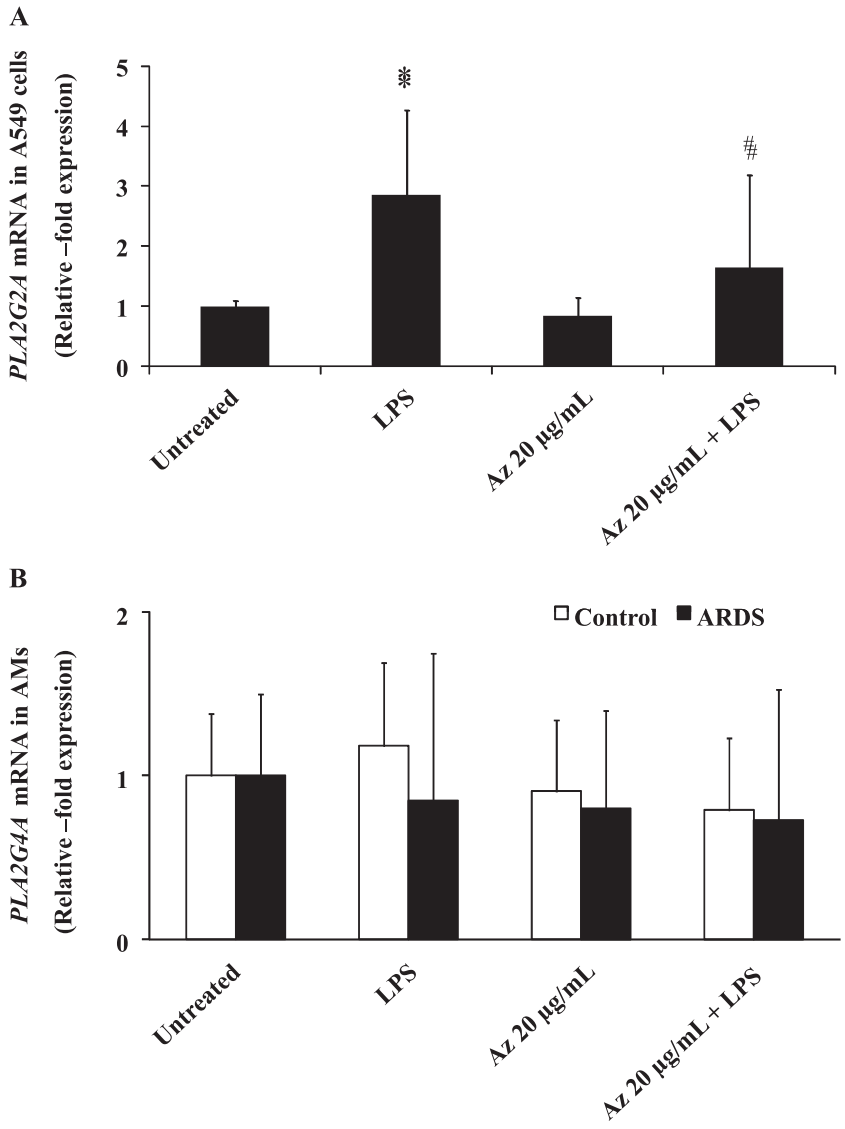

Fig. 7. (A) Relative expression of PLA2G2A mRNA in A549 cells. The data are the means ± SD of eight qRT-PCR experiments with duplicate samples from three independent total mRNA isolations. (B) Relative expression of PLA2G4A mRNA in AM. The data are the means ± SD of five qRT-PCR experiments with duplicate samples from three independent total mRNA isolations. $^{*}P < 0.05$: significant difference from untreated; ($^{\#}P < 0.05$): significant difference from samples treated with LPS.

[23] and other studies where macrolides were used [31,32] and provide a possible explanation for above observations.

Alveolar macrophages are key effector cells, representing the first line of pulmonary defense. They exert pathogen recognition, antigen processing and phagocytosis and produce inflammatory mediators, among which $sPLA_2$-IIA is a major contributor. LPS induced the production and secretion of $sPLA_2$-IIA in AM from control patients, as expected. However, in cells from ARDS patients, stimulated ex vivo with LPS, the enzyme was detected only in the extracellular medium and not within the cells. This finding, combined with the fact that $sPLA_2$-IIA was detected in BAL fluid from ARDS patients only, and not in BAL fluid from control patients, supports the concept that under activation the injured lung secretes $sPLA_2$-IIA.

