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Redox-sensitive regulation of macrophage-inducible nitric oxide synthase expression *in vitro* does not correlate with the failure of apocynin to prevent lung inflammation induced by endotoxin

Daniela Viačková[a], Michaela Pekarová[a], Tomáš Crhák[a,b], Martina Búcsaiová[a], Ján Matiašovic[c], Antonín Lojek[a], Lukáš Kubala[a,*]

[a] *Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, CZ-612 65 Brno, Czech Republic*
[b] *Institute of Experimental Biology, Department of Physiology and Immunology of Animals, Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic*
[c] *Department of Immunology, Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic*

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ABSTRACT

Reactive oxygen and nitrogen species are among the crucial mediators in the development of the pathological inflammatory process in the lungs and contribute to the damage of lung epithelium. The aim of the present study was to evaluate the potential of selected antioxidants or inhibitors of NADPH oxidase (glutathione, N-acetyl cysteine, trolox, apocynin, and diphenyleneiodonium chloride) to modulate nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression by mouse macrophages induced by lipopolysaccharide (LPS) *in vitro* and to evaluate the potential of apocynin to modulate the course of LPS-induced lung inflammation *in vivo*. All the tested drugs revealed inhibitory effects on LPS-induced NO production and iNOS expression in RAW 264.7 macrophages. Further, apocynin significantly inhibited activation of nuclear factor kappa B induced by LPS. *Ex vivo,* diphenyleneiodonium chloride and apocynin significantly reduced ROS production by inflammatory cells isolated from bronchoalveolar lavage fluid. In contrast, *in vivo* intranasal application of apocynin did not exert any significant effect on the course of lung inflammation in mice induced by LPS that was evaluated based on the accumulation of cells, interleukine-6, interleukine-12, RANTES, tumor necrosis factor-alpha, and protein concentration in bronchoalveolar lavage fluid and expression of iNOS in lung tissue. Only effected were the levels of nitrites 36 h after induction of lung inflammation that were reduced in the apocynin-treated group. In conclusion, our data suggest that the inhibitors of NADPH oxidase possess inhibitory potential against LPS-induced NO production by mouse macrophages; however, apocynin failed to reduce LPS-induced lung inflammation in mice.

Introduction

The adverse health effects of the chronic exposure of farm animals and farm workers to organic dust have a high importance for the occurrence of chronic lung diseases (Douwes et al. 2003; Omland 2002). Symptoms reported in studies investigating organic-dust environments often comprise a variety of non-specific symptoms associated with a transient increase in non-specific airway responsiveness and airway inflammation in organic-dust environments (Omland 2002). Among many agents present in organic dust, bacterial endotoxin is a major candidate for the induction of an inflammatory reaction in the lungs (Omland 2002; Thorn 2001). It has been demonstrated that symptoms similar to those reported in studies investigating organic-dust environments can be observed after acute inhalation of endotoxin (Jagielo et al. 1996; Larsson et al. 1994). Therefore, it has been suggested that inhalation challenges with endotoxin mimic those found among animals or humans exposed to endotoxin in organic-dust environments (Rylander et al. 1989; Thorn 2001). Lipopolysaccharide (LPS), the main component of endotoxin found in the outer membrane of various Gram-negative bacteria, binds to the surface receptors of epithelial cells and inflammatory cells present in the lungs, mainly macrophages (Thorn 2001). In response to LPS, these cells produce a wide range of inflammatory mediators leading to an intensive influx of leukocytes, particularly neutrophil granulocytes. Neutrophil granulocytes, together with macrophages, release

Abbreviations: APO, apocynin; BALF, bronchoalveolar lavage fluid; DMSO, dimethyl sulfoxide; DPI, diphenyleneiodonium chloride; GSH, glutathione; IL, interleukine; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NAC, N-acetyl cysteine; NF-κB, nuclear factor-κB; NO, nitric oxide; OZP, opsonized zymosan particles; PBS, phosphate buffered saline; PMA, phorbol-12-myristate-13-acetate; ROS, reactive oxygen species; qRT-PCR, real-time reverse transcription polymerase chain reaction.

* Corresponding author. Tel.: +420 541 517 117; fax: +420 541 211 293.
E-mail address: kubalal@ibp.cz (L. Kubala).

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antimicrobial and inflammatory intermediaries, including reactive oxygen species (ROS) and nitric oxide (NO), proteases, and cytokines, further promoting the inflammatory process and contributing to the damage of host tissue (Azenabor et al. 2009; Thorn 2001; Welbourn and Young 1992).

These inflammatory mediators not only trigger an acute inflammatory response but also enhance oxidative stress. ROS together with NO modulate both the extra and intracellular redox environments that play a key role in the regulation and potentiation of inflammatory responses in lung cells. The imbalance of the redox system may lead to abnormal signaling associated with lung-tissue damage (Rahman 2005). Furthermore, intracellular signaling pathways triggered upon the activation of cells by endotoxin are sensitive to the intracellular redox environment (Genestra 2007). Thus, it has been suggested that intracellularly formed ROS by NADPH oxidases, in phagocytes particularly by isoform NOX2, and NO produced NO synthases, are important mediators augmenting the response of cells to LPS. Redox-sensitive signaling pathways activated by LPS lead to the activation of the nuclear factor-κB (NF-κB) transcription factor, which results in various gene expressions. One of these is inducible nitric oxide synthase (iNOS), a constitutively active enzyme that is responsible for the exhaustive production of NO in macrophages and other cell types (Azenabor et al. 2009; Pekarova et al. 2009a; Zelnickova et al. 2008). Redox regulation of iNOS expression in macrophages and other cell types was suggested previously by various authors (Blesa et al. 2003; Gerhauser et al. 2003; Lanone et al. 2005; Mendes et al. 2001; Prasanna et al. 2007; Victor et al. 2003; Wu et al. 2008). Interestingly, in a complex environment NO could positively promote NOS expression and further increase NO production through the positive-feedback mechanism. Thus, expression of iNOS and overproduction of NO by inflammatory cells could be suggested as crucial in the development of acute lung inflammation. Studies focusing on the modulation of lung inflammation through iNOS selective inhibition have produced dichotomous results, but a significant number of data suggest that iNOS contributes to the pathogenesis of acute lung inflammation induced by endotoxin (Baron et al. 2004; Okamoto et al. 2004; Zeidler et al. 2004).

