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Azithromycin has an antiproliferative and autophagic effect on airway smooth muscle cells

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ABSTRACT: Azithromycin is used in long-term, low-dose treatment of airway diseases where airway wall remodelling is present. Since it improves total score symptom and respiratory function of such patients, we hypothesise that azithromycin's additional clinical benefits are due to an inhibition of airway smooth muscle cell (SMC) proliferation.

Rabbit tracheal SMCs were treated with azithromycin (10^{-5} to 10^{-6} M) in the presence or absence of 10% fetal bovine serum (FBS). The proliferation was estimated using the Cell Titer 96® AQ_{ueous} One Solution Assay (Promega, Madison, WI, USA). Cell viability was assessed with Trypan blue staining and flow cytometry after 7-aminoactinomycin D (7-AAD) staining. Induction of autophagy was studied by indirect immmunofluorescence and/or Western blotting with antibodies against human smooth muscle α-actin, beclin 1, light chain 3 and caspase 3. The involvement of the phosphoinositide 3-kinase pathway was investigated with the inhibitors LY294002 and wortmannin.

Incubation with azithromycin for 72 h in the presence of FBS reduced SMC proliferation and viability in a dose-dependent manner. Azithromycin treatment was accompanied by the formation of cytoplasmic vacuoles, characteristic of autophagy. All these effects were reversible after azithromycin removal and prevented by the autophagy inhibitor, 3-methyladenine, or LY294002, but not by wortmannin.

In conclusion, azithromycin reduces proliferation and causes autophagy of airway SMCs.

KEYWORDS: Airway smooth muscle cells, autophagy, azithromycin, proliferation

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Macrolide antibiotics are widely used for the treatment of airway diseases. Low-dose, long-term macrolide therapy has been reported to be very effective in patients with chronic airway diseases, such as diffuse panbronchiolitis, chronic bronchitis and bronchial asthma [1–5]. In many of these diseases, airway wall remodelling is present. Airway wall remodelling includes thickening of the reticular membrane, proliferation of the smooth muscle cells (SMCs) and increase in both number and size of vessels [6, 7].

The improvement of pulmonary function, total score symptom and quality of life with macrolides is mainly attributed to their antimicrobial and anti-inflammatory activity. However, data available from studies on airway smooth muscle show an effect of macrolides on the contractility of airway smooth muscle. Specifically, erythromycin inhibits cholinergic neuroeffector transmission in the airways [8, 9] and azithromycin has a direct relaxant effect on precontracted rabbit airway smooth muscle [10].

It is possible that azithromycin has an additional effect in airway SMC proliferation. As has been shown previously, erythromycin inhibits hypertrophic and metaplastic changes of goblet cells in rat nasal epithelium [11], roxithromycin inhibits proliferation of human coronary artery SMCs [12], and rapamycin and its analogue SAR943 inhibit proliferation of human epithelial and SMCs [13], while clarithromycin and azithromycin induce apoptosis of activated lymphocytes [14]. In order to investigate whether azithromycin exerts a similar effect on airways, we studied the effect of azithromycin on airway SMC proliferation and the pathways involved. In this study, we present evidence that azithromycin causes autophagy and a reversible reduction of SMC proliferation isolated from trachea of adult rabbits.

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MATERIALS AND METHODS

Animals

Adult rabbits were maintained in individual cages in a controlled environment and were provided with food and water before use for the study. Animals were treated in compliance with ethical and institutional guidelines. Rabbits were euthanised by an overdose of intravenously administrated pentothal (Abbott, Abbott Park, IL, USA).

Study design

Isolated tracheal SMCs were serum starved for 72 h and treated with azithromycin 10^{-5} to 10^{-6} M (Pfizer Inc., New York, NY, USA) dissolved in Dulbecco's modified Eagle's medium/Ham/F12 (DMEM-F12) for 24, 48 and 72 h. The cell proliferation was estimated using the Cell Titer 96® AQ_{ueous} One Solution Assay (Promega, Madison, WI, USA), while the cell morphology was observed using immunofluorescence with anti-human smooth muscle α-actin antibody or optical microscopy. Cell viability was assessed using Trypan blue staining and flow cytometry after 7-aminoactinomycin D (7-AAD) staining.

In the cell proliferation assay, the optical density (OD) of cells incubated in serum-free medium (24 h) was used as control (set at 100%). Results are presented as percentage of OD of the control cells. In the experiments investigating the reversible effect of azithromycin, OD values were presented as percentage of relative OD values at the time of azithromycin removal, which were set as 100%.

The effect of azithromycin on lysosomes was observed by Lysotracker Red staining. The autophagy pattern was studied using immunofluoerescence and Western blot analysis with anti-beclin 1 and anti-light chain 3 (anti-LC3) antibodies. The caspase activation was studied performing Western blotting with anti-caspase 3 antibody.

Methods

Isolation and culture of airway SMCs

Isolation of airway SMCs from adult rabbit trachea was carried out as previously described [15]. Cells were grown in DMEM-F12 containing 10% fetal bovine serum (FBS), 100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin (Gibco BRL, Invitrogen Corp., Eugene, OR, USA) at 37°C in a humified incubator under 5% CO_2. SMCs were characterised by immunofluorescence using an anti-human smooth muscle α-actin monoclonal antibody. All experiments were performed with cells on passages three to six and cells were cultured in serum-free medium for 72 h before further treatment to achieve quiescence.

Treatment of cells

Serum-starved cells were exposed to azithromycin (10^{-5} to 10^{-6} M; Pfizer) in serum-free medium or medium containing 10% FBS for 24–72 h, as indicated. Control cells remained in serum-free medium. In the experiments regarding the reversible effect of azithromycin, cells were cultured in serum-free medium for 72 h and further incubated in azithromycin (10^{-5} M) for 48 h. Subsequently, azithromycin was washed out with PBS, and DMEM-F12 containing 10% FBS was added to the culture. The phosphoinositide 3-kinase (PI3K) pathway inhibitors LY294002 (5 μM) or wortmannin (500 nM; Sigma-Aldrich Chemie, Steinheim, Germany) were added to the medium 15 min prior to the addition of 10^{-5} M azithromycin for 24, 48 or 72 h. The solvent for LY294002 and wortmannin was dimethylsulphoxide, which in the used concentrations does not affect SMCs.

The autophagy inhibitor 3-methyladenine (3-MA; Sigma-Aldrich Chemie; 10 mM) was added to the medium 3 h prior to the addition of azithromycin. 3-MA was dissolved in water.

The cells were observed under a Nikon Diaphot 300 (Nikon Inc., Melville, NY, USA) inverted microscope using a Leica DFC 480 camera (Leica Cameras, Solms, Germany).

Cell proliferation and viability

Cells were seeded into 96-well Elisa plates at a density of 2,000 $cells \cdot well^{-1}$, allowed to adhere overnight, washed twice with PBS and incubated in serum-free medium for 72 h before further treatment. Replicates of three were used for each point. Cell proliferation was assessed using the Cell Titer 96® AQ_{ueous} One Solution Assay. Briefly, MTS, a tetrazolium reagent, was added directly to each well and incubated for 2 h at 37°C. The MTS tetrazolium compound is converted to formazan by living cells [16, 17]. Absorbance was measured at 490 nm with a

