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Generation of nitric oxide by neutrophils isolated from heifers in the course of acute and chronic Bovine Respiratory Disease under the influence of IL-8

Wytwarzanie tlenku azotu przez neutrofile wyizolowane od jałówek w przebiegu ostrej i przewlekłej postaci zespołu oddechowego pod wpływem IL-8

Summary. Nitric oxide (NO) generated by neutrophils (PMN) is involved in tissue damage in the course of respiratory inflammation. However, little is known about participation of IL-8 in NO mediated lung injury in the course of Bovine Respiratory Disease (BRD). The purpose of this study was to evaluate how IL-8 influenced the generation of NO by neutrophils isolated from heifers in the course of acute and chronic BRD. Neutrophils isolated from blood were incubated with 0–1000 ng/ml of IL-8 and NO level was determined by Griess reaction after 0.5–72 h of incubation. Generation of NO increased with the growing concentration of IL-8. This augmentation was the greatest in acute BRD, less intense in chronic BRD and the least in healthy heifers. Both in healthy heifers and in both BRD groups, the maximal production of NO was observed in the presence of 1000 ng/ml IL-8. This observation revealed that generation of NO by neutrophils increased in dependence on the concentration of IL-8, time of incubation and group of animals. Therefore, augmented production of NO by neutrophils from heifers with BRD may lead to lung injury and worsening the course of disease.

Key words: neutrophil, heifer, nitric oxide, IL-8

INTRODUCTION

Bovine respiratory disease (BRD) is a polyetiological disease, which causes many economical problems in bovine farms. Although neutrophils are essential for the host defence, under certain conditions, prolonged or enhanced activation and degranulation of neutrophil mediate tissue damage contributed to the worsening of the course of disease

[Ruchaud-Sparagano *et al.* 1998, Wessely-Szponder and Bobowiec 2005]. Neutrophils cause tissue destruction by release of proteolytic enzymes such as elastase, myeloperoxidase and alkaline phosphatase, and production of free radicals such as nitric oxide. Nitric oxide, generated by neutrophils during inflammation, influences both acute and chronic inflammatory reactions. It is also involved in tissue destruction by initiating lipid peroxidation, DNA damage, or inactivation of enzymes and proteins during airway inflammation [Stockley 1995, Roy *et al.* 1996, Abu-Sound *et al.* 2000, Wessely-Szponder 2006]. Apart from these deleterious actions, NO rapidly reacts with superoxide anion yielding peroxynitrite. This compound is a strong weapon against invading microorganisms, but excessive formation of peroxynitrite is an important factor in the tissue damage during inflammatory process [Rodenas 1995, Ishida-Okawara *et al.* 1996, Muijsers *et al.* 1997, Dorger *et al.* 2002]. Release of NO by neutrophils can be regulated by several cytokines [Pechlowsky *et al.* 1996, Tsukahara *et al.* 2001]. However, the role of IL-8 stimulation of NO production in the course of BRD still remains unresolved. The aim of this study was to evaluate the influence of IL-8 on generation of NO by neutrophils isolated from heifers in the course of acute and chronic BRD.

MATERIALS AND METHODS

The study was conducted on 90 heifers divided into three groups on the basis of physical examination, which was performed before the collection of blood. Peripheral blood was collected with the following protocol: from 30 animals with acute BRD (three times in 4 days' intervals), from 30 animals with chronic BRD (in 14 days' intervals), and from 30 healthy heifers (n = 15 in 4 days' intervals, and n = 15 in 14 days' intervals). These periods correspond to three phases of disease: *stadium incrementi*, *stadium manifestationis* and *sanatio*. Neutrophils were isolated according to the method of Mottola [Hoebden *et al.* 1997, Wessely-Szponder *et al.* 2004]. The remaining pellet was washed with phosphate-buffered saline (PBS) and the final cell pellet was resuspended in 1 ml of Dulbecco's Modified Eagle's Medium (DMEM-Sigma). After isolation, viability of PMNs cells was determinated by trypan blue exclusion. After cells counting and differentiation cell suspensions were adjusted to a final concentration of $2 \cdot 10^6$ cells/ml. Then, cell cultures were incubated at 37°C and 5% CO_2 with 0, 5, 10, 100 and 1000 ng/ml of human recombinant IL-8, control groups were supplemented by PBS in equal volume. Nitric oxide level was determined after 0.5, 24, 48 and 72 hours of incubation by Griess reaction: 50 µl of supernatant was mixed with 200 µl of Griess reagent (1% sulfanilamide, 0.1% naphthylendiamine dihydrochloride and 2.5% H_3PO_4). The obtained values were expressed as concentration of nitrite, the stable product of NO, which accumulates in medium [Nims *et al.* 1995, Robbins and Grisham 1997, Ridnour *et al.* 2000, Seti and Dikshit 2000, Wessely-Szponder 2006]. The examined values were compared using the analysis of variance and Student's t-test and differences were considered as significant at $p < 0.05$.

