Vet. Res. (2010) 41:75
DOI: 10.1051/vetres/2010047

www.vetres.org

Original article

Rosette nanotubes inhibit bovine neutrophil chemotaxis

Minh Hong Anh Le[1], Sarabjeet Singh Suri[1], Felaniaina Rakotondradany[2], Hicham Fenniri[2*], Baljit Singh[1*]

[1] Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK S7N 5B4, Canada
[2] National Institute of Nanotechnology and Department of Chemistry, University of Alberta, 11421 Saskatchewan Drive, Edmonton, AB T6G 2M9, Canada

(Received 12 November 2009; accepted 27 July 2010)

Abstract – Migration of activated neutrophils that have prolonged lifespan into inflamed organs is an important component of host defense but also contributes to tissue damage and mortality. In this report, we used biologically-inspired RGD-tagged rosette nanotubes (RNT) to inhibit neutrophil chemotaxis. We hypothesize that RGD-RNT will block neutrophil migration through inhibition of MAPK. In this report, RNT conjugated to lysine (K–RNT) and arginine-glycine-aspartic acid-serine-lysine (RGDSK-RNT) were co-assembled in a molar ratio of 95/5. The effect of the resulting composite RNT (RGDSK/K–RNT) on neutrophil chemotaxis, cell signaling and apoptosis was then investigated. Exposure to RGDSK/K–RNT reduced bovine neutrophil migration when compared to the non-treated group ($p < 0.001$). Similar effect was seen following treatment with ERK1/2 or p38 MAPK inhibitors. Phosphorylation of the ERK1/2 and p38 MAPK was inhibited at 5 min by RGDSK/K–RNT ($p < 0.05$). The RGDSD/K-RNT did not affect the migration of neutrophils pre-treated with αvβ3 integrin antibody suggesting that both bind to the same receptor. RGDSK/K–RNT did not induce apoptosis in bovine neutrophils, which was suppressed by pre-exposing them to LPS ($p < 0.001$). We conclude that RGDSK/K–RNT inhibit phosphorylation of ERK1/2 and p38 MAPK and inhibit chemotaxis of bovine neutrophils.

neutrophil / cattle / apoptosis / inflammation / MAPK

1. INTRODUCTION

Inflammation is a hallmark of many respiratory diseases of humans and animals. For example, bovine respiratory disease (BRD) complex is the most common and costly inflammatory disease in feedlot cattle [26]. BRD complex accounts for lower average daily gain, increased cost of treatment and mortality [39]. Despite advances in genetic selection, vaccination and the use of antibiotics and anti-inflammatory drugs to reduce inflammation, BRD remains a major concern [26].

Activated neutrophils migrate into the lungs to combat infections through production of various chemicals such as free oxygen species [1]. Neutrophil migration occurs through a series of events that are regulated by chemoattractants and adhesive proteins such as selectins and integrins. Although the role of β2 integrins in neutrophil migration is well recognized, there is some evidence that the αvβ3 integrin expressed on neutrophils and endothelial cells may also play a role in the movement of neutrophils [4, 25]. Importantly, ligation of integrins impacts cell migration through regulation of the extracellular signal regulated protein kinase (ERK) signaling [23, 48]. The ERK1/2 regulate cell motility through phosphorylation of

* Corresponding authors: Baljit.singh@usask.ca, fenniri@ualberta.ca

adhesion complexes including focal adhesion kinase, cytoskeletal proteins, and Ca^{2+}-activated proteolytic enzymes. In addition, active ERK1 in association with αvβ3 integrin is essential for effective functions of this integrin during cell movement and spreading on vitronectin [36]. Currently, there are no data on the role of MAPK cell signaling or the αvβ3 integrin in the migration of bovine neutrophils.

Neutrophils are the first line of defense against bacterial infections and are needed for elimination of infections. However, intensive and excessive sequestration of activated neutrophils in the lungs can exacerbate the inflammatory condition [1]. Furthermore, activation of neutrophils suppresses their constitutive apoptosis, which delays their death and clearance by macrophages leading to increased tissue damage [28, 47]. Because the tissue damage caused by the activated neutrophils in lung is associated with increased morbidity and mortality, we need to understand mechanisms to control migration and lifespan of neutrophils in lung inflammation.