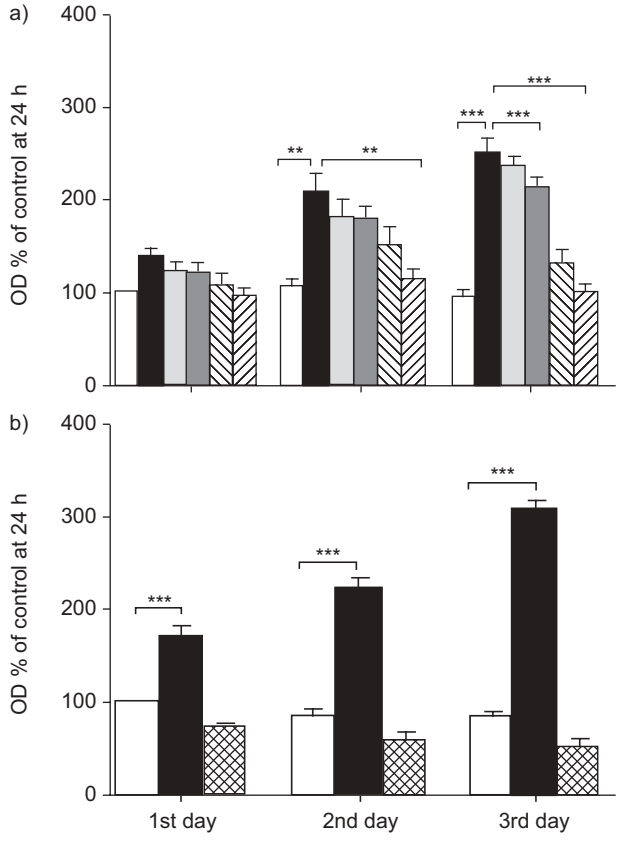

FIGURE 1. The effect of 10^{-6} to 10^{-5} M azithromycin on the proliferation of airway smooth muscle cells incubated in a) 10% fetal bovine serum (FBS)-containing medium or b) serum-free medium. Data are presented as mean±SEM. Data are taken from five independent experiments. □: control; ■: 10% FBS; ▩: 10% FBS and azithromycin 10^{-6} M; ▩: 10% FBS and azithromycin 3×10^{-6} M; ▩: 10% FBS and azithromycin 6×10^{-6} M; ▩: 10% FBS and azithromycin 10^{-5} M; ■: azithromycin 10^{-5} M. OD: optical density. **: $p<0.01$; and ***: $p<0.001$ compared with control or 10% FBS.

reference at 630 nm in an ELISA plate reader. There was a linear response between cell number and absorbance at 490 nm (data not shown).

In order to assess cell viability, we used the vital dye Trypan blue (Sigma-Aldrich Chemie). This chromophore is negatively charged and interacts with cells when the cytoplasmic membrane is damaged. Therefore, all the cells which exclude the dye are viable. 0.4% Trypan blue solution was added in the cell suspension at a concentration of 20% and the cells were counted on a haemocytometer under a microscope (Nikon YS2T; Nikon Inc.).

Flow cytometry

The viability of SMC after treatment with azithromycin, was assessed by staining with 7-AAD (Beckman Coulter, Fullerton, CA, USA), a dye that intercalates with the DNA bases cytosine and guanine, and allows a clear discrimination of viable and nonviable cells [18, 19]. In brief, cells after culture with azithromycin for 24–72 h, were collected, washed with 1% FBS–PBS, and 20 μL of 7-AAD was added for 20 min at ambient temperature in the dark. Excess 7-AAD was washed out and cells were fixed in 1% formaldehyde–PBS. Cells were analysed using a FC500 flow cytometer (Beckman Coulter) on the basis of their forward and side scatter characteristics (FSC and SSC) and light emission on the FL3 channel which discriminated viable from nonviable cells. To accommodate alterations of the cell scattering characteristics due to treatment with azithromycin, all analyses were undertaken using ~10,000 events gated on similar FSC and SSC profiles.

Immunofluorescence

Airway SMCs were plated onto glass coverslips and were incubated for 72 h in serum-free medium. Then SMCs were treated as described above. After incubation, the cells were fixed with PBS–3% formaldehyde, rinsed with PBS and permeabilised with PBS–1% Triton X-100. Following blocking with PBS–0.1% Tween 20–3% bovine serum albumin, the cells were incubated with an anti-human smooth muscle α-actin (A104) mouse monoclonal antibody (1:400; Sigma-Aldrich Chemie), as well as anti-human beclin 1 rabbit polyclonal

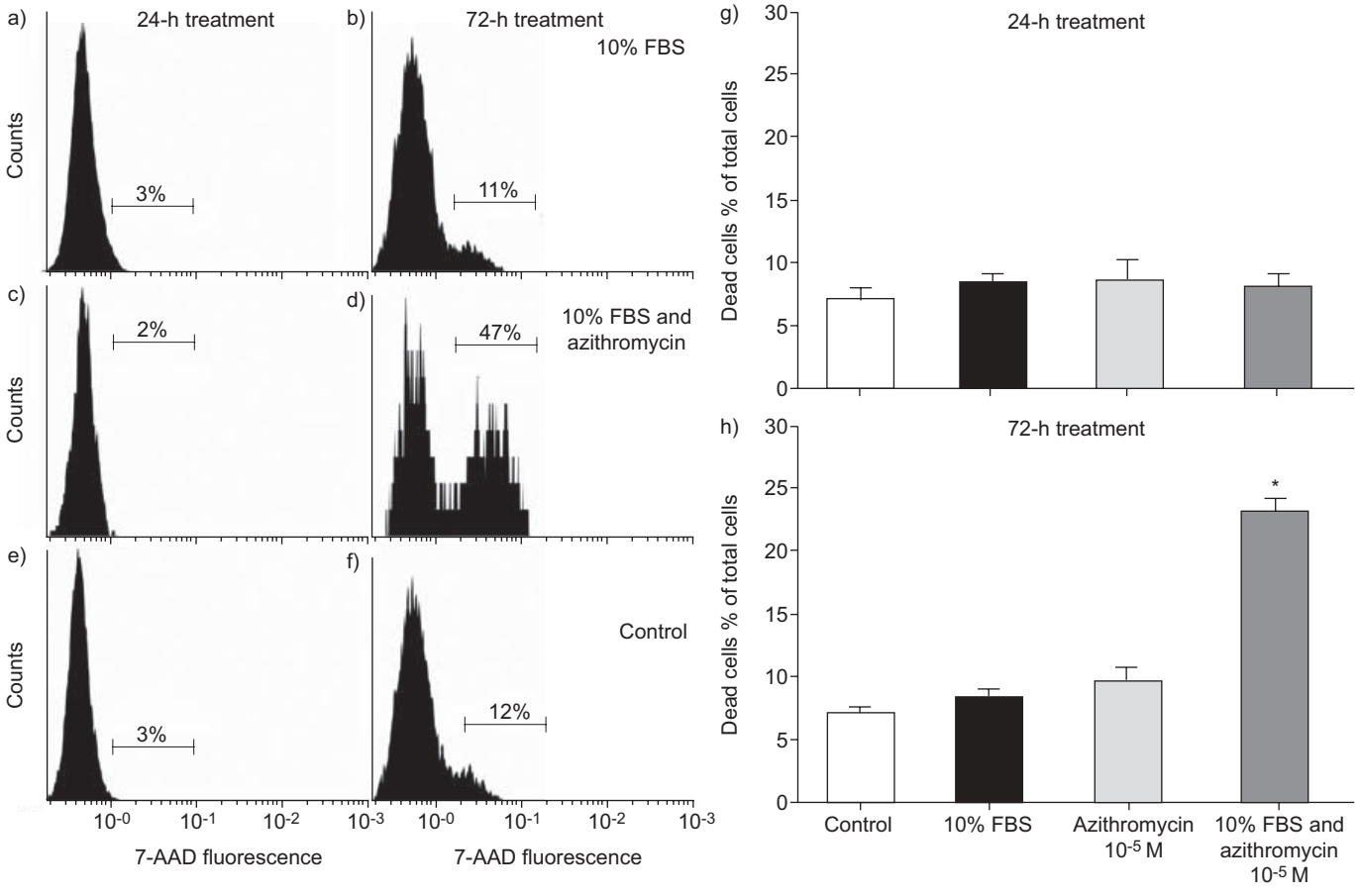

FIGURE 2. The effect of azithromycin (10^{-5} M) on the viability of smooth muscle cells. a–f) Cells were stained with 7-aminoactinomycin D (7-AAD) and analysed using a FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA). 10,000 events (*y*-axis) were collected on the basis of their forward and side scatter characteristics (>95% for each sample) and discriminated with respect to 7-AAD fluorescence (*x*-axis). Histogram plots represent cells treated or not with azithromycin in the presence or absence of 10% fetal bovine serum (FBS). Each experiment was repeated at least twice. g and h) Smooth muscle cells viability in the absence or presence of 10% FBS was assessed by Trypan blue staining 24 h or 72 h after cell treatment with 10^{-5} M azithromycin. Data are presented as mean±SEM from four independent experiments. *: $p<0.05$ compared with 10% FBS.

