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Original article

Tilmicosin-induced bovine neutrophil apoptosis is cell-specific and downregulates spontaneous LTB_4 synthesis without increasing Fas expression

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Abstract – The pathology of bacterial pneumonia, such as seen in the bovine lung infected with *Mannheimia haemolytica*, is due to pathogen virulence factors and to inflammation initiated by the host. Tilmicosin is a macrolide effective in treating bacterial pneumonia and recent findings suggest that this antibiotic may provide anti-inflammatory benefits by inducing polymorphonuclear neutrophilic leukocyte (PMN) apoptosis. Using an in vitro bovine system, we examined the cell-specificity of tilmicosin, characterized the changes in spontaneous leukotriene B_4 (LTB_4) synthesis by PMN exposed to the macrolide, and assessed its effects on PMN Fas expression. Previous findings demonstrated that tilmicosin is able to induce PMN apoptosis. These results were confirmed in this study by the Annexin-V staining of externalized phosphatidylserine and the analysis with flow cytometry. The cell-specificity of tilmicosin was assessed by quantification of apoptosis in bovine PMN, mononuclear leukocytes, monocyte-derived macrophages, endothelial cells, epithelial cells, and fibroblasts cultured with the macrolide. The effect of tilmicosin on spontaneous LTB_4 production by PMN was evaluated via an enzyme-linked immunosorbent assay. Finally, the mechanisms of tilmicosin-induced PMN apoptosis were examined by assessing the effects of tilmicosin on surface Fas expression on PMN. Tilmicosin-induced apoptosis was found to be at least partially cell-specific, as PMN were the only cell type tested to die via apoptosis in response to incubation with tilmicosin. PMN incubated with tilmicosin under conditions that induce apoptosis spontaneously produced less LTB_4, but did not exhibit altered Fas expression. In conclusion, tilmicosin-induced apoptosis is specific to PMN, inhibits spontaneous LTB_4 production, and occurs through a pathway independent of Fas upregulation.

macrolide / neutrophil / apoptosis / pasteurellosis / inflammation

1. INTRODUCTION

The pathophysiology of bovine pneumonic pasteurellosis is initiated by bacterial virulence factors and the host immune response [8, 18, 22, 30]. During infection, *Mannheimia haemolytica* secretes a heat-labile leukotoxin, which stimulates polymorphonuclear neutrophilic leukocytes (PMN) to release the pro-inflammatory mediator leukotriene B_4 (LTB_4) and forms transmembrane pores in these cells, leading

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to cell death [18, 34]. This process is exacerbated by pro-inflammatory cytokines produced by the host [30]. The release of LTB_4 results in the recruitment of additional PMN to the lung. Reactive oxygen species and proteolytic enzymes released by the infiltrating PMN damage the bronchial epithelium, causing a self-perpetuating inflammatory response that in turn leads to pulmonary failure and death.

The discovery that certain macrolide antibiotics possess anti-inflammatory properties independent of their anti-bacterial effects was made over 20 years ago [21, 37]. However, only recently have investigators begun to elucidate the mechanisms by which such antibiotics counteract inflammation. These mechanisms may include the reduction of PMN and mononuclear leukocyte infiltration [25, 31], the inhibition of pro-inflammatory cytokine expression in PMN, monocytes, and epithelial cells [24, 27, 32, 44], the inhibition of PMN adhesion to epithelial cells [24, 26], and the suppression of the PMN oxidative burst [15, 32, 45]. In addition, recent studies have demonstrated the ability of several macrolides and azalides, including erythromycin and azithromycin, to induce PMN apoptosis in vitro [7, 16, 20, 28]. PMN apoptosis, and the subsequent uptake of these cells by macrophages, is an important mechanism for the resolution of inflammation [9, 39]. PMN apoptosis may be initiated by various internal or external stimuli [1], including the activation of surface Fas (CD95) [3]. Unlike death via necrosis, membrane integrity is maintained throughout the apoptotic process, ensuring that the histotoxic contents of PMN are not released into the extracellular environment [43]. Cells dying via apoptosis are rapidly phagocytosed by nearby macrophages, a process that is regulated by a number of receptor-mediated recognition events, including the surface expression of phosphatidylserine on apoptotic cells [9, 11]. The phagocytosis of apoptotic PMN actively inhibits the synthesis of various pro-inflammatory cytokines by macrophages [12]. Together, these observations have established that PMN apoptosis is an important prerequisite for the return of inflammatory sites to normal physiological conditions.