Nanotechnology enables us to develop and exploit the novel properties of materials at the nanometer scale [31], a scale at which most living systems operate. The rosette nanotubes (RNT) are a new class of biologically-inspired nanotubes which are self-assembled in water from low molecular weight synthetic organic modules [12, 13]. The formation of RNT is characterized by the self-assembly of the G∧C motif, which mimics the complementary hydrogen bonding arrays of DNA bases guanine and cytosine. G∧C self-assembles under physiological conditions into a six-membered supermacrocycle (called rosette) held together by 18 H-bonds, which in turn self-organizes into a helical stack with an outer diameter of 3.5 nm (for K–RNT), a 1.1 nm channel and up to several μm in length [10, 12, 14]. Functional groups covalently attached to the G∧C motif are expressed on the RNT surface and impart physical and chemical properties suitable for many applications [45]. The stability of the RNT depends on several factors including density of functional groups, electrostatics (net charge), hydrophobic and stacking interactions, as well as pH and temperature [12]. We have shown that RGDSK/K-RNT do not induce inflammation in vivo or cause over activation of lung epithelial and macrophage cells lines in vitro [20–22]. More recently, we have shown that RGDSK/K-RNT activate p38 MAPK cell signaling pathways leading to induction of apoptosis in human bronchiolar adenocarcinoma cell line [41].

In this report, RNT conjugated to a lysine amino acid (K–RNT) and RNT conjugated to arginine-glycine-aspartic acid-serine-lysine (RGDSK-RNT) were co-assembled in a ratio of 95/5. The effects of the composite RNT (RGDSK/K–RNT) on bovine neutrophil chemotaxis, cell signaling and apoptosis were then investigated in vitro. The data show that RGDSK/K–RNT inhibit MAPK cell signaling and chemotaxis, most probably via the integrin αvβ3, without inducing apoptosis in neutrophils.

2. MATERIALS AND METHODS

2.1. Isolation of bovine blood neutrophils

Blood from healthy adult (3–4 years old), non-pregnant cattle that were without any clinical signs of disease and with normal hemograms was collected from the jugular vein with prior approval of the University of Saskatchewan's Animal Research Ethics Board. The blood (20 mL/animal) collected from individual animals was not pooled. Neutrophils were isolated by density gradient centrifugation with lymphocyte separation media (LSM, MP Biomedicals, Solon, USA) after using ammonium chloride for erythrocyte lysis [37]. After isolation, neutrophils were suspended in RPMI-1640 medium (Invitrogen, Carlsbad, USA) modified with 10% fetal bovine serum and glutamine. The viability of isolated PMN was assessed immediately by trypan blue (Sigma-Aldrich, St. Louis, USA) exclusion. Cell cytospin preparation stained with Diff-Quik (EMD Chemicals, Gibbstown, USA) was used for differential cell count. PMN viability was greater than 97% and their purity was more than 90%.

2.2. Neutrophil chemotaxis

2.2.1. Neutrophil chemotaxis assay

Chemotaxis of bovine neutrophils was assessed in 48-well Boyden chambers as described previously [11]. Briefly, neutrophil suspensions (50 μL)

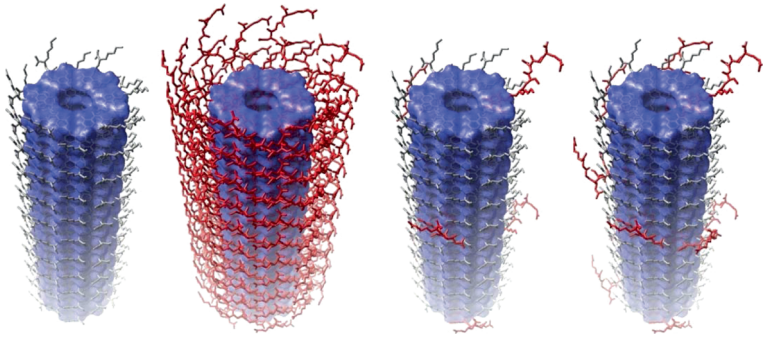

Figure 1. Model of the RNT investigated in this study. (A color version of this figure is available at www.vetres.org.)

with or without treatments were loaded in upper wells of the chamber in triplicate for each treatment with 5×10^4 cells per well. Isolated neutrophils were exposed to RGDSK/K–RNT ((RGD–RNT) = 0.1 μM, (K–RNT) = 2 μM; Fig. 1) and collected at 0, 5, 10, 15, 30 and 60 min. Cells in upper wells were allowed to migrate toward chemoattractant fMLP in lower wells (114 nM) at 37 °C in humidified air with 5% CO_2 for 30 min. After wiping the non-migrated cells off, the filters were dried and stained with Diff-Quik (EMD Chemicals, Gibbstown, USA). The stained filter was mounted on glass slides and the cells within the filter pores were then counted in 5 random fields under light microscopy at 400× magnification. The results are presented as the number of migrated neutrophils per microscopic field.