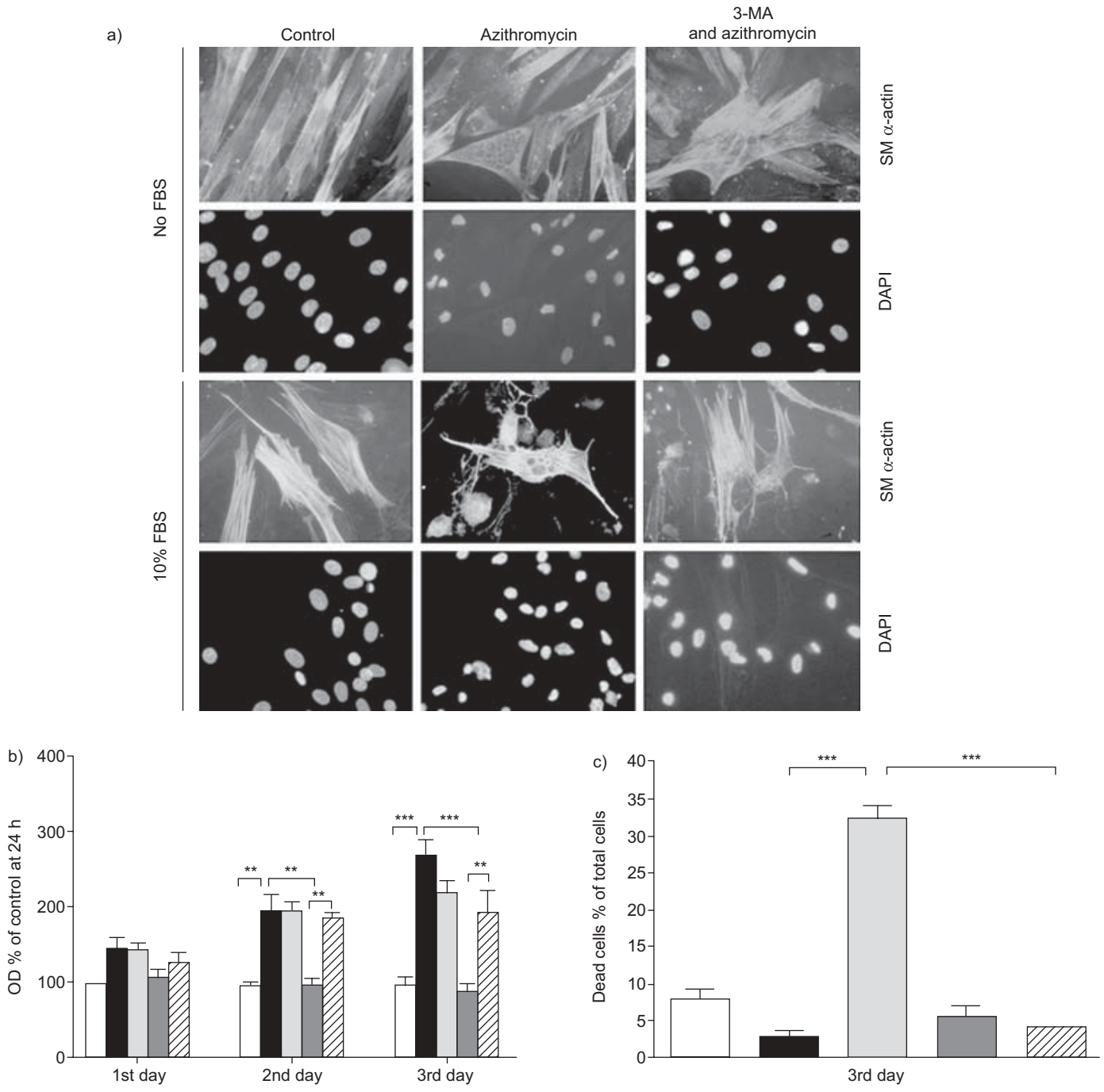

FIGURE 3. 3-Methyladenine (3-MA; 10 mM) prevents the effect of azithromycin (10^{-5} M) on a) the formation of autophagic vacuoles in airway smooth muscle cells cultured in serum free or 10% fetal bovine serum (FBS)-containing medium, observed by indirect immunofluorescence with smooth muscle (SM) α-actin antibody, b) the suppression of smooth muscle cell proliferation estimated with Cell Titer 96® AQ_{ueous} One Solution Assay (Promega, Madison, WI, USA) and c) the decrease of cell viability assessed by Trypan blue staining. Data are presented as mean±SEM from three or four independent experiments. □: controls; ■: 10% FBS; ▩: 10% FBS and 3-MA; ▨: 10% FBS and azithromycin 10^{-5} M; ▨: 10% FBS, 3-MA and azithromycin 10^{-5} M. DAPI: 4′,6-diamidino-2-phenylindole; OD: optical density. **: $p<0.01$; and ***: $p<0.001$ compared with control, 10% FBS alone or in the presence of azithromycin (10^{-5} M).

antibody (1:200; Cell Signaling Technology Inc., Danvers, MA, USA) and anti-human LC3 rabbit polyclonal antibody (1:200; Cell Signalling). The cells were then incubated with an anti-mouse immunoglobulin (Ig)G antibody coupled to fluorescein isothiocyanate or an anti-rabbit IgG antibody coupled to Cy3. Vectrashield solution containing 4′,6-diamidino-2-phenylindole (DAPI) for DNA staining was used for mounting of coverslips. The cells were observed under a Zeiss Axioskop 40 microscope (Carl Zeiss Inc., New York, NY, USA), using a Leica DFC 480 camera.