Tilmicosin is a 16-membered macrolide used effectively to treat *M. haemolytica* infection in cattle. The effectiveness of tilmicosin in the treatment of pneumonic pasteurellosis has been attributed to its pharmacodynamic distribution in appropriate tissues. A single subcutaneous injection of 10 mg/kg tilmicosin in cattle leads to pulmonary tissue concentrations above the MIC for *M. haemolytica* at least up to 72 h post-inoculation [33]. A recent study indicated that this macrolide induced PMN apoptosis and reduced LTB_4 levels in bronchoalveolar lavage samples of calves with *M. haemolytica*-infected lungs [6]. Further experiments demonstrated that tilmicosin causes PMN apoptosis in vitro regardless of the presence or absence of live bacteria, clearly distinguishing the pro-apoptotic effects of this antibiotic from its anti-microbial properties [7]. In addition, exposure of purified PMN to tilmicosin induced surface expression of phosphatidylserine and significantly enhanced their phagocytic uptake by monocyte-derived macrophages [7].

Together, the findings suggest that induction of PMN apoptosis by tilmicosin confers anti-inflammatory properties to this antibiotic. However, it is not known whether the pro-apoptotic effects of tilmicosin are cell-specific nor is it understood whether this macrolide directly modulates the synthesis of pro-inflammatory mediators by PMN. In addition, the molecular mechanisms responsible for tilmicosin-induced PMN apoptosis remain unclear. The aims of the present study were: (i) to determine whether tilmicosin-induced apoptosis is cell-specific, (ii) to assess the effects of tilmicosin-induced apoptosis on LTB_4 production by PMN, and (iii) to identify the effects of tilmicosin on Fas receptor expression in PMN.

2. MATERIALS AND METHODS

2.1. PMN purification

Peripheral blood was drawn from the jugular veins of healthy Holstein calves into Acid Citrate Dextrose vacutainers (ACD solution A; Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). The blood was pooled into a 50 mL polypropylene centrifuge tube and spun at 1 200 *g* in a Beckman J-6B centrifuge for 20 min at 4 °C without braking. The plasma, buffy coat, and top of the erythrocyte pack were removed. Contaminating erythrocytes were removed with hypotonic lysis steps, as previously described [7]. After the final lysis step, the leukocyte cell pellet was resuspended in 10 mL of 25 mM HEPES-buffered RPMI 1640 cell culture medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 2 mM L-glutamine (Sigma). The concentration and viability of cells was calculated with a haemocytometer and trypan blue exclusion (Flow Laboratories Inc., McLean, VA, USA). Differential cell counts were performed on cytospin preparations stained with Diff-Quik (Baxter Healthcare Corp., Miami, FL, USA) to assess PMN purity. The isolated cells consisted of > 90% PMNs with a viability of > 95%.

2.2. Mononuclear leukocyte purification

Blood from Holstein calves was collected as above. Whole blood was centrifuged at 1 200 *g* for 20 min at room temperature without braking. Mononuclear leukocytes were isolated and purified from the buffy coat using a polysucrose and sodium diatrizoate gradient (Histopaque® 1077, Sigma Diagnostics, St. Louis, MO, USA), as previously described [7]. Contaminating erythrocytes were eliminated by resuspension in pyrogen-free sterile water (MTC Pharmaceuticals, Cambridge, ON, Canada) three times for 1 min each and osmolarity was restored with 2× HBSS (Gibco BRL, Life Technologies, Inc., Grand Island, NY, USA). Following one additional wash with 1x HBSS, purified cells were resuspended in Iscove's Modified Dulbecco's Medium (Gibco BRL) to a final concentration of 6×10^6 cells/mL. The mononuclear cells consisted of 25–35% monocytes and 65–75% lymphocytes, with an overall viability of > 90%.

