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Short communication

Characterization of arginase expression by equine neutrophils

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ABSTRACT

Neutrophils are the predominant cells recruited in the airways of horses suffering from heaves. These cells have been shown to express arginase in some species. The metabolism of L-arginine is thought to be involved in chronic inflammation, and airway obstruction and remodeling. The aim of this study was to assess the expression, regulation, activity, and functional role of arginase isoforms in equine neutrophils. Arginase I, arginase II, ornithine decarboxylase (ODC) and ornithine aminotransferase (OAT) expression were assessed in resting and stimulated (IL-4, LPS/fMLP, PMA; 5 and 18 h) blood neutrophils using quantitative PCR. Arginase expression was also studied by Western blot and enzyme activity assay. The effect of nor-NOHA (1 mM), a specific arginase inhibitor, was assessed on arginase activity *in vitro* and *ex vivo* on neutrophil's inflammatory gene expression and viability. Results showed that equine neutrophils constitutively express arginase isoform 2, ODC and OAT. Neutrophil *ex vivo* stimulation did not induce arginase I or influence arginase II mRNA expression. *Ex vivo* inhibition of arginase activity by nor-NOHA had no effect on neutrophils inflammatory gene expression induced by LPS/fMLP (5 h) but significantly reversed the cell loss observed after this stimulation.

1. Introduction

Heaves is a chronic lower airway inflammatory disease of mature horses. It features bronchospasm, airway neutrophilia, and systemic inflammation following stabling and hay dust exposure (reviewed in Leclere et al., 2011b). Neutrophils are thought to contribute to the airway wall remodeling associated with heaves by prolonged survival due to reduced apoptosis (Turlej et al., 2001) and the release of mediators including proteinases, oxidative species, leukotrienes and pro-inflammatory cytokines (Brazil et al., 2005; Bureau et al., 2000; Lindberg et al., 2004).

Over the last decade, the arginase pathway was highlighted as a possible key factor in driving the pathologic changes associated with chronic airway diseases. Indeed, the metabolites of L-arginine promote tissue fibrosis through collagen deposition as well as cell proliferation and differentiation (reviewed in Maarsingh et al., 2009). Arginase converts L-arginine into urea and L-ornithine, the latter being metabolized through the ornithine aminotransferase (OAT) and ornithine decarboxylase (ODC) pathways respectively into L-proline, an essential constituent of collagen fibers, and into polyamines (putrescine, spermidine and spermine)(Wu and Morris, 1998) required for cell proliferation and differentiation (Pegg, 1986). Moreover, the arginase pathway competes with constitutive isoforms of nitric oxide synthase (NOS) for cellular uptake of L-arginine (Meurs et al., 2000). Thus, increased arginase activity limits the production of NO, an important endogenous bronchodilator (Ricciardolo et al., 2004) that also

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possesses anti-inflammatory properties (Bogdan, 2001). The two arginase isoforms are encoded by different genes (Cederbaum et al., 2004). Arginase I is cytosolic and highly expressed in the liver while arginase II is mitochondrial and is constitutively expressed in many tissues. Both enzymes have similar metabolic functions as they share 100% sequence homology in amino acid areas critical for enzymatic function (Cederbaum et al., 2004).

Studies performed using rodents suggest that the chronic asthmatic airway inflammation can be reversed using arginase inhibitors (Kenyon et al., 2008; Maarsingh et al., 2008; North et al., 2009). In these models, macrophages are one of the primary sources of increased arginase activity in the lungs (North et al., 2009; Zimmermann et al., 2003). Arginase I gene expression is STAT6-dependant (Zimmermann et al., 2003) and part of the murine alternatively activated macrophages (AAMϕ) transcriptome signature induced by Th2 cytokines (Gordon, 2003). In humans however, arginase I is constitutively expressed by neutrophils. They are also the only leukocytes expressing arginase I both *in vitro* (Luckner-Minden et al., 2010; Munder et al., 2005) and *in vivo* (King et al., 1986; Kropf et al., 2007; Munder et al., 2006; Rodriguez et al., 2009). While neutrophils have not been yet been identified as the source of arginase in asthmatic subjects, arginase I was shown to be up-regulated in the lungs and peripheral blood of these patients (Morris et al., 2004; North et al., 2009; Ogino et al., 2011).