2.2.2. Blocking the αvβ3 integrin by antibody

Isolated bovine neutrophils were suspended in modified RPMI-1640 medium, resting for 1 h before any treatments. Neutrophils were incubated with the anti-human αvβ3 monoclonal antibody or isotype-matched antibody (R&D Systems, Minneapolis, USA) at the concentration of 1 μg/mL for 1 h at room temperature [2, 18]. The cells were then used for chemotaxis assay.

2.2.3. Blocking MAPK by MAPK inhibitors

Isolated bovine neutrophils were incubated with 20 μM of each MAPK inhibitors, either ERK1/2 inhibitor UO126 (Cell Signaling Technology, Danvers, USA) or p38 MAPK inhibitor SB239063 (Calbiochem, Temecula, USA), for 1 h at 37 °C in humidified air with 5% CO_2. As UO126 and SB239063 were dissolved in DMSO (dimethyl sulfoxide), DMSO was used as a negative control. The concentration of DMSO in all treatments was 0.2% (v/v) [17]. These cells were used for chemotaxis assay.

2.3. Enzyme linked-immunosorbent assay (ELISA) for MAPK phosphorylation

Isolated neutrophils were exposed to RGDSK/K–RNT and collected at 0, 5, 10, 15, 30 and 60 min. fMLP (5 μM) was also used as a positive control to induce phosphorylation of MAPK for 1 min at 37 °C [9]. Stimulation was stopped by cell sedimentation and discarding of supernatants followed by freezing pellets in liquid nitrogen. Subsequently, cell pellets were stored at −80 °C for later use. Cellular extracts were prepared by solubilizing pelleted cells at 5×10^6 cells/mL in lysis buffer comprised of EDTA (1 mM), Triton X-100 (0.5%), NaF (5 mM), urea (6 M), leupeptin (10 μg/mL), pepstatin (10 μg/mL), PMSF (100 μM), aprotinin (3 μg/mL), sodium pyrophosphate (2.5 mM) and activated sodium orthovanadate (1 mM) in PBS, pH 7.2–7.4. After vortex and ice incubation, supernatants were collected. Sample protein concentration was quantified using a protein microassay based on the Bradford dye-binding procedure (Bio-Rad, Mississauga, Canada). Cell lysates in duplicate for each time points were then used for sandwich ELISA (DuoSet® IC kit, R&D Systems, Minneapolis, USA) to measure phosphorylated levels of ERK1/2 and p38 MAPK. Results are expressed by the amount of phosphorylated ERK1/2 or p38 MAPK (ng) per μg of total protein quantified.

2.4. Detection of neutrophil apoptosis

2.4.1. Cell treatments

Isolated neutrophils at 5×10^6 cells/mL were pre-incubated at 37 °C in humidified air with 5% CO_2 with or without LPS (lipopolysaccharide, 1 μg/mL). After 30 min, LPS was discarded and cells were resuspended in modified RPMI-1640. Cells were then treated with 0.1 μM of RGDSK peptide (an Arg-Gly-Asp-Ser-Lys containing peptide, Peptides International, USA; $RGDSK^5/K^{95}$–RNT or $RGDSK^{10}/K^{90}$–RNT (RGDSK–RNT) = 0.2 μM, (K–RNT) = 2 μM) or modified RPMI-1640 only for 18, 24 and 36 h at 37 °C in humidified air with 5% CO_2. Cells were centrifuged at 400 *g* followed by the removal of supernatant, snap-freezing and storage at −80 °C.

2.4.2. Caspase-3 quantification

Cell pellets were applied for quantitative determination of caspase-3 using caspase-3 colorimetric assay kit (Assay Designs, Inc., Ann Arbor, USA). Cell lysates were used for caspase-3 colorimetric detection. The conversion was then measured kinetically at 405 nm. The activity of caspase-3 in samples was calculated as unit/mL.