2.3. Macrophage differentiation

Isolated mononuclear cells were incubated (37 °C, 5% CO_2) in Iscove's Modified Dulbecco's Medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 80 µg/mL tylosin (all from Sigma) for 60 min in 24-well plates. Non-adherent cells were removed by three washes with warm 1× HBSS. The remaining adherent monocytes were incubated for seven days and allowed to differentiate into macrophages with the medium being replaced every two days. We have previously established that this procedure yields > 95% pure mature macrophages [7].

2.4. Cell lines

The bovine pulmonary endothelial cell line CPA47 (ATCC, Manassas, VA, USA) was grown in F-12K media, Kaighn's modification (Gibco BRL) containing 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 80 µg/mL tylosin. The bovine kidney epithelial cell line MDBK (NBL-1, ATCC) was grown in minimum essential medium (Eagle's modification) containing 2 mM L-glutamine and Earle's balanced salt solution with 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate (all from ATCC) and 10% FBS. The bovine tracheal fibroblast cell line EBTr (NBL-4, ATCC) was grown in minimum essential medium (Eagle's modification) containing 2 mM L-glutamine and Earle's balanced salt solution with 1.5 g/L sodium bicarbonate,

0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 10% FBS. All cell lines were cultured at 37 °C and 5% CO_2.

2.5. Effects of tilmicosin on other cell types

The cellular specificity of tilmicosin-induced apoptosis was determined with the use of various types of bovine cells. Mononuclear leukocytes, monocyte-derived macrophages, CPA47 endothelial cells, MDBK epithelial cells, and EBTr tracheal fibroblasts were cultured in 24-well plates as described above. On the day of the experiment, the culture medium was discarded and the cells were incubated in 0.5 μg/mL of tilmicosin (Micotil, Provel, Guelph, ON, Canada) or PBS for 2 h. This concentration is consistent with the known blood levels of the antibiotic during treatment and with previous studies [7, 33]. After the incubation, the supernatant was discarded, and the plate was snap-frozen and stored at –70 °C. Cell apoptosis was determined using a cell death detection enzyme-linked immunosorbent assay (ELISA) (Roche Molecular Biochemicals, Laval, QC, Canada) according to the manufacturer's instructions. This quantitative sandwich enzyme immunoassay specifically measures the histone regions (H1, H2A, H2B, H3, and H4) of mono- and oligonucleosomes, which are released during apoptosis. Photometric development was monitored by reading the plate (THERMOmax microplate reader; Molecular Devices Corp., Menlo Park, CA, USA) (405 nm) at 5 min intervals for a total of 60 min. Apoptosis was measured in triplicate from 10^5 cells of each group and expressed as the absorbance ratios of the experimental cell lysates versus the mean absorbance from PBS-treated control cells, which was arbitrarily set at 1.0, as previously described [6, 7].

2.6. Phosphatidylserine translocation

As a marker of apoptosis, we assessed the effect of tilmicosin on the translocation of phosphatidylserine in PMN and mononuclear leukocytes by fluorescence microscopy [7]. Briefly, cells were isolated and incubated with either PBS control or 0.5 μg/mL tilmicosin for 2 h at 37 °C and 5% CO_2. Following incubation, the cells were spun at 200 *g* for 5 min (Hermle Z180M, National Labnet Co., Woodbridge, NJ, USA). The supernatant was discarded and the pellet was resuspended with 0.1 mL of a staining solution containing 20 μL of Annexin-V fluorescein, 20 μL propidium iodide, and 1 mL of HEPES buffer, all from Roche. The cells were incubated in the dark for 15 min at room temperature. After incubation, the cells were washed with 0.1 mL of HEPES buffer and were spun at 200 *g* for 5 min. The pellet was resuspended in HEPES buffer and a wet mount of 15 μL of the stained cells was visualized under fluorescent microscopy using a Zeiss Axiovert 25 CFL inverted microscope. Apoptotic cells appeared solid green (fluorescein isothiocyanate filter, excitation = 450 nm, emission = 490 nm) and necrotic cells appeared green (fluorescein isothiocyanate filter) with red nuclei (CY-3 filter, excitation = 535 nm, emission = 550 nm). The mean percentages of apoptotic and necrotic cells in each sample were obtained under 400× magnification from 10 randomly selected fields containing 25–30 cells.