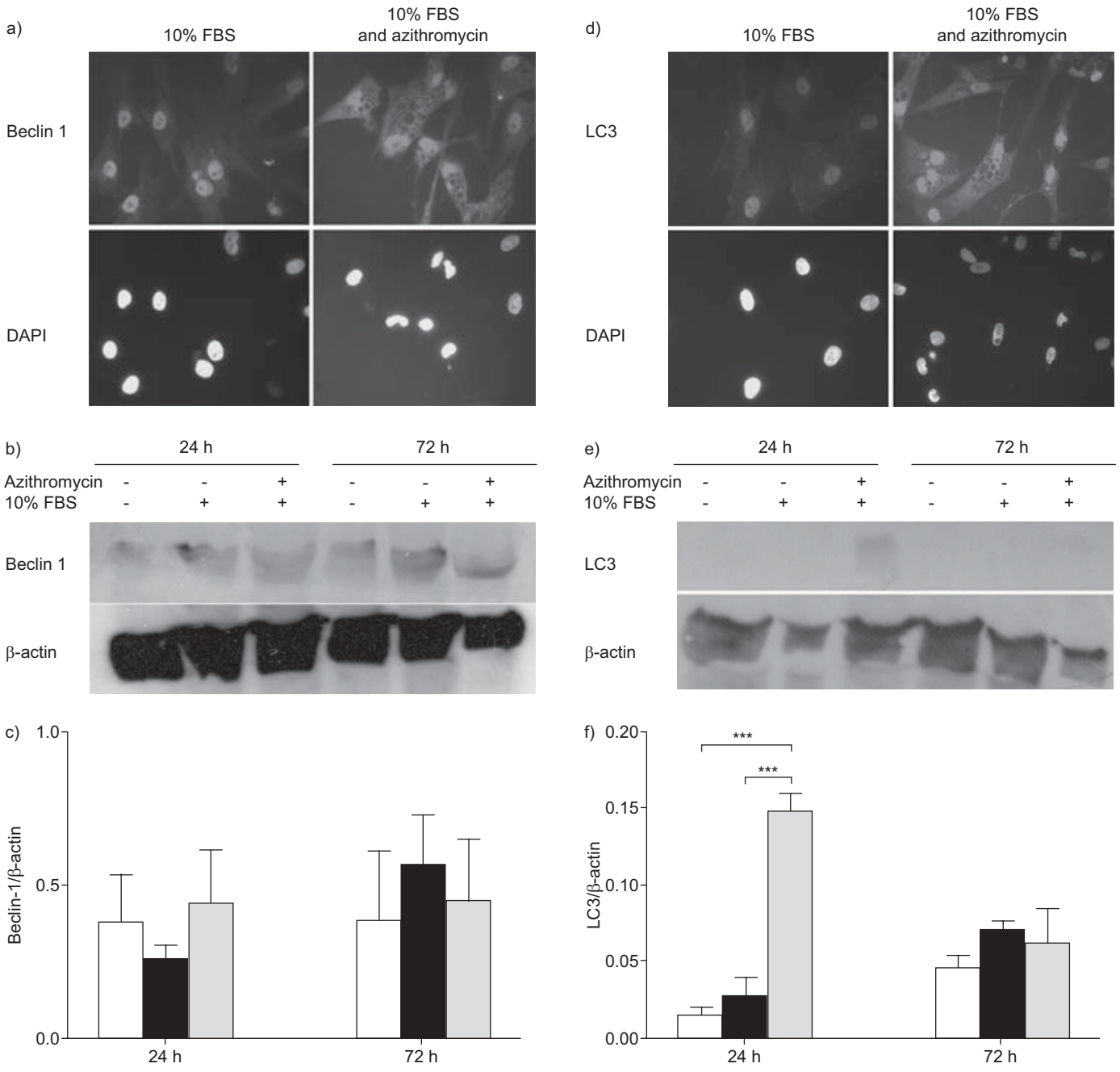

FIGURE 4. a) Beclin 1 protein is detected by indirect immunofluorescence with anti-beclin 1 antibody in smooth muscle cells treated with azithromycin (10^{-5} M). b) Western blot analysis with anti-beclin 1 and anti-β-actin (loading control) antibodies did not reveal activation of beclin 1 in azithromycin-treated smooth muscle cells, since the ratio of beclin 1 to β-actin does not change compared with the controls (c). Data are taken from three independent experiments. d) Light chain 3 (LC3) autophagic protein is detected by indirect immunofluorescence with anti-LC3 antibody in smooth muscle cells treated with azithromycin (10^{-5} M) and e) by Western blot analysis. f) The ratio of LC3 to β-actin, in cells treated with azithromycin (10^{-5} M), is significantly greater. DAPI: 4′,6-diamidino-2-phenylindole. ***: $p<0.001$ compared to the controls. Data are taken from three independent experiments. □: controls; ■: 10% fetal bovine serum (FBS); ■: 10% FBS and azithromycin 10^{-5} M.

Lysotracker Red staining

Cells were stained with Lysotracker Red (Molecular Probes, Invitrogen Corp.) [20]. Briefly, cells were incubated in 75 nM Lysotracker Red for 30 min in 37°C, 5% CO_2, mounted in a drop of PBS and observed under a Zeiss Axioskop 40 microscope, using a Leica DFC 480 camera.

Western blot analysis

Cells were lysed in 20 mM Tris·Cl pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, and 100 μg·mL^{-1} phenylmethylsulphonyl fluoride. Total cell extracts were cleared by centrifugation (10,000 × *g* for 20 min at 4°C). 40 μg of protein were analysed in 10% sodium dodecyl sulphate–polyacrylamide