RESULTS

NO generation by unstimulated neutrophils was the greatest in acute disease at the first phase and diminished in the second and third phases (3.0 ±0.57, 2.72 ±0.61, and

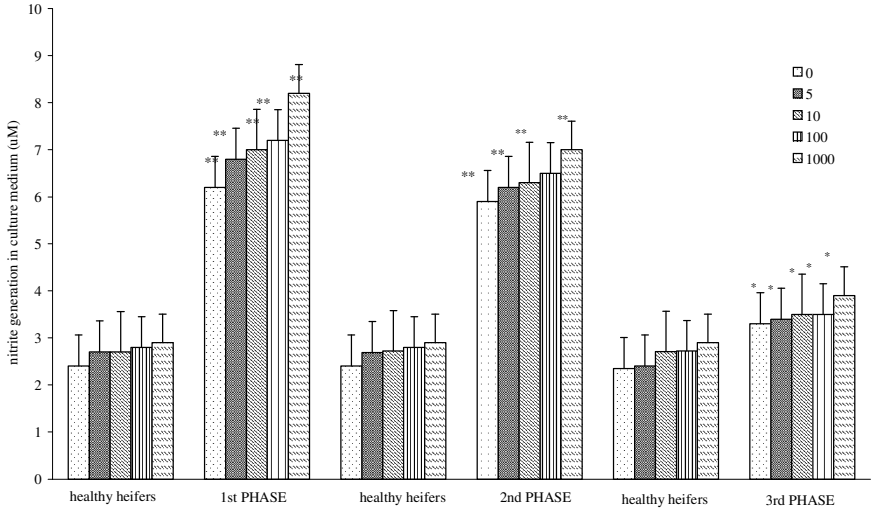

Fig. 1. The influence of different concentrations of IL-8 (in ng/ml) on NO generation by neutrophils isolated from heifers during three phases of acute BRD after 72 h of incubation. $^{**}p < 0.01$ versus neutrophils from healthy heifers; $^{*}p < 0.05$ versus neutrophils from healthy heifers (mean ±SD)

Rys. 1. Wpływ różnych stężeń IL-8 (w ng/ml) na wytwarzanie przez neutrofile izolowane od jałówek w trzech fazach ostrej BRD po 72 godz. inkubacji; $p < 0.05$, $^{**}p < 0.01$ w porównaniu z neutrofilami od zdrowych jałówek

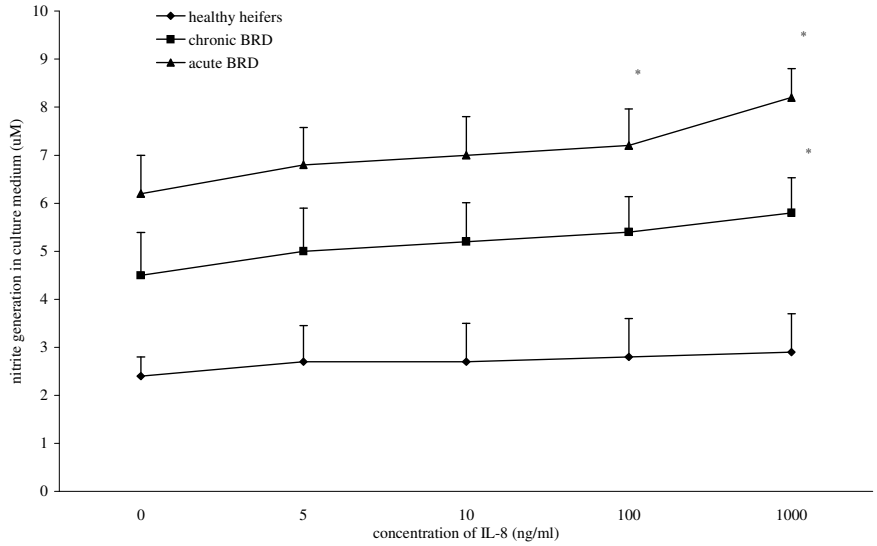

Fig. 2. The effect of different IL-8 (in ng/ml) concentrations on NO production by neutrophils from acute and chronic BRD heifers after 72 h of incubation;
$^{*}p < 0.05$ versus neutrophils from healthy heifers (mean ±SD)