To further confirm the pro-apoptotic properties of tilmicosin, translocation of phosphatidylserine in PMN was also assessed using a flow cytometer (Becton Dickinson FacScan, San Jose, CA, USA). PMN were prepared and stained as above, but without adding propidium iodide. As a positive control, an extra group of PMN was incubated with staurosporine (200 nM, Sigma), a specific inducer of apoptosis [2]. After staining, cells were washed, and fixed with 2% paraformaldehyde for 1 h at room temperature. The cells were then pelleted and the fixative was discarded. The pellet was resuspended in HEPES buffer and stored in the dark at 4 °C until analysis. When assessing PMN apoptosis, 10 000 granulocytes were gated based on their light scatter

properties (data not shown), and the fluorescence of these cells was measured. The binding of Annexin-V was assessed by measuring the mean fluorescence intensity (MFI) of tilmicosin and staurosporine treated PMN, and the results were compared to fluorescence levels of PMN exposed to saline vehicle.

2.7. LTB_4 quantification

To assess the effects of tilmicosin on spontaneous LTB_4 synthesis, 6×10^6 PMN were isolated and incubated with PBS, 0.5 µg/mL tilmicosin, or 0.5 µg/mL oxytetracycline (Sigma) for 0.5, 1, 2, or 4 h at 37 °C and 5% CO_2. Antibiotic concentrations were consistent with the pharmacokinetics of both compounds and with previous studies using similar experimental approaches [7, 33]. Oxytetracycline, another antibiotic used to treat bovine pneumonic pasteurellosis, does not induce PMN apoptosis [7]. Following incubation, supernatants were collected, aliquoted, and stored at –70 °C. The concentration of LTB_4 in supernatants was determined using an enzyme-linked immunosorbent assay (ELISA) (Cayman Chemicals, Ann Arbor, MI, USA).

2.8. Surface Fas expression

Additional experiments attempted to determine whether the pro-apoptotic effect of tilmicosin was associated with altered Fas expression on PMN. Isolated PMN (10^6 cells/mL) were incubated (37 °C, 5% CO_2) with either tilmicosin (0.5 µg/mL) or PBS for 2 h. Following incubation, the cells were pelleted by centrifugation (200 *g*, 5 min) and washed once with PBS. The cells were fixed in 2% paraformaldehyde for 1 h. The fixative was aspirated and the cells were washed twice with PBS prior to staining. PBS with 1% BSA was used to dilute the primary and secondary antibodies and as a control. Fas was labeled by incubating the PMN with 5 µg/mL murine anti-human Fas which is cross-reactive in bovine systems (Upstate Biotech, Lake Placid, NY, USA) or control for 1 h at 37 °C, as in a previously published study that validates this assay [49]. Cells were then pelleted and washed three times with 1× PBS for 5 min. Cells were incubated in the dark at room temperature in a 1:200 dilution of FITC-conjugated goat anti-murine IgM (Calbiochem, San Diego, CA, USA) for 1 h. Following incubation, the cells were again pelleted and washed three times with PBS for 5 min. The pellet was resuspended in 500 µL PBS and stored in the dark at 4 °C. PMN were gated based on their light scatter properties (data not shown) and the parameters of 10 000 gated PMN were measured with flow cytometry. The mean fluorescence intensity of tilmicosin-treated PMN was compared to mean fluorescence levels of control PMN.