Despite the fact that our RNA preparation was quite pure and we have tried 3 different sets of primers, i.e., two synthetic pairs, 5′ GGCA CCAAATTTCTGAGCTACA 3′ (Forward)/5′ TTATCACACTCACACAGTTGAC TTCTG 3′ (Reverse), 5′ CAACAGATCCAGGGAGCATT 3′ (Forward)/5′ AGGCCGTCTTGTTTGTTCTG 3′ (Reverse) and one set purchased from Qiagen, which we finally used in this study, we did not detect the *PLA2G2A* mRNA transcript in AM cells by qRT-PCR. On the contrary, the mRNA transcript of *PLA2G4A* was readily detectable in either A549 cells or AMs. The above differences could be due to possible high turnover of the *PLA2G2A* mRNA especially in AMs. Synthesis of $sPLA_2$-IIA is regulated in a complex manner and is highly depended on the target cell type and the stimulus employed [33]. This could be reflected by altered *PLA2G2A* mRNA turnover rates in different cells in response to various stimuli, through different signaling pathways. Such a phenomenon has been reported for the mRNA of *PLA2G4A* in A549 cells, where an inflammatory signal results in activation and binding of a transcription factor to the *PLA2G4A* transcript thereby leading to its stabilization and expression and prevents its degradation [34]. Besides, high levels of protein expression despite undetectable mRNA levels have been reported in other cases as well [35]. Thus, we could hypothesize that, in LPS-treated cells, a high turnover of the mRNA transcript of *PLA2G2A*, following its translation, might prevent its detection by qRT-PCR. In any case, the conclusions drawn in the present study are based on specific activity assays, as well as on consistent analysis of the protein levels by immunoblotting, and provide evidence for the expression levels of this enzyme both at the activity and the protein level.

The reason why we could not detect $sPLA_2$-IIA intracellular levels can be explained by the possibility that it is retained loosely in formations near the extracellular membrane [36], which are detached during the cell harvesting and collected with the cell supernatant. The difference in the response of AM from control or ARDS patients to LPS could be explained by immune tolerance of cells from ARDS patients to the prompt formation of new pools of the enzyme within the cells. This is in agreement with a previous work where we found impaired $sPLA_2$-IIA production by stimulated macrophages from patients with ARDS [18].

The fact that azithromycin in our primary AM cultures did not suppress LPS-induced $sPLA_2$-IIA levels intracellularly or extracellularly even in high concentration of 40 μg/mL signifies that these cells are allowed to function even in the presence of the antibiotic. This finding is in consistency with other studies where the ability of macrolides did not suppress, but rather enhanced phagocytosis of apoptotic epithelial cells and neutrophils by macrophages [37,38]. Finally, azithromycin did not affect the $cPLA_2$ expression at mRNA or protein levels in AMs. This fact could be of interest, since $cPLA_2$ participates in signal transduction and regulates innate immune responses. However, in a previous work [18], we had found that LPS increases p-$cPLA_2$ levels in alveolar macrophages from control patients but this could have been due to the more potent LPS stimulus, which was a higher LPS concentration (25 μg/mL, instead of 1 μg/mL used in the present study). Other investigators suggested that azithromycin inhibits $cPLA_2$ activity in LPS-stimulated J774A.1 cells, based on indirect experimental evidence, where inhibition of arachidonic acid release was observed. However, they did not observe any suppression on $cPLA_2$ mRNA levels, which is in agreement with our results, where azithromycin did not affect neither the protein nor the mRNA levels of $cPLA_2$, and it did not inhibit total PLA_2 activity in vitro [39].