Rys. 2. Działanie różnych stężeń IL-8 (w ng/ml) na wytwarzanie NO przez neutrofile wyizolowane od jałówek z ostrą lub przewlekłą postacią BRD po 72 godz. inkubacji;
$^{*}p < 0,05$, $^{**}p < 0,01$ w porównaniu z neutrofilami od zdrowych jałówek

1.64 ±0.44 μM of nitrite, respectively, after 0.5 h of incubation). Less intense NO production was observed in chronic disease (2.12 ±0.3 μM of nitrite in the first phase after 0.5 h of incubation). The values obtained in this group diminished in subsequent phases of the disease. In this experiment the production of NO increased with growing concentration of IL-8 up to the greatest response at 1000 ng/ml in all studied groups of animals and all phases of disease (Fig. 1–3). The level of nitrite in medium also gradually increased with time of incubation from 0.5 to 72 h. The greatest augmentation was observed in cultures from the group of acute disease (from 3.0 ±0.57 to 6.2 ±0.8 for unstimulated cells, and from 4.5 ±0.67 to 8.2 ±0.6 μM of nitrite for 1000 ng/ml, $p < 0.05$ in comparison with unstimulated cells) – Fig. 4.

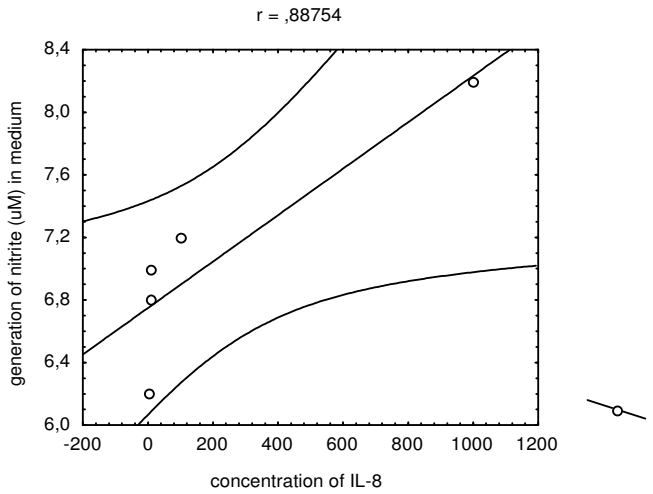

Fig. 3. Correlation between concentration of IL-8 and NO production by neutrophils from heifers with acute BRD after 72 h of incubation
Rys. 3. Zależność pomiędzy stężeniem IL-8 i wytwarzaniem NO przez neutrofile od jałówek z ostrą postacią BRD po 72 godz. inkubacji

DISCUSSION

Cytokines play a crucial role in the inflammatory process by neutrophil activation, which may lead to pulmonary tissue changes. They specifically modulate human neutrophil chemotaxis, adherence, degranulation, respiratory burst, antimicrobial activity, and cytotoxicity. IL-8 as a proinflammatory cytokine, promote neutrophil activation and up-regulate most functional responses of these cells [Pechlowsky *et al.* 1996].

The stimulating effect of IL-8 on generation of NO by neutrophils is related to the concentration of this cytokine, and varied in dependence on group of animals.