2.9. Statistical analyses

Results were expressed as the mean ± standard errors of the means. One-way analysis of variance tests were performed, followed by Tukey's test for multiple-comparison analysis where applicable and the non-parametric Kruskal-Wallis statistical test when appropriate. Flow cytometry histograms were compared with the Kolmogorov-Smirnov goodness of fit test, as previously described [50], using the FACScan software. *P*-values of less than 0.05 were considered significant.

3. RESULTS

3.1. Detection of apoptosis via flow cytometry

The externalization of phosphatidylserine by PMN incubated with 0.5 µg/mL tilmicosin was measured with FITC-conjugated Annexin-V staining and quantified by flow cytometry. Incubation with tilmicosin or with the known pro-apoptotic compound staurosporine significantly ($P <$ 0.05) increased MFI compared to controls

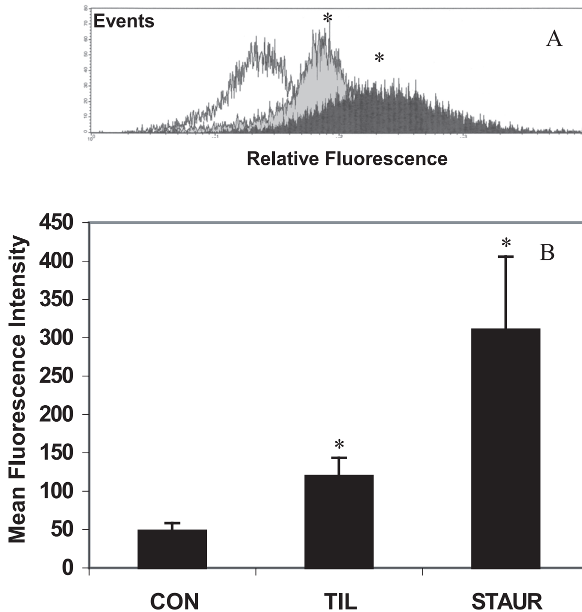

Figure 1. Apoptosis in bovine peripheral neutrophils incubated for 2 h with 0.5 μg/mL tilmicosin as determined by annexin V staining of externalized phosphatidylserine. (A) Representative cell fluorescence profile of peripheral bovine PMN incubated with either PBS (control; □), 0.5 μg/mL tilmicosin (■), or 200 nM staurosporine (■) for 2 h as analyzed by flow cytometry. (B) Mean fluorescence intensity ± SEM of PMN incubated with PBS (CON), 0.5 μg/mL tilmicosin (TIL) or 200 nM staurosporine (STAUR). $n = 7$ for CON and TIL groups, $n = 6$ for STAUR group. * $P < 0.05$ versus PBS control.

(Figs. 1A and 1B), reflecting the induction of apoptosis by both products.

3.2. Cell specificity of tilmicosin-induced apoptosis

The ability of tilmicosin to induce apoptosis of different bovine cell types was assessed in vitro (Fig. 2). PMN incubated with tilmicosin for 2 h were used as a positive control. Circulating bovine mononuclear leukocytes exposed to tilmicosin for 2 h did not show any difference in levels of apoptosis when compared to sham-treated cells using either Annexin-V FLUOS staining (Fig. 2A) or cell death ELISA (Fig. 2B). Bovine monocyte-derived macrophages, pulmonary endothelial cells, kidney epithelial cells, and tracheal fibroblasts exposed to tilmicosin for 2 h did not show any difference in levels of apoptosis when compared to controls (Fig. 2B). In contrast, tilmicosin significantly increased apoptosis in PMN (Fig. 2B).