Our results show a different response of LPS-treated alveolar macrophages and A549 cells to azithromycin as far as $sPLA_2$-IIA expression is concerned. Apart from the different cell types, this phenomenon may be due to the function of alveolar epithelial type II cells as immune boosters in activating AMs through an autocrine loop involving the production of inflammatory molecules [40]. NF-κB signaling pathway, which plays a prominent role in the regulation of $sPLA_2$-IIA gene expression [41], could be involved in this event. Recently, it was shown that the anti-inflammatory activity of azithromycin in epithelial and endothelial cells, was associated with the inhibition of NF-κB activation after LPS instillation in the lung [42]. This was not the case for AMs since azithromycin did not inhibit the NF-κB pathway in a model of LPS-induced pulmonary neutrophilia [43]. In summary, in A549 cells azithromycin inhibits the LPS-induced $sPLA_2$-IIA expression possibly through inhibition of the NF-κB signaling pathway, contrary to AMs where azithromycin does not affect the NF-κB pathway.

5. Conclusions

Based on our experimental data, we suggest that azithromycin exhibits a protective anti-inflammatory activity on epithelial cells through the suppression of LPS-induced $sPLA_2$-IIA expression and secretion, an enzyme known to be implicated in the pathogenesis of lung injury. These results could explain the beneficial effect of azithromycin in certain diseases, such as panbronchiolitis and acute lung injury.

Transparency Document

The Transparency document associated with this article can be found, in the online version.

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References

[1] H.A. Kirst, Introduction to the macrolide antibiotics, in: W. Schönfeld, H.A. Kirst (Eds.), Macrolide Antibiotics, Milestones in Drug Therapy, Birkhauser Verlag, Basel/Switzerland, 2002, pp. 1–13.
[2] W.S. Champney, R. Burdine, Macrolide antibiotics inhibit 50S ribosomal subunit assembly in *Bacillus subtilis* and *Staphylococcus aureus*, Antimicrob. Agents Chemother. 39 (1995) 2141–2144.
[3] T.R. Shryock, J.E. Mortensen, M. Baumholtz, The effects of macrolides on the expression of bacterial virulence mechanisms, J. Antimicrob. Chemother. 41 (1998) 505–512.
[4] A. Equi, I.M. Balfour-Lynn, A. Bush, M. Rosenthal, Long term azithromycin in children with cystic fibrosis: a randomised, placebo-controlled crossover trial, Lancet 360 (2002) 978–984.
[5] M.H. Gotfried, Macrolides for the treatment of chronic sinusitis, asthma, and COPD, Chest 125 (Suppl.) (2004) 52–60.
[6] K. Tateda, R. Comte, J.C. Pechere, T. Kohler, K. Yamaguchi, C. Van Delden, Azithromycin inhibits quorum sensing in *Pseudomonas aeruginosa*, Antimicrob. Agents Chemother. 45 (2001) 1930–1933.
[7] B.K. Rubin, M.O. Henke, Immunomodulatory activity and effectiveness of macrolides in chronic airway disease, Chest 125 (Suppl. 2) (2004) 70–78.
[8] F.J. Martinez, J.L. Curtis, R. Albert, Role of macrolide therapy in chronic obstructive pulmonary disease, Int. J. COPD 3 (2008) 331–350.
[9] Y.S. Lopez-Boado, B.K. Rubin, Macrolides as immunomodulatory medications for the therapy of chronic lung diseases, Curr. Opin. Pharmacol. 8 (2008) 286–291.
[10] S. Kanoh, B.K. Rubin, Mechanisms of action and clinical application of macrolides as immunomodulatory medications, Clin. Microbiol. Rev. 23 (2010) 590–615.