2.4.3. Flow cytometry

For flow cytometry, the Annexin V-FITC apoptosis detection kit II from BD Biosciences, Mississauga, Canada [46]. Briefly, the cells were suspended in 100 μL of 1× Annexin V binding buffer at the concentration of 1×10^6 cells/mL followed by addition of 5 μL of Annexin V-FITC and 5 μL of propidium iodide, and incubation for 15 min at room temperature in the dark. Finally, 400 μL of 1× Annexin V binding buffer was added. Cells were analyzed with flow cytometer and the results were expressed as percentages.

2.5. Data analysis

Data was analyzed using SigmaStat® statistical software. All-pairwise comparisons were performed followed by analysis of variance to compare differences between treatment groups. Results of at least three separate experiments are displayed as mean ± standard error of the mean (SEM). Differences are considered statistically significant when the probability (p) < 0.05.

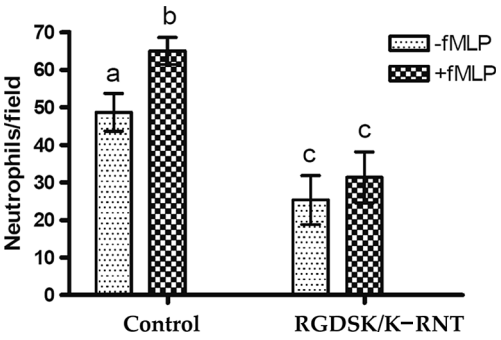

Figure 2. Effect of RGDSK/K–RNT on bovine neutrophil chemotaxis. While fMLP significantly increased the migration of neutrophils, exposure to RGDSK/K–RNT for 5 min, inhibited migration of control or fMLP-exposed neutrophils. Results are mean ± SEM of three separate experiments. Different letters above bars indicate significant differences ($p < 0.01$).

3. RESULTS

3.1. Effect of RGD-RNT on neutrophil chemotaxis

Control neutrophils exposed to RGDSK/K–RNT showed reduced migration compared to the non-treated group ($p < 0.01$, Fig. 2). Neutrophil migration towards fMLP was also inhibited by RGDSK/K–RNT at 5 min compared to the control.

3.2. Effect of RGD-RNT on MAPK phosphorylation

To understand the molecular effects of RGD-RNT on neutrophil migration, cells were exposed to RGDSK/K–RNT with or without fMLP followed by quantification of the phosphorylated ERK1/2 and p38 MAPK. Neutrophils exposed to fMLP showed significant increase in phosophorylation of ERK1/2 (Fig. 3A) and p38 (Fig. 3B) at 5 min of the exposure. There was a difference between treatment groups for ERK1/2 ($p < 0.001$, Fig. 3C) and p38 MAPK ($p < 0.01$, Fig. 3D). The phosphorylation of both the ERK1/2 and p38 was inhibited at 5 min ($p < 0.05$) of exposure to RGDSK/K–RNT followed by an increase at 10 min, which was sustained until 60 min.