3.3. Spontaneous LTB_4 synthesis

In an attempt to further characterize the anti-inflammatory effects of tilmicosin, we assessed the levels of LTB_4 released spontaneously by PMN incubated in vehicle, oxytetracycline, or tilmicosin. Two hours post-incubation, PMN exposed to PBS alone or oxytetracycline released significant amounts of LTB_4 (Fig. 3). In contrast, concentrations of LTB_4 measured in the culture medium of tilmicosin-treated PMN remained at baseline levels throughout the entire experimental time.

3.4. PMN surface Fas expression

To investigate the mechanism by which tilmicosin enhances PMN apoptosis, surface Fas expression on PMN was determined by flow cytometry (Fig. 4). PMN incubated with the secondary antibody (FITC-IgG) alone exhibited baseline staining in both groups. In addition, the primary antibody did not auto-fluoresce (data not shown). Incubation with both antibodies significantly enhanced staining, both in cells exposed to tilmicosin and in controls, consistent with the specific detection of constitutive Fas expression in both cell populations. Mean fluorescence was not

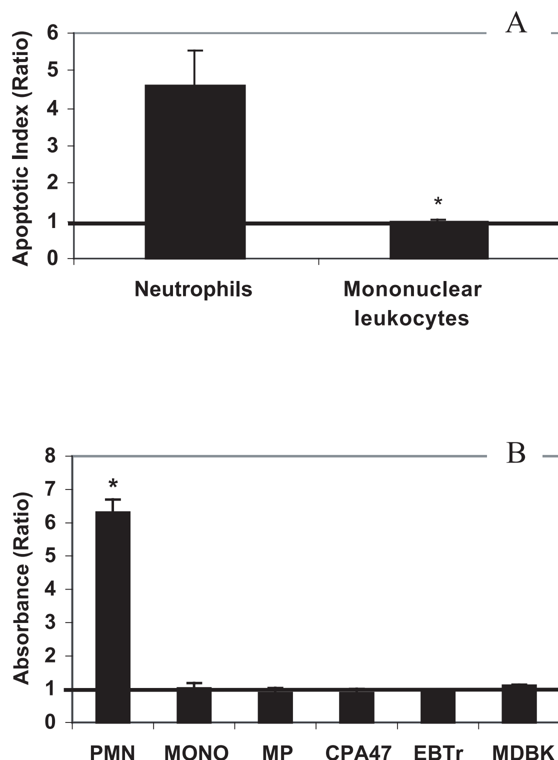

Figure 2. Effect of a 2 h incubation with 0.5 μg/mL tilmicosin on apoptosis in several cell types. (A) Apoptosis of neutrophils and mononuclear leukocytes as determined by annexin V staining. Apoptotic index was calculated as percentage apoptosis versus values from cells incubated in PBS, which were arbitrarily set at 1.0 (thick line). Values are means ± SEM; $n = 6$ per group. * $P < 0.05$ versus PMN group. (B) Apoptosis of neutrophils (PMN), mononuclear leukocytes (MONO), monocyte-derived macrophages (MP), pulmonary endothelial cells (CPA47), kidney epithelial cells (MDBK), and tracheal fibroblasts (EBTr) as determined by cell death ELISA. Data were calculated as absorbance ratios versus values from cells incubated in PBS, which were arbitrarily set at 1.0 (thick line). Values are means ± SEM; $n \geq 11$ per group. * $P < 0.05$ versus control.

different between sham-treated and tilmicosin-treated PMN, suggesting that tilmicosin-induced PMN apoptosis is not associated with an upregulation of Fas expression.

4. DISCUSSION

Results from this study confirmed previous in vivo [6] and in vitro [7] observations that the macrolide tilmicosin induces apoptosis in bovine peripheral PMN. In addition, the findings indicated that this effect was at least in part cell specific, as tilmicosin did not induce apoptosis in bovine mononuclear leukocytes, macrophages, fibroblasts, endothelial, or epithelial cells. The results also showed that tilmicosin may directly inhibit the synthesis of pro-inflammatory LTB_4 in PMN. Finally, the present study demonstrated that tilmicosin-mediated PMN apoptosis occured in the absence of increased Fas receptor expression on these cells. Together, the findings further support the hypothesis

