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Research paper

# Expression of annexin-1 in equine leucocytes and the effects of the N-terminal annexin-1 peptide, Ac2-26, on equine neutrophil superoxide production

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ABSTRACT

N-terminal peptides derived from the anti-inflammatory peptide, annexin-1, inhibit neutrophil function but can also induce pro-inflammatory effects. Although equine annexin-1 has been sequenced, its cellular expression and properties have not been reported. This study has examined whether annexin-1 is present in equine leucocytes and how the N-terminal peptide, Ac2-26, affects equine neutrophil superoxide production.

Annexin-1 expression in equine neutrophils and mononuclear cells and the ability of Ac2-26 to activate neutrophil p42/44 MAPK were determined by immunoblotting. Equine neutrophil superoxide production was measured by the reduction of cytochrome (cyt) C following stimulation with Ac2-26 and the formyl peptide receptor (FPR) agonists, FMLP, WKYMVm and WKYMVM. Responses were examined in the presence of the pan-FPR antagonist, BOC-2, and the role of p42/44 MAPK in agonist-induced effects was determined using PD98059. The effect of Ac2-26 on superoxide production in response to serum-treated zymosan (STZ) was also investigated, and the roles of FPR and p42/44 MAPK ascertained.

Annexin-1 was detected in both equine neutrophils and mononuclear cells using a polyclonal rabbit anti-human annexin-1 antibody. Ac2-26 ($5 \times 10^{-5}$ M) induced superoxide production in cytochalasin B-primed ($48 \pm 8$ versus $21 \pm 9$ (unstimulated cells) nmol cyt C/$10^6$ neutrophils) and un-primed cells ($37 \pm 10$ versus $11 \pm 5$ nmol cyt C/$10^6$ neutrophils). FMLP and WKYMVm, but not WKYMVM, also caused superoxide production in primed neutrophils, suggesting the response was mediated by FPR receptor binding. This was supported by the marked inhibitory effect of BOC-2 on the responses to Ac2-26 and FMLP although, interestingly, the effects of WKYMVm were not significantly reduced ($50 \pm 5$ (WKYMVm) versus $45 \pm 5$ (WKYMVm + BOC-2) nmol reduced cyt C/$10^6$ neutrophils). Inhibition of p42/44 MAPK activation with PD98059 significantly attenuated superoxide production in response to Ac2-26, FMLP and WKYMVm and Western blotting showed that Ac2-26 induced p42/44 MAPK activation. At a concentration which did not cause superoxide production, Ac2-26 ($10^{-5}$ M) significantly reduced the response to STZ ($84 \pm 17$% inhibition). This inhibitory effect was attenuated by both BOC-2 and PD98059.

These results suggest that if activation of equine leucocytes *in vivo* leads to the release and subsequent cleavage of annexin-1, the N-terminal peptides formed could bind to neutrophil FPR and decrease free radical production in response to particulate stimuli. This

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could help to reduce local tissue damage but, as Ac2-26 can also stimulate superoxide production at higher concentrations in an FPR-dependent manner, the amount of free radical production may depend on the concentration of peptide present.



## 1. Introduction

Annexin-1 is a 37 kD, glucocorticoid-regulated, endogenous anti-inflammatory peptide that is present in large amounts in neutrophils as well as in other blood cells (Morand et al., 1995). Although the effects of annexin-1 on neutrophil function are largely inhibitory, cleavage at the N-terminal domain results in the production of peptides that have anti-inflammatory properties but which can additionally induce pro-inflammatory effects (Movitz and Dahlgren, 2000; Perretti and Gavins, 2003).

Annexin-1 and the N-terminal peptides, Ac2-26 and Ac9-25, have been shown to bind to members of a family of G-protein coupled receptors known as formyl peptide receptors (FPR; Perretti et al., 2001; Ernst et al., 2004; Karlsson et al., 2005), leading to activation of the intracellular kinase p42/44 MAPK (ERK; Walther et al., 2000; Hayhoe et al., 2006). Human and rabbit neutrophils express FPR and FPR2, which is also known as FPR like-1 (FPRL-1) or ALXR (lipoxin $A_4$ receptor), to which the formyl peptide, f-Met-Leu-Phe (FMLP), binds with high and low affinity, respectively. However, there is inter-species variability in neutrophil FPR populations and in their responsiveness to FMLP (Brazil et al., 1998; Perretti and Gavins, 2003). A single FPR population has been identified on horse neutrophils to which FMLP binds with a similar $K_d$ to that of the high affinity site on human cells although, interestingly, equine neutrophils only respond functionally to low (nanomolar) concentrations of FMLP once primed (Brazil et al., 1998).