[11] S.J. Skerrett, H.D. Liggitt, A.M. Hajjar, Respiratory epithelial cells regulate lung inflammation in response to inhaled endotoxin, Am. J. Physiol. Lung Cell. Mol. Physiol. 287 (2004) L143–L152.
[12] M. Triggiani, F. Granata, G. Giannattasio, G. Marone, Secretory phospholipases A2 in inflammatory and allergic diseases: not just enzymes, J. Allergy Clin. Immunol. 116 (2005) 1000–1006.
[13] E. Kitsiouli, G. Nakos, M.E. Lekka, Phospholipase A2 subclasses in acute respiratory distress syndrome, Biochim. Biophys. Acta 1792 (2009) 941–953.
[14] G. Nakos, E. Kitsiouli, E. Hatzidaki, V. Koulouras, L. Touqui, M.E. Lekka, Phospholipases A2 and platelet-activating-factor acetylhydrolase in patients with acute respiratory distress syndrome, Crit. Care Med. 33 (2005) 772–779.
[15] M. Triggiani, F. Granata, A. Frattini, G. Marone, Activation of human inflammatory cells by secreted phospholipases A2, Biochim. Biophys. Acta 1761 (2006) 1289–1300.
[16] E. Letsiou, E. Kitsiouli, G. Nakos, M.E. Lekka, Mild stretch activates cPLA2 in alveolar type II epithelial cells independently through the MEK/ERK and PI3K pathways, Biochim. Biophys. Acta 1811 (6) (2011) 370–376.
[17] V.M. Ranieri, G.D. Rubenfeld, B.T. Thompson, N.D. Ferguson, E. Caldwell, E. Fan, L. Camporota, A.S. Slutsky, Acute respiratory distress syndrome: the Berlin Definition ARDS Definition Task Force, J. Am. Med. Assoc. 307 (2012) 2526–2533.
[18] E. Hatzidaki, G. Nakos, E. Galiatsou, M.E. Lekka, Impaired phospholipases A2 production by stimulated macrophages from patients with acute respiratory distress syndrome, Biochim. Biophys. Acta 1802 (2010) 986–994.
[19] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.
[20] E. Kitsiouli, G. Nakos, M.E. Lekka, Differential determination of phospholipase A2 and PAF-acetylhydrolase in biological fluids using fluorescent substrates, J. Lipid Res. 40 (1999) 2346–2356.
[21] A. Karkabounas, E.I. Kitsiouli, G. Nakos, M.E. Lekka, HPLC-fluorimetric assay of phospholipase A2. Application to biological samples with high protein content and various reaction conditions, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 879 (2011) 1557–1564.
[22] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (2001) 2002–2007.
[23] A.J. Walkey, R.S. Wiener, Macrolide antibiotics and survival in patients with acute lung injury, Chest 141 (2012) 1153–1159.
[24] M.A. Dobrovolskaia, S.N. Vogel, Toll receptors, CD14, and macrophage activation and deactivation by LPS, Microbes Infect. 4 (2002) 903–914.
[25] H. Oshiumi, M. Sasai, K. Shida, T. Fujita, M. Matsumoto, T. Seya, TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to Toll-like receptor 4 TICAM-1 that induces interferon-beta, J. Biol. Chem. 278 (2003) 49751–49762.
[26] E.S. Van Amersfoot, T.J.C. Van Berkel, J. Kuiper, Receptors, mediators and mechanisms involved in sepsis and septic shock, Clin. Microbiol. Rev. 16 (2003) 379–414.
[27] Y.J. Jiang, B. Lu, P.C. Choy, G.M. Hatch, Regulation of cytosolic phospholipase A2, cyclooxygenase-1 and -2 expression by PMA, TNF alpha, LPS and M-CSF in human monocytes and macrophages, Mol. Cell. Biochem. 246 (2003) 31–38.
[28] S. Medjane, B. Raymond, Y. Wu, L. Touqui, Impact of CFTR DeltaF508 mutation on prostaglandin E2 production and type IIA phospholipase A2 expression by pulmonary epithelial cells, Am. J. Physiol. Lung Cell. Mol. Physiol. 289 (2005) L816–L824.