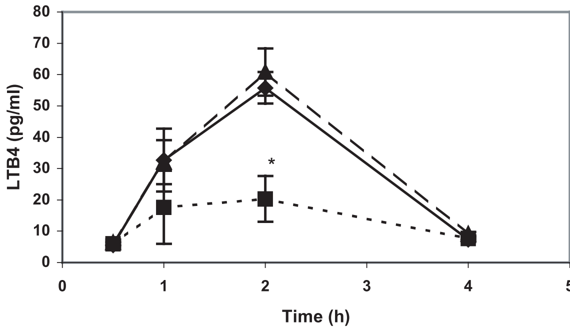

Figure 3. Spontaneous neutrophil leukotriene B_4 synthesis following treatment with PBS (–◆–), 0.5 μg/mL oxytetracycline (--▲--), or 0.5 μg/mL tilmicosin (…■…) as measured by ELISA. Data represent means ± SEM; $n = 6$ per group. * $P < 0.05$ versus PBS and oxytetracycline.

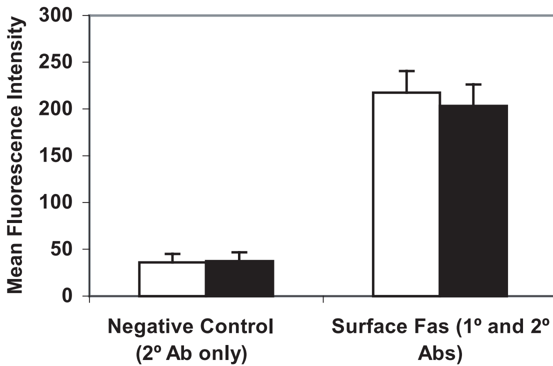

Figure 4. Surface Fas expression of neutrophils treated with PBS (□) or 0.5 μg/mL tilmicosin (■) for 2 h. Mean fluorescence intensities ± SEM are shown for $n = 6$ per group. Neutrophil surface Fas expression was not affected by incubation with tilmicosin for 2 h.

that induction of PMN apoptosis may confer anti-inflammatory properties to antibiotics such as tilmicosin.

PMN infiltration to sites of airway infection is a crucial host response to invasion by bacterial pathogens. However, local necrosis and self-perpetuating recruitment of PMN causes severe pulmonary damage in a number of pneumonic diseases, including bovine pasteurellosis [8, 18, 33]. Indeed, if unchecked, activated PMN produce various proteolytic enzymes and reactive oxygen species that may lead to extensive epithelial injury [18]. In addition, PMN at the site of inflammation recruit and activate additional PMN through the release of potent chemoattractants such as IL-8 and LTB_4 [35, 41]. While tilmicosin does not appear to alter IL-8 gene expression in the inflamed lung of *M. haemolytica*-infected calves [14], reduced LTB_4 concentrations are found in pulmonary samples of tilmicosin-treated animals [6]. This effect is accompanied by increased apoptosis in bronchoalveolar PMN without alteration of their chemotactic, phagocytic, or oxidative functions [6]. Whether increased PMN apoptosis and reduced LTB_4 synthesis are causally linked requires further investigation. Tilmicosin induces PMN apoptosis in vitro in the presence or absence of live bacteria, and stimulates the phagocytic uptake of these cells by macrophages [7], a known contributor to the resolution of inflammation [9]. Therefore, we hypothesized that the clinical efficacy of tilmicosin may be explained at least in part by its pro-apoptotic effects on PMN. The present study was carried out to further investigate this hypothesis.