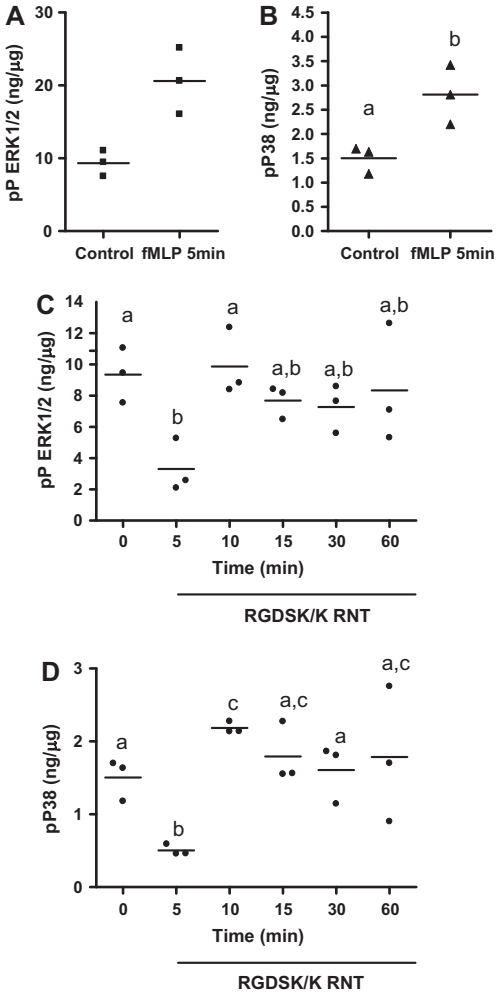

Figure 3. Phosphorylation of ERK1/2 (A, C) and P38 (B, D) MAPK in bovine neutrophils. fMLP induced significant phosophorylation of ERK1/2 (A) and P38 (B) MAPK within 5 min of exposure. RGDSK/K RNT significantly suppressed phosophorylation of ERK1/2 (C) and p38 (D) MPAK within 5 min of treatment. The phosphorylation of ERK1/2 (C) and p38 (MAPK) returned to control values at 10 min and remained so till 60 min. Results of three independent experiments are represented as mean ± SEM. Significant differences between treatment groups are expressed by different letters above bars ($p < 0.001$ and $p < 0.01$ for ERK and P38, respectively).

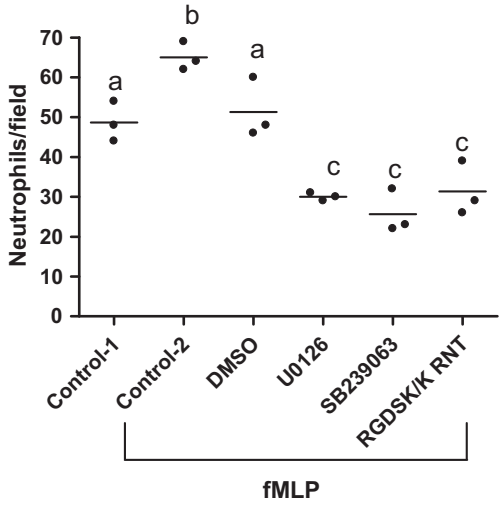

Figure 4. Inhibition of bovine neutrophil chemotaxis induced by RGDSK/K–RNT or MAPK inhibitors. Neutrophil migration, determined by counting the number of neutrophils stuck in filter pores after 30 min of chemotaxis assay, was significantly diminished after exposure to RGDSK/K–RNT for 5 min or MAPK inhibitors for 1 h. Modified RPMI-1640 and fMLP (114 nM) in the lower chamber were used as negative and positive controls, respectively. DMSO (dimethyl sulfoxide), a solvent of MAPK inhibitors, was used as a negative control. Results of three independent experiments are displayed as mean ± SEM. Significant differences between treatment groups are expressed by different letters above bars ($p < 0.001$).

Neutrophils treatment with the ERK1/2 inhibitor (UO126) or p38 inhibitor (SB239063) significantly reduced ($p < 0.001$) their migration in response to fMLP (Fig. 4). The inhibitory effects of RGDSK/K–RNT and MAPK inhibitors on neutrophil chemotaxis were not statistically different (Fig. 4).

3.3. Involvement of the αvβ3 integrin on bovine neutrophil chemotaxis

We treated neutrophils with a monoclonal antibody against the αvβ3 integrin to determine the role of this integrin in the neutrophil

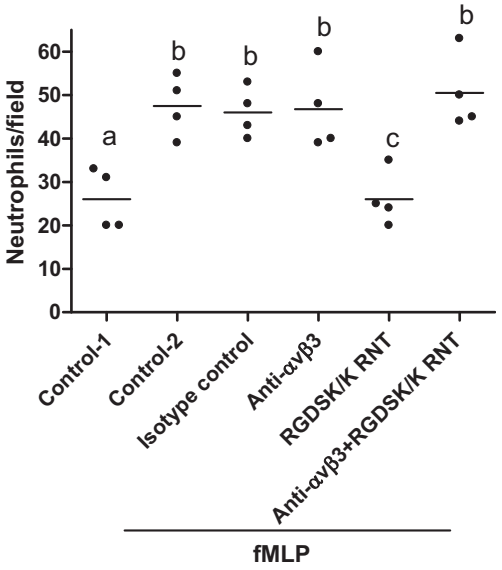

Figure 5. Effect of blockade of the αvβ3 integrin on bovine neutrophil chemotaxis. Compared to the control, neutrophil migration was significantly higher in fMLP alone or with isotype-matched antibody or the αvβ3 integrin antibody but was not different among the three fMLP treatments. While RGDSK/K–RNT alone significantly reduced the fMLP-induced migration, the effect was not noticed in neutrophils pretreated with the αvβ3 integrin mAb. Modified RPMI-1640 and fMLP (114 nM) in the lower chamber were used as negative and positive controls, respectively. Results of three independent experiments are displayed as mean ± SEM. Significant differences between treatment groups are expressed by different letters above bars ($p < 0.001$).

chemotaxis. RGDSK/K–RNT had no effect on the fMLP-induced migration of neutrophils pre-incubated with the integrin antibody. The isotype-matched antibody or the αvβ3 integrin antibody alone did not affect fMLP-induced neutrophil migration ($p < 0.001$, Fig. 5).