[29] S. Masuda, M. Murakami, M. Mitsuishi, K. Komiyama, Y. Ishikawa, T. Ishii, I. Kudo, Expression of secretory phospholipase A2 enzymes in lungs of humans with pneumonia and their potential prostaglandin-synthetic function in human lung-derived cells, Biochem. J. 387 (2005) 27–38.
[30] D.L. Bowton, M.C. Seeds, M.B. Fasano, B. Goldsmith, D.A. Bass, Phospholipase A2 and arachidonate increase in bronchoalveolar lavage fluid after inhaled antigen challenge in asthmatics, Am. J. Respir. Crit. Care Med. 155 (1997) 421–425.
[31] M. Shinkai, Y.S. Lopez-Boado, B.K. Rubin, Clarithromycin has an immunomodulatory effect on ERK-mediated inflammation induced by *Pseudomonas aeruginosa* flagellin, J. Antimicrob. Chemother. 59 (2007) 1096–1101.
[32] C. Cigana, B.M. Assael, P. Melloti, Azithromycin selectively reduces tumor necrosis factor alpha levels in cystic fibrosis airway epithelial cells, Antimicrob. Agents Chemother. 51 (2007) 975–981.
[33] M. Konieczkowski, J.R. Sedor, Cell-specific regulation of type 11 phospholipase A2 expression in rat mesangial cells, J. Clin. Invest. 92 (1993) 2524–2532.
[34] W.L. Liao, W.C. Wang, W.C. Chang, J.T. Tseng, The RNA-binding protein HuR stabilizes cytosolic phospholipase A2 mRNA under interleukin-1a treatment in non-small cell lung cancer A549 cells, J. Biol. Chem. 286 (41) (2011) 35499–35508.
[35] Z. Shinwari, H. Al-Hindi, E. Al-Shail, Y. Khafaga, A. Al-Kofide, N. El-Kum, A. Aboussekhra, Response of medulloblastoma cells to vincristine and lomustine: role of TRKC, CTNNB1 and STK15, Anticancer Res. 31 (5) (2011) 1721–1733.
[36] M. Murakami, T. Kambe, S. Shimbara, S. Yamamoto, H. Kuwata, I. Kudo, Functional association of type IIA secretory phospholipase A2 with the glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan in the cyclooxygenase-2-mediated delayed prostanoid-biosynthetic pathway, J. Biol. Chem. 247 (1999) 22927–22936.
[37] S. Hodge, G. Hodge, S. Brozyna, H. Jersmann, M. Holmes, P.N. Reynolds, Azithromycin increases phagocytosis of apoptotic bronchial epithelial cells by alveolar macrophages, Eur. Respir. J. 28 (2006) 486–495.
[38] T. Yamaryo, K. Oishi, H. Yoshimine, Y. Tsuchihashi, K. Matsushima, T. Nagatake, Fourteen-member macrolides promote the phosphatidylserine receptor-dependent phagocytosis of apoptotic neutrophils by alveolar macrophages, Antimicrob. Agents Chemother. 47 (2003) 48–53.
[39] M. Banjanac, V.K. Munić, K. Nujić, M. Vrančić, D. Belamarić, S. Crnković, M. Hlevnjak, H.V. Eraković, Anti-inflammatory mechanism of action of azithromycin in LPS-stimulated J774A.1 cells, Pharmacol. Res. 66 (4) (2012) 357–362.
[40] S. Kannan, H. Huang, D. Seeger, A. Audet, Y. Chen, C. Huang, H. Gao, S. Li, M. Wu, Alveolar epithelial type II cells activate alveolar macrophages and mitigate *P. aeruginosa* infection, PLoS One 4 (3) (2009) e4891.
[41] L. Touqui, M. Alaoui-El-Azher, Mammalian secreted phospholipases A_2 and their pathophysiological significance in inflammatory diseases, Curr. Mol. Med. 1 (2001) 739–754.
[42] F.F. Stellari, A. Sala, G. Donofrio, F. Ruscitti, P. Caruso, T.M. Topini, K.P. Francis, X. Li, C. Carnini, M. Civelli, G. Villetti, Azithromycin inhibits nuclear factor-κB activation during lung inflammation: an in vivo imaging study, Pharmacol. Res. Perspect. 2 (5) (2014) e00058.
[43] M. Bosnar, S. Čužić, B. Bošnjak, K. Nujić, G. Ergović, N. Marjanović, I. Pašalić, B. Hrvačić, D. Polančec, I. Glojnarić, H.V. Eraković, Azithromycin inhibits macrophage interleukin-1β production through inhibition of activator protein-1 in lipopolysaccharide-induced murine pulmonary neutrophilia, Int. Immunopharmacol. 11 (2011) 424–434.