Previous studies have established that tilmicosin induces apoptosis in bovine PMN

[6, 7]. Consistent with these observations, a recent study demonstrated that treatment with tilmicosin, but not with ceftiofur, significantly increases the level of apoptosis in bronchoalveolar PMN of calves with subacute or chronic airway diseases [46]. In contrast, another study was unable to detect significant levels of apoptosis in circulating PMN of tilmicosin-treated calves experimentally infected with *M. haemolytica* [13]. However, these results remain difficult to interpret as apoptosis has not been measured in bronchoalveolar cells in that study [13]. Moreover, the authors also failed to detect apoptosis in circulating PMN of animals given camptothecin, a known pro-apoptotic agent [13]. Regardless, the findings reported here confirm the pro-apoptotic properties of tilmicosin. In addition, results from this study indicate that tilmicosin-induced PMN apoptosis is at least in part cell-specific, as drug concentrations that are encountered by host cells during treatment did not induce apoptosis in bovine mononuclear leukocytes, monocyte-derived macrophages, endothelial cells, epithelial cells, or fibroblasts. It has been well established that tilmicosin expresses a high affinity for intracellular uptake in PMN and macrophages [40]. Moreover, upon removal of extracellular tilmicosin, high concentrations of the antibiotic are maintained within PMN but not macrophages [40]. The apparent cell-specificity of the pro-apoptotic effects of tilmicosin on PMN is consistent with these observations.

LTB_4 is an arachidonic acid metabolite synthesized via the 5-lipoxygenase pathway [10]. LTB_4 is a potent PMN chemoattractant and stimulates PMN to release elastase and generate superoxide radicals [5, 36, 47]. The role of LTB_4 in promoting self-perpetuating inflammatory injury at various mucosal surfaces is well established [17, 19, 41]. Although PMN are the principal source of LTB_4 in the bovine lung infected with *M. haemolytica* [8, 18] the direct effects of tilmicosin on LTB_4 synthesis by bovine PMN remain incompletely understood. The results from the present study demonstrate that circulating bovine PMN incubated with tilmicosin, but not oxytetracycline, have an impaired ability to synthesize LTB_4. Upon injection with tilmicosin, PMN will first encounter the antibiotic in the circulation, which further underscores the clinical significance of these results. Consistent with the present observations, recent findings suggest that tilmicosin reduces prostaglandin E_2 synthesis by activated bovine alveolar macrophages [29]. The effects of tilmicosin on PMN phospholipase expression and activity, both in tissues and in the circulation, require further investigation. We postulate that in combination with its pro-apoptotic properties, the ability of tilmicosin to inhibit spontaneous PMN LTB_4 synthesis may contribute to the anti-inflammatory properties of this macrolide.

The apoptosis of PMN can be induced upon direct activation of the tumor necrosis factor (TNF-α) receptor or Fas. After binding to Fas ligand, Fas oligomerizes and associates with the Fas-associated death domain, which recruits caspase-8, ultimately leading to the DNA breakdown characteristic of apoptotic death [4, 42]. In bronchoalveolar lavage samples of tilmicosin-treated calves infected with *M. haemolytica*, PMN apoptosis may be detected in the absence of altered TNF-α levels [6]. Several drugs have been shown to induce apoptosis by upregulating Fas receptor expression on the cell surface [23, 38, 48]. Therefore, the present study assessed whether increased PMN expression of Fas receptors may contribute to tilmicosin-induced apoptosis. The results from flow cytometric analysis indicate that tilmicosin, at concentrations known to enhance apoptosis, does not increase Fas expression on bovine PMN. Further investigation is warranted to determine whether tilmicosin may induce PMN apoptosis via activation of pro-apoptotic pathways dependent on mitochondria.

In summary, tilmicosin-induced PMN apoptosis is at least partially cell-specific, occurs concomitantly with an inhibition of

PMN LTB_4 synthesis, and is not associated with an upregulation of Fas expression on PMN. The fact that pro-apoptotic concentrations of tilmicosin do not upregulate Fas expression on PMN may indicate that its pro-apoptotic effects are induced via an intrinsic pathway. Together, the findings lend further support to the hypothesis that, in addition to classical anti-microbial properties, induction of PMN apoptosis and reduction of LTB_4 synthesis contribute to the clinical efficacy of macrolides such as tilmicosin.

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