3.4. Effects of RGD-RNT on bovine neutrophil apoptosis

Neutrophils were incubated without (controls) or with RGDSK peptide, $RGDSK^{5}/K^{95}$–RNT or $RGDSK^{10}/K^{90}$–RNT for 0, 18, 24 and 36 h. As shown in Figure 6, caspase-3 activity was increased over time in LPS-stimulated and non-stimulated neutrophils. However, the activity was not different at 18, 24 and 36 h of treatment with the same agent. The RGDSK peptide and RGDSK/K-RNT did not induce an increase in caspase-3 activity, except for the significant induction of caspase-3 activity by $RGDSK^{10}/K^{90}$–RNT compared to the control at 18 h ($p < 0.05$). In addition, LPS caused significant suppression of caspase-3 activity in all treatments at all time points ($p < 0.001$). There was no effect on neutrophil apoptosis at 24 h of treatment with RGDSK peptide, $RGDSK^{5}/K^{95}$–RNT or $RGDSK^{10}/K^{90}$–RNT. However, apoptosis was markedly suppressed in cells treated with LPS for 30 min ($p < 0.001$, Fig. 7).

4. DISCUSSION

Neutrophil migration into sites of infections is a critical component of the host response [33]. However, there is growing evidence that dysregulated migration of activated neutrophils leads to tissue damage which results in morbidity and mortality [35]. This creates a need to develop approaches to fine-tune their migration into inflamed organs such as the lungs in BRD in cattle. Here we show that RGDSK/K–RNT inhibit fMLP-induced chemotaxis of neutrophils and MAPK signaling without affecting their apoptosis.

4.1. RGDSK/K–RNT suppress MAPK phosphorylation and neutrophil chemotaxis

Our data show that RGDSK/K–RNT inhibit neutrophil chemotaxis in response to fMLP. The role of signaling molecules such as MAPK and ERK1/2 in cell migration is well established [19]. We observed increased phosphorylation of MAPK and ERK1/2 in the neutrophils exposed to the fMLP. However, treatment of neutrophils with RGD-RNT caused significant suppression of ERK1/2 and p38 MAPK activation. Integrin engagement of RGD-ligand induces various intracellular signals including MAPK pathways that regulate cell migration via activation of focal adhesion