Despite the sequence of equine annexin-1 having been reported some time ago (Bryant et al., 2002), there are no published studies on the expression of the peptide in equine leucocyte sub-populations, nor showing how annexin-1 or its N-terminal peptides affect equine neutrophil activation. In this study we investigated expression of annexin-1 in equine leucocytes and examined the effects of the N-terminal peptide, Ac2-26, and other FPR agonists on superoxide production by equine neutrophils. The effect of Ac2-26 on superoxide production in response to another stimulus, opsonised zymosan, has also been examined. The FPR antagonist BOC-2 was used to establish if the effects of Ac2-26 were FPR mediated. Ac2-26 activates p42/44 MAPK following receptor binding, and this intracellular kinase is known to be involved in FMLP-induced superoxide production (Dewas et al., 2000; Shin et al., 2005; Hayhoe et al., 2006). Therefore, the effects of inhibiting p42/44 MAPK activation on equine neutrophil superoxide production were also investigated.

## 2. Materials and methods

### 2.1. Reagents

A rabbit anti-human polyclonal annexin-1 antibody was purchased from Zymed (Cambridge Biosciences, Cambridge, UK). Antibodies recognising total and phosphorylated p42/44 MAPK and isotype matched horseradish peroxidase (HRP-conjugated) secondary antibodies were purchased from New England Biolabs (Hitchin, UK). The MEK-1 inhibitor, PD98059, which was used to inhibit activation of p42/44 MAPK, was purchased from Calbiochem (Merck Chemicals Ltd., Nottingham, UK). The annexin-1 N-terminal peptide, Ac2-26 (Ac-Ala-Met-Val-Ser-Glu-Phe-Leu-Lys-Gln-Ala-Trp-Phe-Ile-Glu-Asn-Glu-Glu-Gln-Glu-Tyr-Val-Gln-Thr-Val-Lys) and the synthetic peptides WKYMVm (Trp-Lys-Tyr-Met-Val-d-Met), an agonist at FPR and FPRL-1, and WKYMVM (Trp-Lys-Tyr-Met-Val-Met), a selective FPRL-1 agonist, were purchased from Tocris (Bristol, UK). The pan-FPR receptor antagonist, BOC-2 (Boc-Phe-Leu-Phe-Leu-Phe), was purchased from Phoenix Pharmaceuticals (Karlsruhe, Germany). FMLP and, unless otherwise stated, all other chemicals were purchased from Sigma–Aldrich (Poole, UK).

### 2.2. Isolation of neutrophils and mononuclear cells

The study was carried out under a Home Office licence with ethical approval from the Royal Veterinary College Ethics and Welfare Committee and the East London Local Research Ethics Committee. Blood (up to 200 ml from horses and 60 ml from one human volunteer) was collected into EDTA ($10^{-2}$ M), and neutrophils and mononuclear cells isolated by density gradient centrifugation as previously described (McKelvie et al., 1998; Rickards et al., 2001).

### 2.3. Expression of annexin-1 in equine leucocytes

Equine neutrophils and mononuclear cells ($5 \times 10^6$ from 6 and 5 horses, respectively) and human neutrophil and mononuclear cells ($5 \times 10^6$) were lysed (lysis buffer: 7.65 mM Tris HCl, 10% (v/v) glycerol and 2% (w/v) SDS supplemented with protease inhibitors ($10^{-3}$ M AEBSF (4-(2-aminoethyl) benzenesulphonyl fluoride hydrochloride)), 50 μg/ml leupeptin and 1 mM $NaVO_3$) for 20 min on ice. Following removal of 25 μl for protein estimation using a BCA protein assay kit (Perbio Ltd., Cramlington, UK), 4× Lammelli's buffer was added and samples stored at −80 °C prior to analysis.

Annexin-1 expression was determined by immunoblotting. Briefly, samples (2.5 μg protein per lane) were separated by SDS-PAGE using 10% polyacrylamide gels and a Biorad Mini Protean II system (BioRad, Hemel Hempstead, UK). Proteins were transferred overnight onto a polyvinylidene fluoride (PVDF) membrane and immunodetection of annexin-1 performed using a 1:1500 dilution of the annexin-1 antibody and a 1:2000 dilution of secondary antibody. Protein bands were detected using an enhanced chemiluminescence (ECL) kit (GE Healthcare UK Limited, Chalfont St Giles, UK).

### 2.4. Effect of Ac2-26 on p42/44 MAPK phosphorylation in equine neutrophils

Equine neutrophils ($5 \times 10^6$ in 1× HBSS containing $Ca^{2+}/Mg^{2+}$ (Invitrogen, Paisley, UK)) were exposed to $10^{-5}$ M Ac2-26 for 2–10 min as this concentration was shown to cause p42/p44 MAPK phosphorylation following activation of cloned FPR receptors in HEK cells (Hayhoe et al., 2006) or to PAF ($10^{-6}$ M) for 2.5 min as a positive control (Cunningham, unpublished data). At the end of the incubation, samples were placed on ice and lysed for 20 min. Following removal of 25 μl for protein estimation, 4× Lammelli's buffer was added and samples stored at −80 °C prior to analysis.