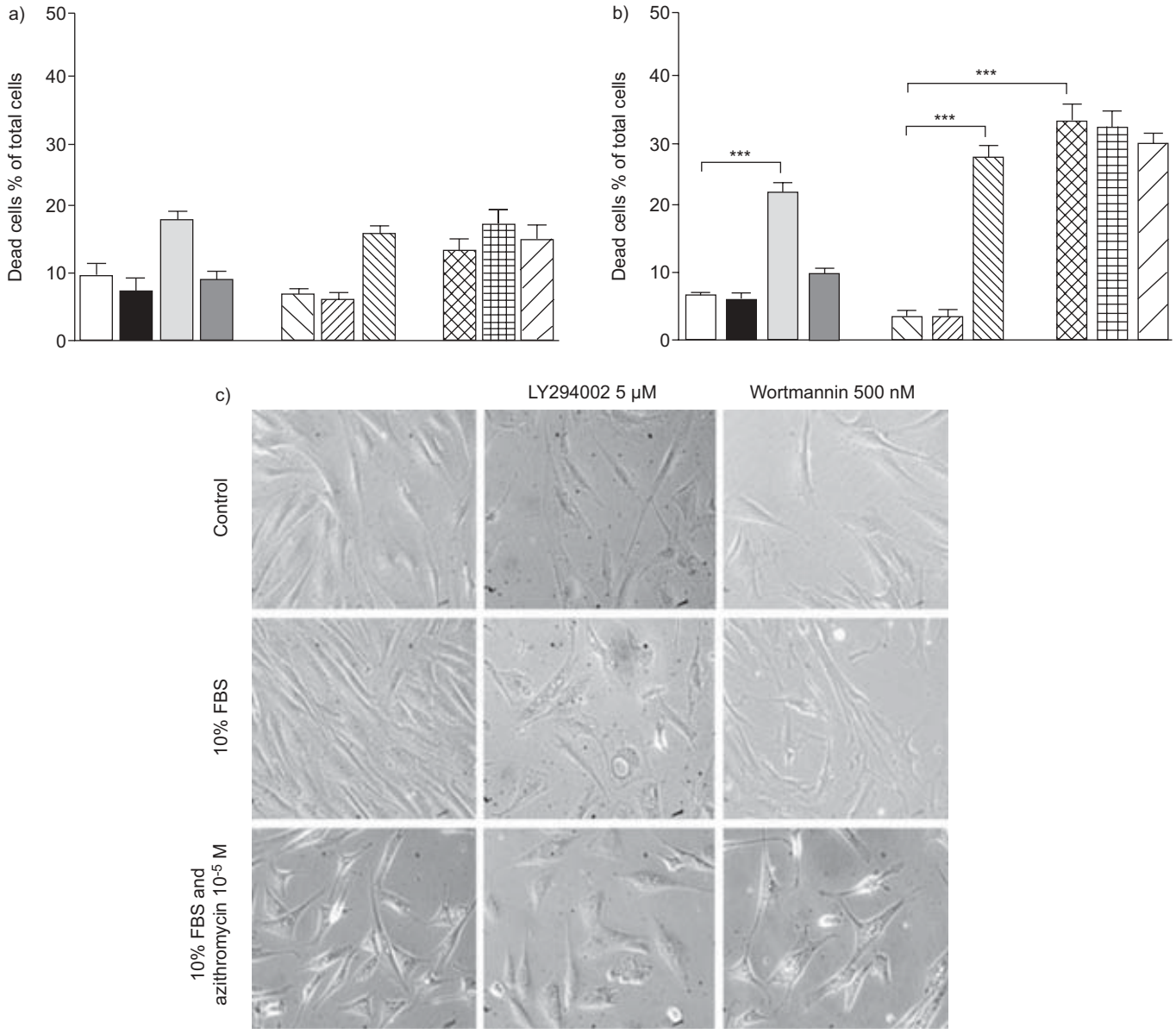

FIGURE 5. The phosphoinositide 3-kinase (PI3K) pathway may be involved in the effect of azithromycin (10^{-5} M). The PI3K pathway inhibitor wortmannin (500 nM) does not affect the decrease of smooth muscle cell viability caused by azithromycin, while azithromycin has no additive effect on the decrease of cell viability caused by LY294002 (5 μM), estimated by Trypan blue staining after 24 h (a) or 72 h (b). c) Optical microscopy shows that LY294002 or wortmannin do not affect the formation of vacuoles caused by azithromycin. Data are presented as mean±SEM from four or ten independent experiments. □: controls (n=10); ■: wortmannin 500 nM (n=4); ■: LY294002 5 μM (n=4); ■: azithromycin 10^{-5} M (n=4); ▨: 10% fetal bovine serum (FBS; n=10); ▨: 10% FBS and wortmannin 500 nM (n=4); ▨: 10% FBS and LY294002 5 μM (n=4); ■: 10% FBS and azithromycin 10^{-5} M (n=10); ▦: 10% FBS, azithromycin 10^{-5} M and wortmannin 500 nM (n=4); ▨: 10% FBS, azithromycin 10^{-5} M and LY294002 5 μM (n=4). ***: $p<0.001$ compared with control or 10% FBS.

electrophoresis gel and transferred to a nitrocellulose membrane. Western blot analysis was performed with anti-human smooth muscle β-actin mouse monoclonal antibody (1:5,000; Cell Signalling), anti-human beclin 1 rabbit polyclonal antibody (1:1,000), anti-human LC3 rabbit polyclonal antibody (1:1,000) and anti-human caspase 3 rabbit polyclonal antibody (1:1,000; Cell Signalling). Membranes were then incubated with horseradish peroxidase conjugated anti-rabbit IgG (1:3,000) or anti-mouse IgG (1:3,000), then signals visualised by enhanced luminosity.

Analysis

In cell proliferation experiments, each point was performed in triplicate and the values presented are the mean of independent experiments. All data are expressed as means±SEM and n refers to the number of experiments. Differences between means were analysed by ANOVA with statistically significant differences between groups being determined by Bonferroni's *post hoc* test. A comparison was considered significant when $p<0.05$. The statistical analysis was performed using SPSS version 11 (SPSS Inc., Chicago, IL, USA). Image analysis of the

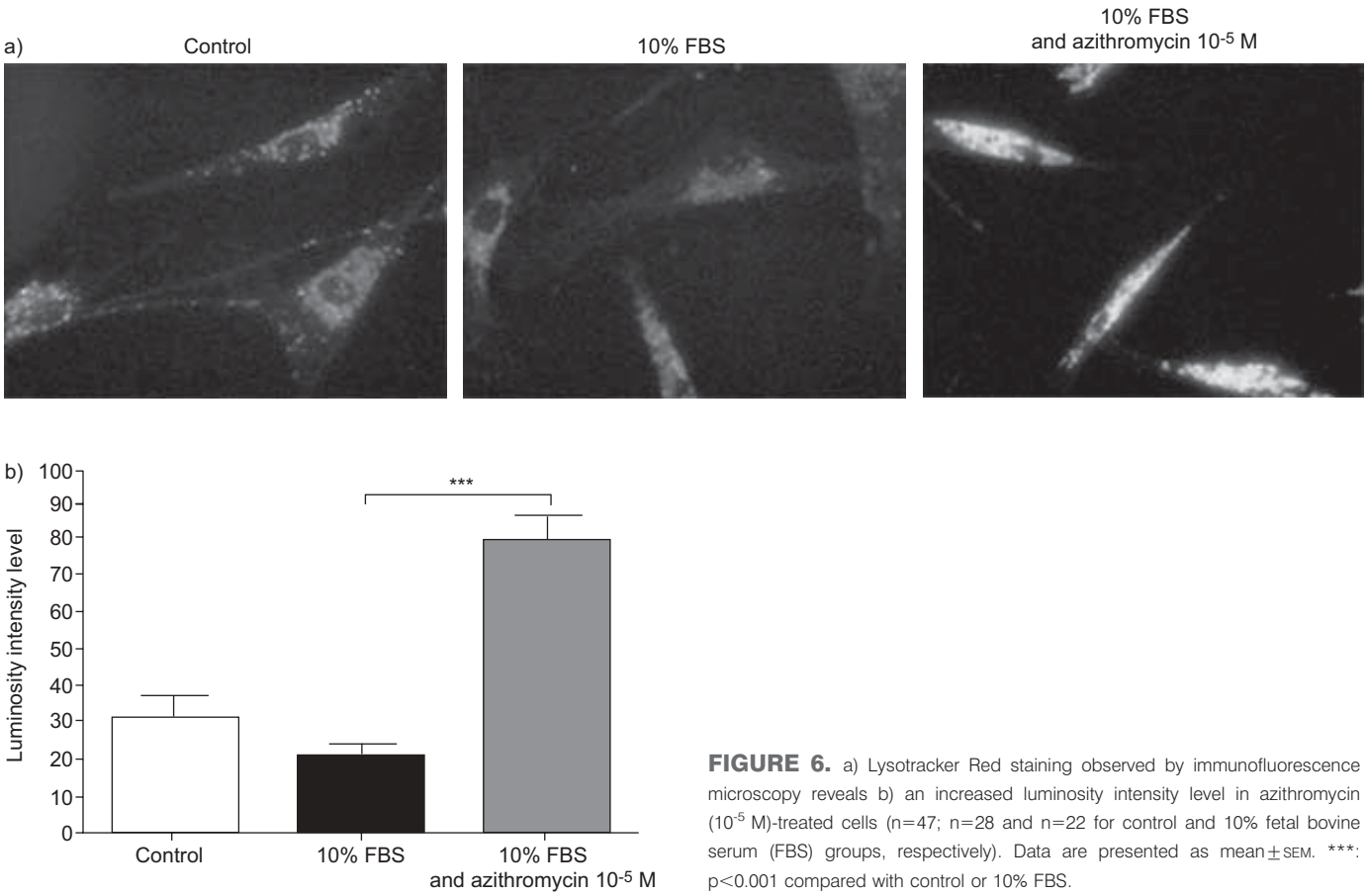

FIGURE 6. a) Lysotracker Red staining observed by immunofluorescence microscopy reveals b) an increased luminosity intensity level in azithromycin (10^{-5} M)-treated cells (n=47; n=28 and n=22 for control and 10% fetal bovine serum (FBS) groups, respectively). Data are presented as mean±SEM. ***: $p<0.001$ compared with control or 10% FBS.

images obtained from the Western blotting and the Lysotracker Red staining was conducted with the use of MacBiotronics Image J programme for Light Microscopy (National Institutes of Health, Bethesda, MD, USA) and results were expressed as intensity values.

RESULTS

Azithromycin decreased, in a dose-dependent manner, the proliferation of rabbit airway SMCs incubated in FBS-containing medium for 48 h or 72 h. The estimated cell proliferation in 10% FBS was 206.5% and 248.5% of control at 48 h and 72 h, respectively ($p<0.01$ compared with control; fig. 1a). Treatment with 10^{-6} M azithromycin did not affect significantly their proliferation. Conversely, the proliferation of SMCs treated for 48 h with 10^{-5} M azithromycin was suppressed to 114.1% of control ($p<0.01$ compared with 10% FBS; fig. 1a). Treatment, for 72 h, with 6×10^{-6} M or 10^{-5} M azithromycin reduced the proliferation to 130.9% and 99.5% of control, respectively ($p<0.001$ compared with 10% FBS; fig. 1a).

Incubation of quiescent SMC in serum-free medium in the presence of 10^{-5} M azithromycin for 24–72 h did not significantly affect SMC number (fig. 1b).