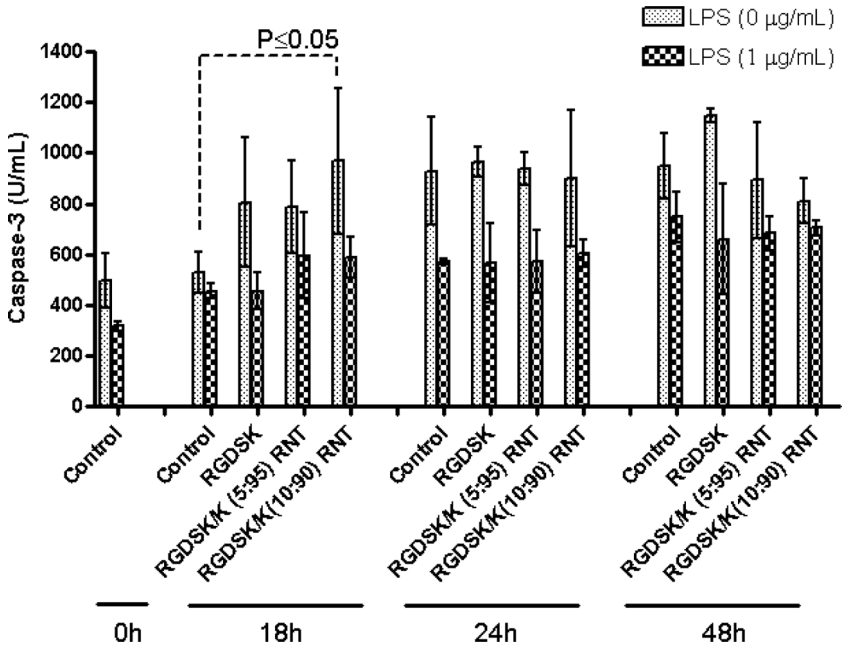

Figure 6. Effect of RGDSK/K–RNT on caspase-3 activity. Neutrophils with or without pre-exposure to LPS (1 μg/mL) were treated with RGDSK, or $RGDSK^{5}/K^{95}$–RNT or $RGDSK^{10}/K^{90}$–RNT for 0, 18, 24 and 36 h. Cell lysates were used for measurement of caspase-3 activity. Results of three different experiments are displayed as mean ± SEM. $p < 0.001$ when compared LPS and non-LPS treated groups.

kinases [15, 19]. In turn, activity of ERK is important for focal adhesion disassembly and direct phosphorylation of myosin light chain kinase that lead to cell migration [24, 34]. Even though ERK enables direct and rapid regulation of cytoskeletal dynamics in migrating cells, the integrin ligation is required for induction and persistence of cell migration with regard to MAPK regulation [23, 34, 40, 48]. The impact of MAPK and ERK1/2 suppression mediated by RGD-RNT in neutrophil migration is further supported by the fact that chemical inhibitors of p38 MAPK and ERK1/2 and the RGDSK/K–RNT were equally potent in inhibiting neutrophil chemotaxis. Therefore, RGDSK/K–RNT may inhibit neutrophil chemotaxis through inhibition of p38 MAPK and ERK1/2.

Neutrophil motility is governed by integrin signaling [23] including that by integrin αvβ3 [25, 30]. Integrins binding to RGD motif changes the conformation that in turn regulates integrin affinity to ligands and integrin redistribution on the cell membrane [7, 43]. The potential advantage of RGDSK/K–RNT compared to cyclic RGD peptides to modulate neutrophil migration is that the nanotubes may be highly effective at lower concentrations owing to their multivalent interactions with the integrins on the neutrophil surface as has been shown recently [29]. RGDSK/K–RNT had no effect on neutrophils pre-treated with anti–αvβ3 antibody. It appears, therefore, that both the antibody and the RGDSK/K–RNT bind to the same protein, the αvβ3 integrin. The inhibition of human neutrophil migration has been observed when neutrophils were treated with flavoridin (FL), an RGD-disintegrin ligand of αvβ3 and α5β1 [3, 30]. Intriguingly, the fMLP-induced neutrophil migration was not affected by the integrin αvβ3 antibody or the isotype-matched antibody alone. We do not know the precise reasons for these