Activation of p42/44 MAPK was examined by immunodetection of the phosphorylated form of the protein using phospho-p42/44 MAPK and total-p42/44 MAPK antibodies known to cross react with the equine protein (Titterton et al., 2004). Protein bands were detected using an enhanced chemiluminescence (ECL) kit. Films were then scanned, band densities determined using Image J image analysis software (http://www.rsb.info.nih.gov/ij/) and expressed as arbitrary units ± SEM ($n = 4$ horses).

### 2.5. Superoxide production by equine neutrophils in response to Ac2-26

Superoxide production was measured colorimetrically as a reduction in cytochrome (cyt) C (Foster and Cunningham, 1997). Briefly, neutrophils ($0.25 \times 10^6$/ml; $n = 3$ horses) in HBSS/cyt C (2.5 mg/ml) were incubated at 37 °C for 5 min with cytochalasin (cyt) B (5 μg/ml) to induce priming (Marr et al., 1997; Bylund et al., 2003; Franck et al., 2009) before addition of agonists or vehicle. To the authors knowledge there are no reports describing the effects of Ac2-26 on neutrophil superoxide production *in vitro*. Thus the concentrations used ($10^{-5}$ M and $5 \times 10^{-5}$ M) were chosen on the basis of a study on human neutrophils carried out by Karlsson et al. (2005) in which another N-terminal annexin 1 peptide, Ac9-25, was shown to induce a response in the micromolar range. FMLP was used at the highest concentration tested by Brazil et al. (1998) in a chemiluminescence assay of free radical production by equine neutrophils and at a 100-fold higher concentration ($10^{-6}$ M and $10^{-4}$ M). WKYMVm and WKYMVM are more potent FPR agonists than FMLP and were used at $10^{-7}$ M as this concentration has been shown to induce superoxide production by human neutrophils (Karlsson et al., 2005). Whilst the lack of superoxide production by FMLP in un-primed equine neutrophils has been reported (Brazil et al., 1998), the effects of Ac2-26 are unknown. Therefore responses to the agonists in vehicle treated cells were examined in parallel. After 30 min neutrophils were removed by centrifugation (875 × *g*, 10 min, 4 °C), supernatant (250 μl) transferred to a 96-well plate and read at 550 nm using a Spectra Max Pro plate reader (Molecular Devices, Wokingham, UK) attached to an Apple Macintosh running Softmax pro (version 1; Molecular Devices). Results are expressed as mean nmol reduced cyt C/$10^6$ cells ± SEM.

In order to determine whether the effects of Ac2-26 were mediated by FPR binding, BOC-2 ($10^{-5}$ M; Hayhoe et al., 2006) or vehicle (0.125% methanol) was added to neutrophils ($n = 3$ horses) 10 min prior to cyt B. After a further 5 min, Ac2-26 ($5 \times 10^{-5}$ M), FMLP ($10^{-6}$ and $10^{-4}$ M), WKYMVm ($10^{-7}$ M) or vehicle were added and superoxide production measured 30 min later as described above.

The effect of inhibiting p42/44 MAPK activation on FPR-mediated neutrophil superoxide production ($n = 3$–5 horses) was determined by pre-incubating neutrophils with PD98059 ($10^{-5}$ M; Houliston et al., 2001). After 25 min at 37 °C, cyt B was added and 5 min later, Ac2-26 ($2 \times 10^{-5}$ M), FMLP ($10^{-4}$ M) or WKYMVm ($10^{-7}$ M). Superoxide production was measured after a further 30 min incubation. An interim study showed that $2 \times 10^{-5}$ M Ac2-26 caused significant superoxide production by equine neutrophils and this concentration was therefore used in this and subsequent studies for reasons of cost.

### 2.6. Effects of Ac2-26 on opsonised zymosan-induced superoxide release by neutrophils

To examine whether Ac2-26 could reduce the response to another stimulus, neutrophils ($n = 6$ horses) were pre-incubated with the peptide at a concentration that did not induce superoxide production ($10^{-5}$ M) or vehicle for 15 min prior to addition of serum-treated {opsonised} zymosan (STZ; 1 mg/ml; Foster and Cunningham, 1997). Superoxide production was measured 30 min later. The effect of adding Ac2-26 at the same time as STZ was also studied. To determine whether the inhibitory effect of Ac2-26 was FPR mediated, neutrophils ($n = 3$ horses) were pre-incubated with BOC-2 ($10^{-5}$ M) or vehicle for 15 min prior to addition of Ac2-26 or vehicle. STZ was added 15 min later and superoxide production measured after a further 30 min.