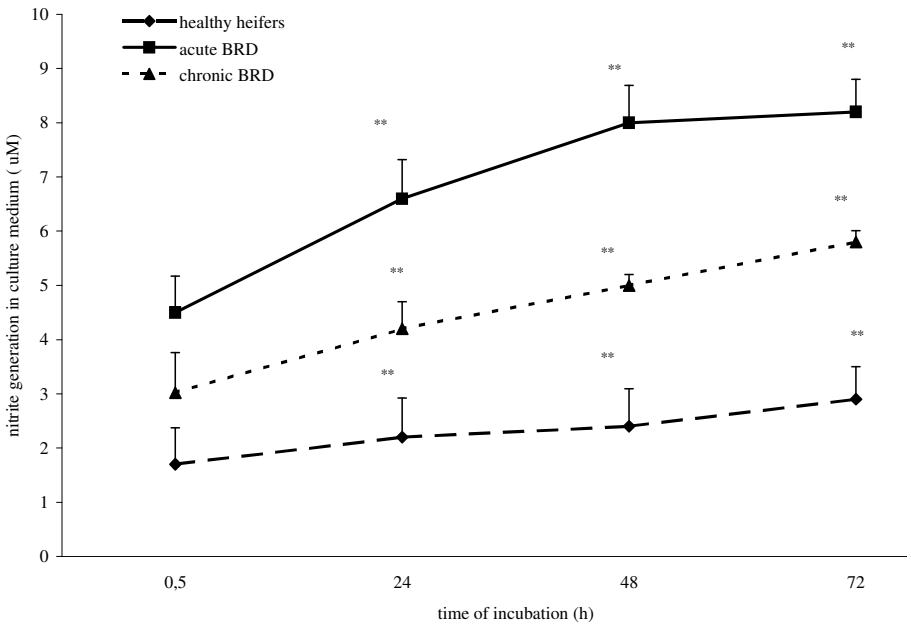

Fig. 4. Changes in generation of NO by neutrophils under influence of 1000 ng/ml IL-8, in acute and chronic BRD during 72 h incubation;
**p < 0,01 versus neutrophils from healthy heifers (mean ±SD)
Rys. 4. Zmiany w wytwarzaniu NO przez neutrofile pod wpływem IL-8 w stężeniu 1000 ng/ml w ostrej i przewlekłej postaci BRD w ciągu 72-godzinnej inkubacji;
**p < 0,01 w porównaniu z neutrofilami od zdrowych jałówek

The strongest effect was observed in acute BRD, less intense in chronic BRD and the least in group of healthy heifers. The generation of NO decreased during recovery in both studied groups. There are some discrepancies in reports concerning the influence of IL-8 on NO generation by neutrophils. Bratt and Palmblad [1997] estimated that IL-8 plays an important role in activation of PMN. Leff, in turn, discovered that IL-8 inhibit LPS-induced increase of inducible NOS (iNOS) which may cause inhibition of NO generation in airway disease. However, Tsuhakara *et al.* [2001] pointed out that IL-8 as well as other cytokines such as TNFα, IL-1, IL-6 is associated with pathogenesis of sepsis. These cytokines in connection with LPS may induce iNOS mRNA expression and NO production by circulating neutrophils in sepsis patients in human. This mechanism also appeared in neutrophils of heifers, which explain higher activity of neutrophils from BRD heifers. Our observations revealed that generation of NO by neutrophils increased in dependence on the concentration of IL-8, time of incubation and group of animals. According to some authors, augmented production of NO by neutrophils may lead to lung injury and worsening the course of disease [Rodenas *et al.* 1995, Roy *et al.* 1996, Abu-Sound and Hazen 2000].

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Streszczenie. Tlenek azotu (NO) wytwarzany przez neutrofile (PMN) ma istotne znaczenie w generowaniu uszkodzeń tkanek w chorobach układu oddechowego, niewiele jednak wiadomo na temat udziału IL-8 w zachodzącym za pośrednictwem NO uszkodzeniu płuc w zespole oddechowym u bydła (BRD). Celem pracy było zbadanie wpływu IL-8 na uwalnianie NO przez neutrofile wyizolowane od jałówek w przebiegu ostrej i przewlekłej postaci BRD. Wyizolowane z krwi neutrofile poddano działaniu IL-8 w stężeniach 0–1000 ng/ml i inkubowano przez 0,5 do 72 godzin w celu oznaczenia poziomu NO za pomocą reakcji Griessa. Doświadczenie wykazało, że poziom NO rósł wraz z rosnącymi stężeniami IL-8, osiągając maksimum przy 1000 ng/ml. Najwyższe wytwarzanie NO przez neutrofile zaobserwowano w ostrej postaci BRD, mniej intensywne w przewlekłej, a najniższe w przypadku zdrowych jałówek. Obserwacje te dowiodły, że ilość NO wzrastała w zależności od stężenia IL-8, czasu inkubacji i grupy zwierząt. Zwiększone wytwarzanie NO przez neutrofile może być przyczyną powstawania uszkodzeń płuc i pogorszenia stanu klinicznego zwierząt podczas choroby.

Słowa kluczowe: neutrofil, jałówka, tlenek azotu, IL-8