Neutrophilic inflammation in heaves is associated with airway wall remodeling, that includes collagen deposition (Leclere et al., 2012; Setlakwe et al., 2009) and increased smooth muscle mass (Herszberg et al., 2006) which appears to be mostly irreversible (Leclere et al., 2011a, 2012). As Th2-type cytokines and alteration of IL-4 expression have been associated with heaves (Cordeau et al., 2004; Horohov et al., 2005; Joubert et al., 2001), we postulated that the arginase pathway may be involved in airway remodeling in this disease. In the present study, we first characterized the expression and regulation of arginase isoforms in normal equine neutrophils and evaluated selected functional aspects of the arginase pathway in equine neutrophils.

2. Materials and methods

2.1. Animals

Blood neutrophils were harvested from 11 healthy adult mixed-breed mares part of the Université de Montréal research herd. All experimental procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care and were approved by the Animal Care Committee of the Faculty of Veterinary Medicine of the Université de Montréal.

2.2. Human samples

Peripheral blood samples were collected from 2 healthy human volunteers. The use of human samples was approved by the Research Ethic Committee of the Faculty of Medicine of the Université de Montréal (Protocol #11-029-CERFM-D).

2.3. Neutrophil isolation from peripheral blood

For experiments in which human and equine neutrophils were compared, centrifugation on a density gradient of Lympholyte-poly® (sodium metrizoate and Dextran 500, Cedarlane Laboratory Limited) was used to isolated neutrophils from blood. Briefly, blood was drawn into sterile heparinized tubes (BD Vacutainer) and the polymorphonuclear-rich cell layer was harvested following centrifugation of whole blood according to manufacturer's instructions. The remaining red blood cells were lysed by hypotonic treatment. For all other experiments, immunomagnetic selection of equine neutrophils was used (MACS system, Myltenyi Biotec), as described previously (Joubert et al., 2001). Cell counting and viability was performed using the ADAM automatic Cell Counter (Montreal-Biotech Inc., Montréal, QC, CA). Cytospin slides were prepared (Cytospin2, Shandon) and stained with Protocol Hema 3 (Fisher Scientific) for differential counting of ≥400 cells to assess neutrophil purity. The purity and viability of neutrophils were 96.4 ± 0.85% and 98.6 ± 0.15%, respectively, when isolated using MACS and 93.5 ± 1.1% and 98.3 ± 0.4% (mean ± SEM) respectively using a density gradient.

2.4. Cell culture conditions

Purified equine neutrophils were suspended at 5×10^6 cells/mL in culture medium RPMI 1640 supplemented with 10% heat inactivated low-endotoxin FBS (Sigma–Aldrich), 2 mM L-glutamine (GIBCO), 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO) and incubated in suspension cell culture plates (Ultident, Canada) for 5 or 18 h at 37 °C, 5% CO2 with or without recombinant equine (req) Interleukine (IL)-4 (20 ng/mL, R&D Systems), lipopolysaccharide (LPS 100 ng/mL; 0111:B4, Sigma–Aldrich) and 10 nM formyl-Met-Leu-Phe (Sigma–Aldrich) or phorbol myristate acetate (PMA, 20 ng/mL; Sigma–Aldrich). The effect of inhibition of arginase activity on neutrophil functions was assessed as followed: neutrophils were pre-incubated with nor-NOHA (1 mM) or the same volume of DMSO (negative control) for 1 h at 37 °C in PBS w/o Ca^{2+} or Mg^{2+} supplemented with 0.5% BSA at 5×10^6 cells/mL then washed twice in PBS before being resuspended in supplemented RPMI for cell culture. In preliminary experiments where supernatants were harvested for polyamine analysis, neutrophils were cultured in RPMI supplemented with 0.5% BSA instead of FBS to prevent degradation of polyamines by serum enzymes. At the end of the culture period, the cells were recovered using a cell scraper in a microcentrifuge tube placed on ice. The cells were gently mixed and an aliquot was used for cell counting and viability assessment.

2.5. RNA extraction and qPCR

RNA extraction from Trizol®Reagent and reverse transcription was performed as described elsewhere