The effect of 10^{-5} M azithromycin on cell viability was assessed by Trypan blue staining and flow cytometry after 7-AAD staining. Azithromycin did not alter the percentage of dead cells 24 h after its addition in serum-free or FBS-containing medium (fig. 2). As evident by 7-AAD uptake, SMCs treated for 72 h with 10^{-5} M azithromycin, displayed a higher number of nonviable cells, as compared to 10% FBS or FBS-depleted cultures (fig. 2a–f). To this end, it must be noted that an increased side and forward scatter profile was observed in the azithromycin-treated cells. Similarly, as shown after Trypan blue staining, the percentage of dead cells in FBS-containing, but not FBS-depleted medium, increased after treatment with azithromycin for 72 h ($p<0.001$ compared with 10% FBS; fig. 2g and h).

The effect of azithromycin on cell morphology was examined by indirect immunofluorescence with an antibody against smooth muscle α-actin. Treatment of SMCs for 24 h with 10^{-5} M azithromycin in medium with or without FBS (fig. 3a), resulted in the vacuolisation of the cytoplasm and shrinkage of the cell nuclei. The vacuoles observed in the cytoplasm of SMCs after the treatment with azithromycin are reminiscent of autophagy. To assess whether azithromycin-induced vacuoles were autophagic, we tested the effect of 3-MA (10 mM), an inhibitor of class-III PI3K that blocks autophagy [21, 22]. Treatment of rabbit tracheal SMCs with 3-MA prevented the formation of vacuoles by azithromycin. The inhibition of vacuolisation was evident both in FBS-deprived (fig. 3a, upper panels) and FBS-fed cells (fig. 3a, lower panels). Treatment with 3-MA, however, led to the appearance of a punctate actin staining and partially reversed the shrinkage of SMC nuclei as shown by DAPI staining (fig. 3a). Furthermore, incubation with 3-MA reversed the reduction of the proliferation induced by azithromycin ($p<0.01$ compared with 10% FBS and

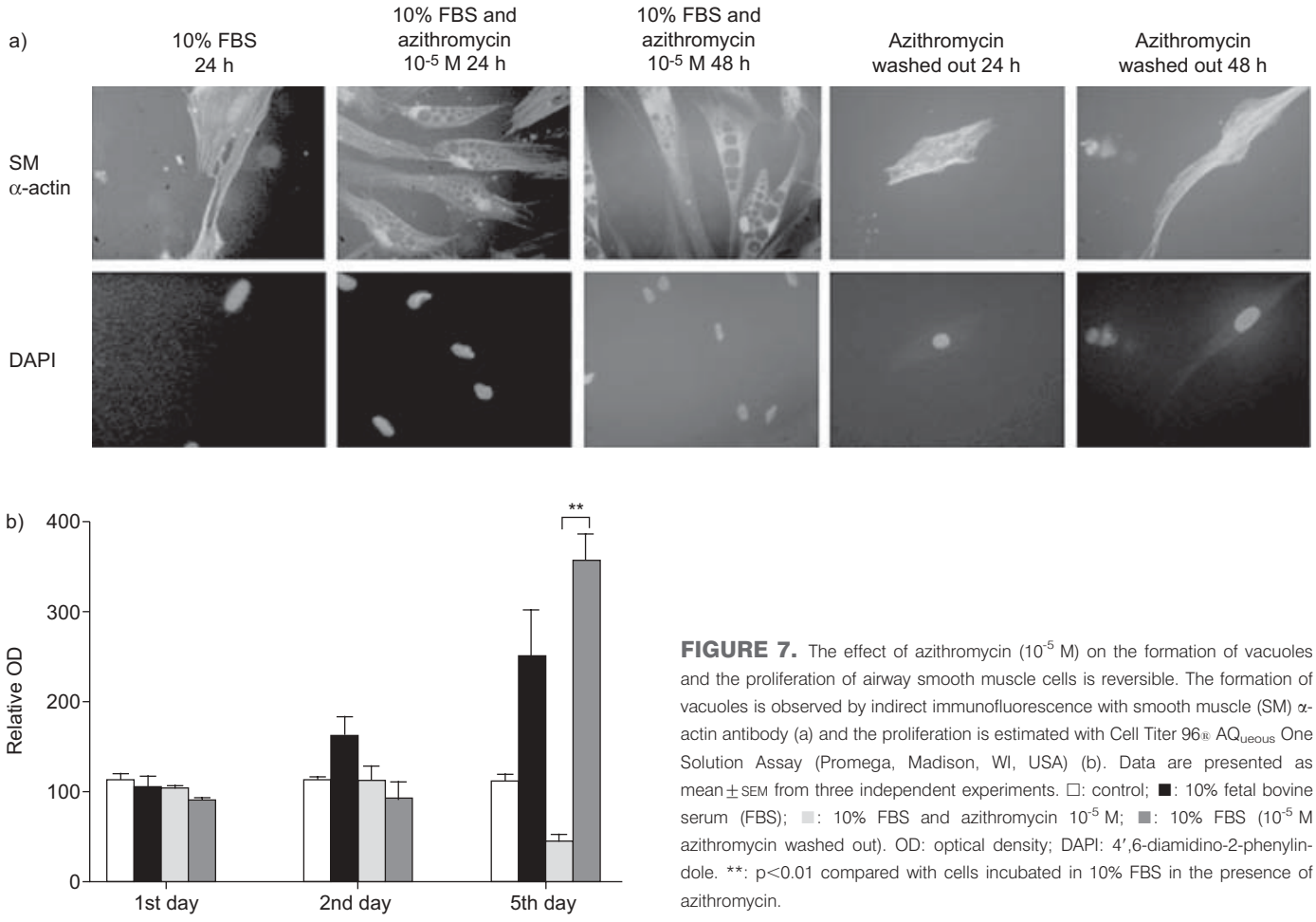

FIGURE 7. The effect of azithromycin (10^{-5} M) on the formation of vacuoles and the proliferation of airway smooth muscle cells is reversible. The formation of vacuoles is observed by indirect immunofluorescence with smooth muscle (SM) α-actin antibody (a) and the proliferation is estimated with Cell Titer 96® AQ_{ueous} One Solution Assay (Promega, Madison, WI, USA) (b). Data are presented as mean±SEM from three independent experiments. □: control; ■: 10% fetal bovine serum (FBS); ■: 10% FBS and azithromycin 10^{-5} M; ■: 10% FBS (10^{-5} M azithromycin washed out). OD: optical density; DAPI: 4′,6-diamidino-2-phenylindole. **: $p<0.01$ compared with cells incubated in 10% FBS in the presence of azithromycin.

azithromycin 10^{-5} M; fig. 3b) and decreased the percentage of dead cells ($p<0.001$ compared with 10% FBS and azithromycin 10^{-5} M; fig. 3c).

In order to further study the azithromycin-induced autophagy, we performed indirect immunofluorescence and Western blot, using anti-beclin 1 and anti-LC3 rabbit polyclonal antibodies. Although the azithromycin treated cells appear to have a more intense anti-beclin 1 signal (fig. 4a), no detectable difference of beclin 1 expression was observed by Western blot analysis in total extracts prepared from azithromycin treated cells (fig. 4b and c). Conversely, LC3 was mainly detected, both by immunofluorescence (fig. 4d) and Western blotting (fig. 4e and f), in cells treated with azithromycin. The presented quantitive data from the ratio of beclin 1 or LC3 to β-actin confirm the above results.

The possibility that the effect of azithromycin on SMC proliferation is mediated *via* the PI3K pathway was assessed using the inhibitors LY294002 (5 μM) or wortmannin (500 nM). Wortmannin did not affect the decrease of cell viability (fig. 5a and b) or the formation of vacuoles (fig. 5c) caused by azithromycin. Conversely, LY294002 alone caused an increase in the percentage of dead cells after 72 h of incubation (fig. 5b) and vacuoles appeared after 24 h of treatment (fig. 5c). When SMCs treated with azithromycin in the presence of LY294002 are compared with cells treated with LY294002 alone, there appears to be no additive effect.