### 2.7. Statistical analyses

Statistical analyses were carried out using Analyse-It version 2.12 for Microsoft Excel (Analyse-It software, Leeds, UK; http://www.analyse-it.com/). One-way analysis of variance followed by Bonferroni's test was used to examine the effects of (i) Ac2-26, FMLP, WKYMVm and WKYMVM on superoxide production, (ii) BOC-2 and PD98059 on agonist-induced responses and (iii) Ac2-26 on STZ-induced superoxide production. Throughout, statistical significance was accepted at $p < 0.05$.

## 3. Results

### 3.1. Expression of annexin-1 in equine neutrophils and mononuclear cells

Equine neutrophils and mononuclear cells expressed a 37 kDa protein that corresponded to the position of a band in human neutrophils and mononuclear cells recognised by the anti-annexin-1 antibody (Fig. 1). Although the peptide was present in all the cell lysates examined, and

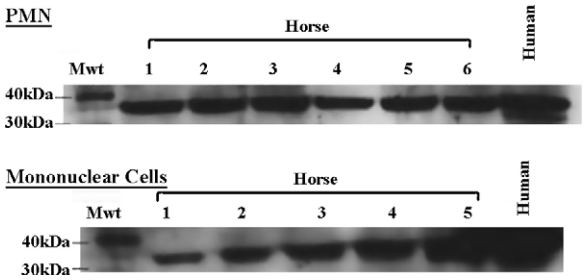


**Fig. 1.** Expression of annexin-1 in (a) equine neutrophils ($n = 6$ horses) and (b) mononuclear cells ($n = 5$ horses) using a polyclonal rabbit anti-human annexin-1 antibody. Human mononuclear cells and neutrophils ($n = 1$) were used as a positive control. Each lane was loaded with 2.5 μg protein.

the level of expression was similar in neutrophils from each of the 6 horses, there appeared to be some inter-animal variability in expression by mononuclear cells. Lymphocytes are reported to contain little annexin-1 in comparison with neutrophils or monocytes (Morand et al., 1995). As the equine mononuclear cell lysates contained both monocytes and lymphocytes, the variation could be due, at least in part, to a difference in the number of monocytes present, which was not ascertained.

### 3.2. Effect of Ac2-26 on p42/44 MAPK phosphorylation in equine neutrophils

Expression of phospho-p42/44 MAPK in human neutrophils significantly increased after incubation with PAF for 2.5 min. Ac2-26 ($10^{-5}$ M) also induced activation of the kinase in a time dependent manner (Fig. 2). The mean increase in phospho-p42 MAPK appeared to be more marked than the increase in phospho-p44 MAPK, although inter-animal variation was evident.

### 3.3. Effect of Ac2-26 on superoxide production by equine neutrophils

Ac2-26 at a concentration of $5 \times 10^{-5}$ M, but not $10^{-5}$ M, induced superoxide production in cyt B-primed neutrophils, as did FMLP and WKYMVm. In contrast the FPRL-1 selective agonist, WKYMVM, was without effect (Fig. 3). At this concentration Ac2-26 also induced superoxide production from un-primed cells ($11 \pm 5$ (basal), $^*37 \pm 10$ ($5 \times 10^{-5}$ M Ac2-26), $18 \pm 8$ ($10^{-5}$ M Ac2-26) nmol reduced cyt C/$10^6$ cells, $^*p < 0.05$; $n = 3$ horses).

BOC-2 alone had no effect on basal superoxide production in primed neutrophils but significantly inhibited responses to both Ac2-26 and FMLP (Fig. 4a). Surprisingly, only a small, non-significant, reduction in WKYMVm-induced superoxide production was observed (Fig. 4a). Superoxide production in response to agonists was lower in this experiment after pre-treatment with PD98059 or Ac2-26 (Figs. 4b and 5). This may be because increasing the length of incubation at 37 °C adversely affected the ability of the cells to respond to stimuli; basal superoxide production did not increase.

Superoxide production induced by Ac2-26 ($2 \times 10^{-5}$ M) in primed cells was attenuated to basal levels by pre-treatment with PD98059 (Fig. 4b). The responses to FMLP