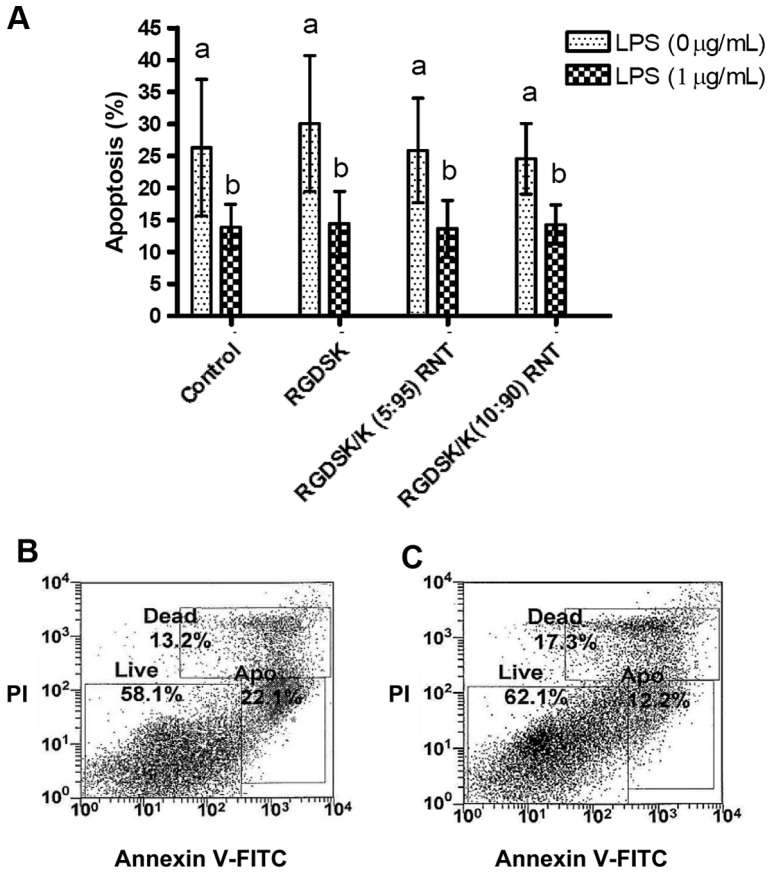

Figure 7. The effect of LPS on bovine neutrophil apoptosis at 24 h in the presence of $RGDSK^{5}/K^{95}$–RNT. The percentage of neutrophil apoptosis is displayed as mean ± SEM of 5 separate experiments (A). The level of apoptosis was measured by Annexin V-FITC staining after 24 h incubation without LPS-pretreatment (B) and with LPS-pretreatment (C). Significant differences between treatment groups are expressed by different letters above bars.

intriguing effects. However, it could be due to conformational changes in the integrin expressed on the neutrophil surface or endocytosis of the antibody-receptor complex to eliminate physical effects of the ligation [16, 44]. We believe that there is a need for additional experiments to clarify the role of the integrin in bovine neutrophil migration. Nevertheless, the data show that RGDSK/K–RNT inhibit neutrophil chemotaxis by suppressing phosophorylation of ERK1/2 and p38 and through inhibition of the αvβ3 integrin.

4.2. Effect of RGDSK/K–RNT on apoptosis of bovine neutrophils

The activation of neutrophils extends their lifespan by inhibiting constitutive apoptosis in neutrophils and the prolonged lifespan of activated neutrophils is believed to cause excessive tissue damage [27]. There is a concerted research effort underway to modulate the lifespan of activated neutrophils. We determined the effect of RGDSK/K–RNT on the neutrophil lifespan by measuring caspase-3

expression in neutrophils as caspase-3 is one of the critical enzymes involved in the terminal events leading to apoptosis. Activation of caspase-3 is induced rapidly in apoptotic granulocytes without early mitochondrial changes [32]. Cyclic RGD peptide upon internalization by the cells binds to RGD-binding motif on procaspase-3 to induce its activation [6, 8]. While treatment of bovine neutrophils with LPS for 30 min significantly suppressed caspase-3 activity, treatment with $RGDSK^{10}/K^{90}$–RNT did not induce the activity of caspase-3 except at 18 h. We further assessed neutrophil apoptosis at 24 h with flow cytometric method using Annexin V and propidium iodide (PI) labeled with flourescein isothiocyanate (FITC) [46]. Although as expected LPS treatment significantly inhibited apoptosis in neutrophils [5, 38, 42], there was no effect of RGDSK peptide, $RGDSK^{5}/K^{95}$ RNT or $RGDSK^{10}/K^{90}$–RNT on neutrophil apoptosis compared to the control. These data are in contrast to our recent observations on the induction of apoptosis by similar RNT in a human adenocarcinoma cell line [41]. One of the reasons could be that while neutrophil is a terminally differentiated cell, the adenocarcinoma cell line is a dividing cell line which may create differences in the actions of RGDSK/K–RNT. Second, considering that the RNT increased caspase-3 expression at 18 h of the treatment, it is possible that a higher concentration of the RNT or higher proportion of K-RNT in the combination or prolonged treatment with it may be needed to induce caspase-3 activity and apoptosis in activated neutrophils.

This study provides the first evidence that RGDSK/K–RNT inhibit phosphorylation of the ERK1/2 and p38 MAPK and chemotaxis of bovine neutrophils. These bovine phosphorylated proteins, ERK1/2 and p38 MAPK, were quantified for the first time by capture ELISA. The data also allude to a complex role of the αvβ3 integrin in bovine neutrophil migration and need for additional experiments. Lastly, RGDSK/K–RNT at the concentrations used in our studies did not induce apoptosis in LPS-treated bovine neutrophils.

Acknowledgements. The work was funded through a grant from Alberta Agricultural Funding Consortium to Dr Baljit Singh and Dr Hicham Fenniri. Dr Le was supported by a scholarship from Government of Vietnam. We thank Dr Takeshi Yamazaki for generating the RNT models.

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