In order to study the effect of azithromycin on lysosomes, Lysotracker Red, a fluorescent acidotropic probe for labelling and tracking of lysosomes in live cells was used. The treatment of SMCs with 10^{-5} M azithromycin for 24 h increased the luminosity intensity level (fig. 6).

The effect of azithromycin on cell morphology was reversed 24 h or 48 h after the removal of azithromycin from the culture medium (fig. 7a). Therefore, we investigated whether the decrease of cell proliferation caused by azithromycin could also be reversed after its removal. To this end, SMCs were incubated for 48 h in serum-free medium containing azithromycin. Then the medium was removed, cells were washed with PBS and incubated in medium containing 10% FBS. Cell number was estimated by Cell Titer 96® AQ_{ueous} One Solution Assay 24 h, 48 h or 5 days after azithromycin removal and compared with their relative number just before azithromycin was washed out. The rate of cell proliferation remained decreased 24 h or 48 h after azithromycin removal, but after 5 days cell proliferation returned to approximately positive control levels (fig. 7b). The estimated cell proliferation was 248.56% in tracheal SMC recovering from azithromycin, 355.53% in 10% FBS-incubated cells and 42.03% in cells incubated in the presence of azithromycin.

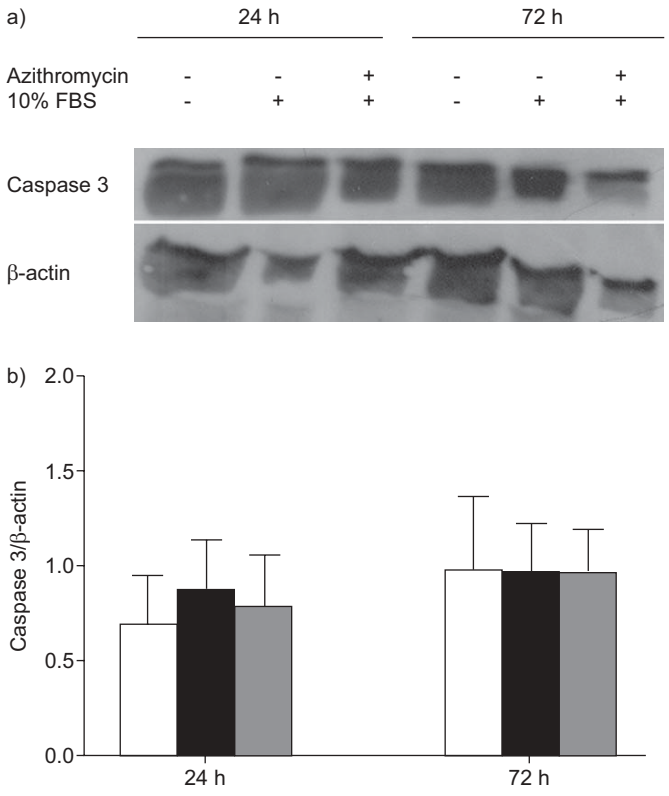

FIGURE 8. Azithromycin (10^{-5} M) does not induce activation of the apoptotic protein caspase 3 detected by Western blot analysis (a), since the ratio of caspase 3 to the β-actin (loading control) of the cell extracts is at the same levels in cells incubated with or without azithromycin (b). Data are presented as mean±SEM from three independent experiments. □: control; ■: 10% fetal bovine serum (FBS); ■: 10% FBS and azithromycin 10^{-5}.

Finally, the possible activation of the apoptotic protein caspase 3, by azithromycin was studied with Western blotting. Treatment of SMCs with azithromycin did not induce any additional activation of caspase 3 (fig. 8).

DISCUSSION

Clinical data show that macrolide therapy has been effective in the treatment of patients suffering from airway diseases in which airway wall remodelling exists, but the anti-inflammatory or antibacterial action of the macrolides is not enough to explain the patients' clinical improvement [23, 24]. A few studies on epithelial cells or SMCs suggest that macrolide antibiotics, apart from their anti-inflammatory and bacteriostatic properties, may affect cell proliferation [12, 13, 25]. In clinical studies, azithromycin is administrated in such concentrations, which result in a lung concentration of 8.93 $mg \cdot L^{-1}$, corresponding to $\sim 10^{-5}$ M azithromycin [26]. Therefore, the concentrations in which azithromycin were used were 10^{-5} to 10^{-6} M. The results from the present study demonstrate that azithromycin affects the proliferation and viability of airway SMCs. Specifically, azithromycin decreases, in a dose-dependent manner, the proliferation of rabbit tracheal SMCs cultured in medium containing 10% FBS. These results suggest that azithromycin has an antiproliferative effect only on actively proliferating cells. Furthermore, azithromycin increased the number of dead SMCs incubated in 10% FBS medium.

Azithromycin treatment also caused the formation of cytoplasmic vacuoles and shrinkage of SMC nuclei. Moreover, the inhibition of the proliferation, the increase of dead cell number and the formation of vacuoles caused by azithromycin were prevented by 3-MA, an inhibitor of autophagy. In addition, inhibition of autophagy by 3-MA partially reversed the shrinkage of SMC nuclei, as shown by DAPI staining. However, 3-MA led to the appearance of a punctate actin staining, possibly due to the accumulation of protein aggregates that are no longer cleared by autophagy. Autophagy is considered to be a major mechanism of nonapoptotic programmed cell death [27, 28]. Apoptosis is characterised by cell shrinkage, chromatin condensation, DNA degradation and fragmentation of the cell to apoptotic bodies. Cells undergoing autophagic cell death, however, are characterised by the formation of membrane-enclosed vesicles in the cytoplasm, which engulf proteins and organelles [28]. This process involves the PI3K pathway [29, 30], as well as beclin 1 and/or LC3 protein activation [28]. These vesicles fuse with lysosomes and degrade their contents by the action of lysosomal enzymes. In this study, the involvement of PI3K pathway in the effect of azithromycin was investigated by treating SMCs with PI3K pathway inhibitors LY294002 or wortmanin. Wortmannin did not alter the effect of azithromycin, while azithromycin in the presence of LY294002 had no additive effect on SMC viability compared with LY294002 alone. These results suggest that both LY294002 and azithromycin may be acting through the same pathways, which may be saturated. The autophagic protein LC3 was induced and Lysotracker Red uptake was increased pointing out an increase of lysosome number in cells treated with azithromycin, as expected for cells undergoing autophagy.

Nuclear changes, such as chromatin condensation, appear later in autophagic cell death than in apoptosis, and there is no DNA fragmentation or formation of apoptotic bodies [30]. Although autophagy is a common feature of mammalian cells undergoing programmed cell death its function in dying cells is not yet clear [30]. Studies provide evidence that autophagy and apoptosis are not mutually exclusive, but extended autophagy can induce apoptosis [31–33]. Interestingly, the antiproliferative effect of azithromycin was reversible, since cells treated with azithromycin for 48 h regain their ability to proliferate after azithromycin removal. The vacuolisation was dependent on the time of incubation with azithromycin, being more intense after 48 h of treatment. This effect was reversed when azithromycin was removed, and the cells were incubated in FBS medium. Furthermore, the lack of additional activation of the apoptotic protein caspase 3 shows that azithromycin does not cause apoptosis of SMC, at least in the experimental conditions described in this study.

Autophagy operates at basal levels in mammalian cells, but is induced under stress conditions (*e.g.* nutrient and growth factor deprivation) [30]. Autophagy is regulated by a number of cellular pathways and hence by molecules that in turn regulate these pathways [30]. Among them, the macrolide rapamycin induces autophagy by binding to and inactivating mTOR (mammalian target of ramamycin), a protein kinase that negatively regulates autophagy, while the macrolide bafilomycin A1 inhibits autophagy by the inhibition of the vacuolar ATPase [34]. To our knowledge, this is the first report of induction of autophagy by the macrolide azithromycin. A possible mechanism might

involve the ability of azithromycin to bind to phospholipids and affect the properties of biological membranes [35], since sphingolipids are regulators of autophagy [30].