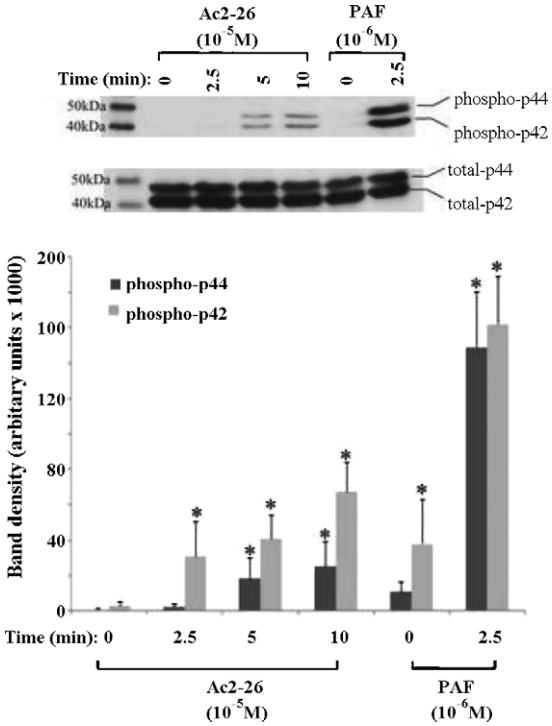


**Fig. 2.** Effect of Ac2-26 on p42/44 MAPK phosphorylation in equine neutrophils. (a) Representative blots showing expression of phospho- and total-p42/44 MAPK in neutrophils from one horse following stimulation at 37 °C with Ac2-26 ($10^{-5}$ M) for up to 10 min or PAF ($10^{-6}$ M) for 2.5 min and (b) mean ± SEM band densities for p42/p44 MAPK expression in neutrophils from 4 horses. $^*p < 0.05$ versus 0 min using one-way ANOVA followed by Bonferroni's test.

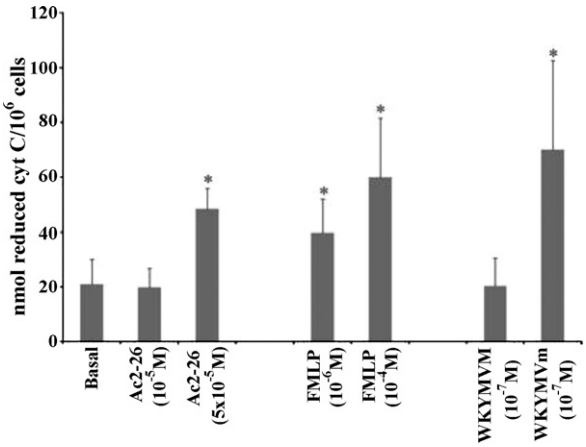


**Fig. 3.** Effect of Ac2-26 on superoxide production by equine neutrophils. Superoxide production by neutrophils pre-incubated for 5 min at 37 °C with cyt B (5 μg/ml) prior to addition of Ac2-26, FMLP, WKYMVM or WKYMVm for 30 min. Values are shown as mean nmol reduced cyt C/$10^6$ cells ± SEM ($n = 3$ horses). $^*p < 0.05$ versus unstimulated cells; one-way ANOVA followed by Bonferroni's test.

and WKYMVm were also markedly reduced, although inhibition was incomplete (Fig. 4b). To determine whether PD98059 was acting on pathways stimulated by cyt B or those triggered by FPR/agonist interactions, the effect of the inhibitor on superoxide production was additionally

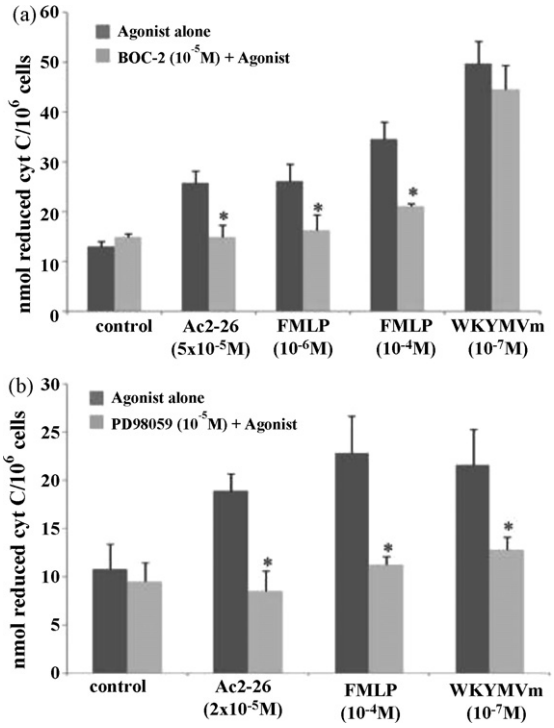


**Fig. 4.** Effect of BOC-2 and PD98059 on agonist-induced superoxide production. (a) The effect of BOC-2 on agonist-induced superoxide production. The receptor antagonist was added 10 min prior to cyt B (5 μg/ml) followed after 5 min by Ac2-26, FMLP or WKYMVm. Superoxide production was measured after a further 30 min. Values are shown as mean nmol reduced cyt C/$10^6$ cells ± SEM ($n = 3$ horses). $^*p < 0.05$ versus the response to agonist alone; one-way ANOVA followed by Bonferroni's test. (b) The effect of PD98059 on agonist-induced superoxide production. The MEK inhibitor was added 25 min prior to cyt B (5 μg/ml) followed 5 min later by Ac2-26, FMLP or WKYMVm. Values are shown as mean nmol reduced cyt C/$10^6$ cells ± SEM ($n = 3$ horses for FMLP and WKYMVm and $n = 5$ horses for Ac2-26). $^*p < 0.05$ versus the response to agonist alone; one-way ANOVA followed by Bonferroni's test.

examined in un-primed cells stimulated with Ac2-26 ($2 \times 10^{-5}$ M). PD98059 was found to completely inhibit superoxide production in un-primed cells following exposure to Ac2-26 (Table 1).

### 3.4. Effect of Ac2-26 on zymosan-induced superoxide production by equine neutrophils

Pre-incubation with Ac2-26, at a concentration that caused no superoxide production in un-primed cells, significantly reduced the response to STZ (Fig. 5) but the peptide had no effect when added at the same time as the stimulus (49 ± 9 (STZ/Ac2-26) versus 51 ± 11 (STZ) nmol reduced cyt C/$10^6$ cells). The inhibitory effect of Ac2-26 was significantly attenuated by pre-treatment of the cells by BOC-2 (Fig. 5). Whilst pre-treatment with PD98059 had no significant effect on STZ-induced superoxide production, it did attenuate the reduction in response caused by Ac2-26 (Table 1).

## 4. Discussion

Regulation of neutrophil function is essential in order to prevent inappropriate activation during the host defence response and, in the otherwise healthy individual, this is achieved by a balance between the actions of endogenously produced pro- and anti-inflammatory mediators (Perretti et al., 2001; Yang et al., 2004; Gavins et al., 2006). The inhibitory effects of the anti-inflammatory peptide, annexin-1, on human neutrophil function are well documented (reviewed in Perretti and Flower, 2004; Perretti and D'Acquisto, 2009). Although equine annexin-1 has been sequenced (Bryant et al., 2002), the present study is the first to demonstrate that the peptide is

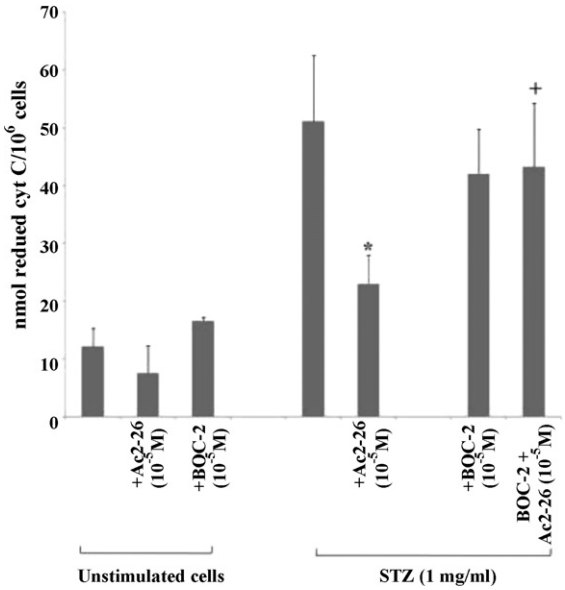


**Fig. 5.** Effect of Ac2-26 on neutrophil superoxide production in response to serum-treated {opsonised} zymosan. Neutrophils were pre-incubated for 15 min with Ac2-26 before addition of STZ and measurement of superoxide production 30 min later. BOC-2 or vehicle (0.125% MeOH) was added 15 min prior to Ac2-26 and STZ added after a further 15 min. Values are means ± SEM ($n = 6$ horses (effect of Ac2-26 on STZ) and $n = 3$ horses (effect of BOC-2 on the inhibitory action of Ac2-26)). $^*p < 0.05$ versus STZ alone; $^+p < 0.05$ versus STZ + Ac2-26. Superoxide production in response to STZ, STZ following exposure to BOC-2 and STZ following exposure to BOC-2 and Ac2-26 was significantly greater than basal; $p < 0.05$.

**Table 1**
Effect of PD98059 ($10^{-5}$ M) on (a) Ac2-26-induced superoxide production by un-primed equine neutrophils and (b) inhibition of STZ-induced superoxide production by Ac2-26.

| Treatment | Superoxide (nmol reduced cyt C/$10^6$ cells) |
|---|---|
| (a) Vehicle | 8 ± 0.2 |
| Ac2-26 ($2 \times 10^{-5}$ M) | 18 ± 1 |
| Ac2-26 ($2 \times 10^{-5}$ M) + PD98059 ($10^{-5}$ M) | 6 ± 2* |
| (b) STZ alone | 42 ± 4 |
| PD98059 ($10^{-5}$ M) alone | 9 ± 0.2 |
| PD98059 ($10^{-5}$ M) + STZ | 38 ± 6 |
| Ac2-26 ($10^{-5}$ M) + STZ | 19 ± 1* |
| PD98059 ($10^{-5}$ M) + Ac2-26 ($10^{-5}$ M) + STZ | 32 ± 3+ |