In summary, our results demonstrate that azithromycin treatment of rabbit SMCs results in a reversible inhibition of cell proliferation, accompanied by the appearance of autophagic vacuoles. In addition to the anti-inflammatory/antibacterial role of azithromycin, this antiproliferative and autophagic effect on the respiratory SCMs might be of clinical significance.

STATEMENT OF INTEREST

Statements of interest for K.I. Gourgoulianis, P-A. Molyvdas and A.A. Hatziefthimiou can be found at www.erj.ersjournals.com/misc/statements.dtl

REFERENCES

1 Kudoh S, Azuma A, Yamamoto M, *et al.* Improvement of survival in patients with diffuse panbronchiolitis treated with low-dose erythromycin. *Am J Respir Crit Care Med* 1998; 157: 1829–1832.
2 Oishi K, Sonoda F, Kobayashi S, *et al.* Role of interleukin-8 (IL-8) and an inhibitory effect of erythromycin on IL-8 release in the airway of patients with chronic airway diseases. *Infect Immun* 1994; 62: 4145–4152.
3 Shirai T, Sato A, Chida K. Effect of 14-membered ring macrolide therapy on chronic respiratory tract infections and polymorphonuclear leukocyte activity. *Intern Med* 1995; 34: 469–474.
4 Miyatake H, Taki F, Taniguchi H, *et al.* Erythromycin reduces the severity of bronchial hyperresponsiveness in asthma. *Chest* 1991; 99: 670–673.
5 Cazzola M, Salzillo A, Diamare F. Potential role of macrolides in the treatment of asthma. *Monaldi Arch Chest Dis* 2000; 55: 231–236.
6 Vignola A, Mirabella F, Costanzo G, *et al.* Airway remodelling in asthma. *Chest* 2003; 123: Suppl. 3, 417S–422S.
7 Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. *Am J Respir Crit Care Med* 2001; 164: S28–S38.
8 Tamaoki J, Tagaya E, Sakai A, *et al.* Effects of macrolide antibiotics on neurally mediated contraction of human isolated bronchus. *J Allergy Clin Immunol* 1995; 95: 853–859.
9 Tagaya E, Tamaoki J, Konno K. Erythromycin inhibits cholinergic neuro-effector transmission in canine airway smooth muscle. *Res Commun Mol Pathol Pharmacol* 1994; 85: 181–192.
10 Daenas C, Hatziefthimiou AA, Gourgoulianis KI, *et al.* Azithromycin has a direct relaxant effect on precontracted airway smooth muscle. *Eur J Pharmacol* 2006; 553: 280–287.
11 Takahashi Y, Shimizu T, Sakakura Y. Effects of indomethacin, dexamethasone, and erythromycin on endotoxin-induced intraepithelial mucus production of rat nasal epithelium. *Ann Otol Rhinol Laryngol* 1997; 106: 683–687.
12 Tomita H, Osanai T, Toki T, *et al.* Roxithromycin is an inhibitor of human coronary artery smooth muscle cells proliferation: a potential ability to prevent coronary heart disease. *Atherosclerosis* 2005; 182: 87–95.
13 Fujitani Y, Trifilieff A. *In vivo* and *in vitro* effects of SAR 943, a rapamycin analogue, on airway inflammation and remodeling. *Am J Respir Crit Care Med* 2003; 167: 193–198.
14 Mizunoe S, Kadota J, Tokimatsu I, *et al.* Clarithromycin and azithromycin induce apoptosis of activated lymphocytes *via* down-regulation of Bcl-xL. *Int Immunopharmacol* 2004; 4: 1201–1207.
15 Chachami G, Simos G, Hatziefthimiou A, *et al.* Cobalt induces hypoxia-inducible factor-1α expression in airway smooth muscle cells by a reactive oxygen species- and PI3K-dependent mechanism. *Am J Respir Cell Mol Biol* 2004; 31: 544–551.
16 Cory AH, Owen TC, Barltrop JA, *et al.* Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Commun* 1991; 3: 207–212.
17 Berridge MV, Tan AS. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Arch Biochem Biophys* 1993; 303: 474–482.
18 Zelenin AV, Poletaev AI, Stepanova NG, *et al.* 7-Amino-actinomycin D as a specific fluorophore for DNA content analysis by laser flow cytometry. *Cytometry* 1984; 5: 348–354.
19 Schmid I, Krall WJ, Uittenbogaart CH, *et al.* Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry. *Cytometry* 1992; 13: 204–208.
20 Shen ZY, Xu LY, Li EM, *et al.* Autophagy and endocytosis in the amnion. *J Struct Biol* 2008; 162: 197–204.
21 Seglen PO, Gordon PB. 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. *Proc Natl Acad Sci USA* 1982; 79: 1889–1892.
22 Petiot A, Ogier-Denis E, Blommaart EF, *et al.* Distinct classes of phosphatidylinositol 3′-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. *J Biol Chem* 2000; 275: 992–998.
23 Ekici A, Ekici M, Erdemoglu AK. Effect of azithromycin on the severity of bronchial hyperresponsiveness in patients with mild asthma. *J Asthma* 2002; 39: 181–185.
24 Yates B, Murphy DM, Forrest IA, *et al.* Azithromycin reverses airflow obstruction in established bronchiolitis obliterans syndrome. *Am J Respir Crit Care Med* 2005; 172: 772–775.
25 Shimizu T, Shimizu S, Hattori R, *et al. In vivo* and *in vitro* effects of macrolide antibiotics on mucus secretion in airway epithelial cells. *Am J Respir Crit Care Med* 2003; 168: 581–587.
26 Danesi R, Lupetti A, Barbara C, *et al.* Comparative distribution of azithromycin in lung tissue of patients given oral daily doses of 500 and 1000 mg. *J Antimicrob Chemother* 2003; 51: 939–945.
27 Clarke PG. Developmental cell death: morphological diversity and multiple mechanisms. *Anat Embryol (Berl)* 1990; 181: 195–213.
28 Gozuacik D, Kimchi A. Autophagy and cell death. *Curr Top Dev Biol* 2007; 78: 217–245.
29 Cui Q, Tashiro S, Onodera S, *et al.* Augmentation of oridonin-induced apoptosis observed with reduced autophagy. *J Pharmacol Sci* 2006; 101: 230–239.
30 Tsujimoto Y, Shimizu S. Another way to die: autophagic programmed cell death. *Cell Death Differ* 2005; 12: Suppl. 2, 1528–1534.
31 Canu N, Tufi R, Serafino AL, *et al.* Role of the autophagic-lysosomal system on low potassium-induced apoptosis in cultured cerebellar granule cells. *J Neurochem* 2005; 92: 1228–1242.
32 Cui Q, Tashiro S, Onodera S, *et al.* Autophagy preceded apoptosis in oridonin-treated human breast cancer MCF-7 cells. *Biol Pharm Bull* 2007; 30: 859–864.
33 Yan CH, Yang YP, Qin ZH, *et al.* Autophagy is involved in cytotoxic effects of crotoxin in human breast cancer cell line MCF-7 cells. *Acta Pharmacol Sin* 2007; 28: 540–548.
34 Shacka J, Klocke B, Shibata M, *et al.* Bafilomycin A1 inhibits cloroquine-induced death of celebellar granule neurons. *Mol Pharmacol* 2006; 69: 1125–1136.
35 Fa N, Ronkart S, Schanck A, *et al.* Effect of the antibiotic azithromycin on thermotropic behavior of DOPC or DPPC bilayers. *Chem Phys Lipids* 2006; 144: 108–116.