Values are means ± SEM nmol reduced cyt C/$10^6$ cells ($n = 4$ horses).
* $p < 0.05$ versus stimulus alone; one-way ANOVA followed by Bonferroni's test.
+ $p < 0.05$ versus STZ in Ac2-26 pre-treated cells; one-way ANOVA followed by Bonferroni's test.

expressed in equine leucocytes. The presence of annexin-1 in leucocytes from other species has been established (Comera and Russo-Marie, 1995; Oliani et al., 2002; Perretti and D'Acquisto, 2009). Moreover, it has been shown that, in unstimulated neutrophils, annexin-1 is co-localised with gelatinase-rich tertiary granules (Perretti et al., 2000) and is rapidly mobilised to the surface on activation, such as occurs when cells adhere to the endothelium (Perretti and Gavins, 2003; Perretti and D'Acquisto, 2009). As in other species, annexin 1 was found to be present in horse neutrophils and mononuclear cells. Lymphocytes have been reported to contain little annexin-1 in comparison with monocytes (Morand et al., 1995) but further studies are required to establish the relative expression in these equine cell populations, as well as the intracellular localisation in both stimulated and unstimulated equine leucocytes.

Following externalisation, annexin 1 acts in an autocrine fashion by binding to FPR receptors, reducing neutrophil extravasation (Perretti and Gavins, 2003; Perretti and D'Acquisto, 2009). However, if N-terminal cleavage occurs, peptides such as Ac2-26 and Ac9-25 are formed (Movitz and Dahlgren, 2000) and, *in vitro*, Ac9-25 has been shown to both inhibit free radical production by human neutrophils in response to other stimuli and to induce the formation of free radicals (Karlsson et al., 2005). The present study has shown that the effects of Ac2-26 on equine neutrophil superoxide production appear to be similar to those of Ac9-25 on human neutrophils.

That Ac2-26-induced superoxide production by both cyt B-primed and un-primed cells was almost completely inhibited by BOC-2 suggests that the stimulatory effect of the annexin-1 peptide was mediated by activation of an FPR. Although BOC-2 is described as a pan-FPR antagonist and should therefore reduce agonist binding to both FPR and FPRL-1 on neutrophils, Stenfeldt et al. (2007) suggested that BOC peptides and cyclosporin H are "fairly specific" FPR antagonists. This, together with the observation that the selective FPRL-1 agonist, WKYMVM, caused no superoxide production by equine neutrophils, and the presence of a single receptor population on these cells that binds FMLP with a similar affinity to human neutrophil FPR (Brazil et al., 1998), suggests that the effects of Ac2-26 are indeed FPR mediated. FMLP and WKYMVm activate both FPR and FPRL-1 and FMLP-induced superoxide production by equine neutrophils was blocked by BOC-2. Why BOC-2 was able to reduce responses to Ac2-26 and FMLP, but caused little attenuation of superoxide production by WKYMVm, is therefore difficult to explain. It is possible that, as WKYMVm is more potent than either FMLP or Ac2-26, the concentration of BOC-2 used was too low to be effective. This seems unlikely, however, as the antagonist was present at a 100-fold higher concentration than WKYMVm, although the $IC_{50}$ has not been established in the horse. At concentrations above $10^{-5}$ M, BOC-2 is reported to lose selectivity of action on neutrophil formyl peptide receptors (Stenfeldt et al., 2007). Therefore higher concentrations were not tested in this study as any functional inhibition observed might not be due to an FPR-mediated effect. As in equine neutrophils, inhibition of FPR had no effect on WKYMVm-induced superoxide production by human neutrophils (Karlsson et al., 2006). A selective inhibitor of FPRL-1 was also without effect when $10^{-7}$ M WKYMVm was used, as in the present study, and was only partially effective at reducing the effects of lower concentrations of the peptide (Karlsson et al., 2006). Although these findings suggested that WKYMVm might be acting on a different type of cell surface receptor, this possibility was excluded as combined inhibition of FPR and FPRL-1 did block the response. The explanation for their findings put forward by the authors related to hierarchical cross-talk between FPR and FPRL-1, both of which are present on human neutrophils. However, this could not explain the lack of effect of BOC-2 on WKYMVm-induced superoxide production by equine cells which express only a single receptor population and the underlying mechanism therefore remains to be determined.

It was also of interest that Ac2-26 promoted the production of a small, but significant, amount of superoxide by un-primed neutrophils whereas FMLP and WKYMVm were without effect. Whether this can be explained by differential activation of one or more intracellular signalling molecules by the agonists may become clear once the pathways activated downstream of the cell surface receptor have been delineated.

Ac2-26 was shown to activate p42/44 MAPK in equine neutrophils, which is in agreement with previously published work in HEK cells transfected with human FPR and FPRL-1 (Hayhoe et al., 2006). Moreover, as superoxide production by both un-primed and primed equine cells in response to Ac2-26 was abolished in the presence of PD98059, it suggests that activation of p42/44 MAPK is required for this response. Activation of p42/44 MAPK is also a necessary step for superoxide production in response to FMLP or WKYMVm in human neutrophils and eosinophils, respectively (Dewas et al., 2000; Shin et al., 2005), and the findings in primed equine neutrophils are consistent with this. Further studies are required to determine whether p42/44 MAPK activation by FPR agonists influences the phosphorylation of NADPH oxidase subunits, as has been shown in human neutrophils stimulated with FMLP (Dewas et al., 2000). Activation of p38 MAPK is also involved in the production of reactive oxygen species by neutrophils stimulated with FMLP (Dang et al., 2006; Sakamoto et al., 2006). However, as p38 MAPK activation was not shown to occur following Ac2-26 binding to FPR or FPRL-1 in HEK cells (Hayhoe et al., 2006), activation of this kinase in equine neutrophils and its role, if any, in superoxide production caused by Ac2-26 was not investigated as a part of this study.

The oxidative burst which occurs following stimulation of equine neutrophils with serum-treated {opsonised} zymosan was significantly attenuated following pre-incubation with Ac2-26 at a concentration which alone caused no significant superoxide production. Addition of Ac2-26 at the same time as STZ had no significant effect, suggesting that the peptide may be affecting downstream signalling events involved in mediating the response to this particulate stimulus. That the inhibition required Ac2-26 binding to FPR and activation of p42/44 MAPK is indicated by the ability of BOC-2 and PD98059 to decrease the inhibitory effect. Studies in rodents have suggested

that the inhibitory effects of Ac2-26 on neutrophil function are mediated by the peptide binding to the murine analogue of FPRL-1 (Gastardelo et al., 2009). Hayhoe et al. (2006), however, found that an FPRL-1 antibody did not affect the inhibitory action of Ac2-26 on human neutrophil rolling *in vitro* although the effect was blocked by BOC-2. In contrast, Karlsson et al. (2005) have suggested that the inhibition of human neutrophil superoxide production obtained with Ac9-25 is mediated by an, as yet unidentified, receptor. As the inhibitory effects of Ac2-26 in murine, human and equine cells all appear to be mediated by members of the family of FPR receptors it suggests that Ac9-25 may behave differently to Ac2-26.

The observation that pre-treatment of equine neutrophils with PD98059 inhibits superoxide production in response to Ac2-26 ($5 \times 10^{-5}$ M) and reverses the inhibitory effect of $10^{-5}$ M Ac2-26 on the response to STZ appears somewhat contradictory as it implies that activation of p42/44 MAPK is required to both induce and inhibit the same response. However, PD98059 does not directly inhibit activation of p42/44 MAPK but rather that of the upstream kinase, MEK. Although PD98059 is a selective MEK inhibitor it can inhibit the activation of related kinases such as MEK5, and hence activation of ERK5 (Bain et al., 2007). Thus it is possible that it is the consequence of inhibition of different, or additional, signalling molecules downstream of MEK activation that is responsible for either the stimulatory or inhibitory effects of Ac2-26 on superoxide production by equine neutrophils.

In summary, neutrophils play a key role in host defence against invading pathogens but do also contribute to the pathogenesis of common equine inflammatory diseases such as laminitis (Black et al., 2006; Hurley et al., 2006), recurrent airway obstruction (Fairbairn et al., 1993) and endotoxaemia (Hedges et al., 2001). The actions of endogenous anti-inflammatory mediators are one means by which inappropriate neutrophil activation can be prevented. The present study supports the hypothesis that annexin-1 may help to down-regulate equine neutrophil superoxide production. There are as yet no reports demonstrating that generation of Ac2-26 occurs in inflamed tissue but Hayhoe et al. (2006) have speculated that it is unlikely high micromolar concentrations of N-terminal annexin 1 peptides will be attained *in vivo*. Thus, although this *in vitro* study has shown that Ac2-26 has the potential to promote tissue damage in equine disease by increasing localised free radical production, the negative regulatory role of annexin 1 peptides may be of greater importance *in vivo*. Indeed, by reducing neutrophil accumulation *in vivo*, intravenous administration of Ac2-26 has been shown to be protective in a rat model of myocardial ischaemia reperfusion injury during which activated cells can contribute to tissue damage by the release of free radicals (La et al., 2001).

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