The intracellular redox status can be pharmacologically modulated by chemical antioxidants that act by donating an electron to a free radical and converting it to a non-radical form, or by the inhibition of enzymes involved in ROS and NO production, including inhibitors of NADPH oxidases (Genestra 2007; Heumuller et al. 2008; Mendes et al. 2001; Stefanska and Pawliczak 2008).

The purpose of this study was to examine the hypothesis that LPS-induced iNOS expression and production of NO by macrophages are reduced by antioxidants and inhibitors of NADPH oxidases and, through this, an application of selected drug results in reduced LPS-induced lung inflammation in mice. To this end, the potency of different inhibitors of NADPH oxidases and antioxidants (apocynin – APO, glutathione – GSH, N-acetyl cysteine – NAC, trolox, diphenyleneiodonium chloride – DPI) to modulate LPS-mediated NO production and iNOS expression by mouse macrophages was evaluated *in vitro*. Based on these results, APO, both a NADPH oxidase inhibitor and an antioxidant significantly inhibiting iNOS expression and NO production *in vitro*, was tested to modulate the course of lung inflammation induced by intranasal LPS *in vivo*.

Materials and methods

In vitro cell cultures and preparation of tested compounds

The murine RAW 264.7 macrophage cell line was obtained from the American Type Culture Collection (ATCC, Manassas, USA). Cells were maintained in a Dulbecco's Eagle medium (PAN – Biotech GmbH, Aidenbach, Germany) supplemented with a 10% fetal bovine serum (PAN) and gentamycin (0.045 mg/L) (Pekarova et al. 2009b). Unless otherwise stated, all chemicals were the highest grade from Sigma–Aldrich (St. Louis, USA). The stock solution of LPS from *Escherichia coli* serotype 026:B6 was 1 mg/ml in phosphate buffered solution (PBS), pH 7.4. The final concentration of LPS for cell treatment was 0.1 μg/ml. The stock solution of DPI was made up at 5 mM in dimethyl sulfoxide (DMSO). APO (4-hydroxy-3-methoxyacetophenone) stock solution (20 mM) was prepared in ethanol. The stock solution of trolox (230 mM) was prepared in DMSO and prediluted in PBS. The stock solution of GSH (1 M) was prepared in PBS. NAC (400 mM) was dissolved in PBS with addition of NaOH to reach pH 7.4. All appropriate vehicle and saline controls were tested in the experiments. For most experiments, RAW 264.7 was seeded at a density 500×10^3 cells/well in 6-well plates (TPP, Trasadingen, Switzerland), grown for 24 h, and treated by LPS and/or tested compounds for another 24 h. For immunofluorometric determination of iNOS protein, RAW 264.7 was diluted to a density of 100×10^3 cells/well of a 96-well microplate (TPP), grown for 24 h and treated by LPS and/or tested compounds for another 24 h.

In vivo model of acute lung inflammation in mice induced by LPS

The experiments were approved by the Animal Care Committee and were in accordance with the EU and NIH Guide for Care and Use of Laboratory Animals. C57BL/6J mice (25–30 g) (Masaryk University, Brno, Czech Republic) were subjected to brief anesthesia with ketamine–xylazine (Gedeon Richter Ltd., Budapest, Hungary and Spofa, Prague, Czech Republic). A stock solution of APO (1 M) in DMSO was diluted in PBS to the concentration 1 mM (0.1% DMSO). Mice were pretreated with 50 μl of 1 mM APO instilled directly into their nostrils (Szarka et al. 1997). Control mice received a similar volume of sterile 0.1% DMSO dissolved in PBS. Further, a intranasal application of the LPS (from *Escherichia coli* serotype 026:B6) solution in PBS (50 μl) was similarly applied to the anaesthetized mice to reach a dose of 0.3 mg/kg 1 h later. At different times – 12, 24, 36 or 48 h after LPS instillation – the mice were overdosed by intraperitoneal administration of ketamyne/xylazin. Blood was collected by cardiac puncture. The trachea was cannulated, and the lungs were lavaged with 2 consecutive washes with 1 ml of PBS to obtain bronchoalveolar lavage fluid (BALF). The recovery of BALF was approximately 85–95% of the used volume. BALF was centrifuged at 700 *g* for 10 min at 4 °C. Supernatants were aliquoted, immediately frozen, and maintained at −80 °C until cytokine and nitrite measurements were taken. Cell pellets were resuspended in 300 μl of PBS, and the total cell counts were determined using a Coulter Counter ZF (Beckman Coulter, Brea, USA). The remaining cell suspension was centrifuged in a cyto-chamber (Fisher Scientific, Pittsburgh, USA). Cytospin slides were stained with the Giemsa stain, and differential counts were determined under a light microscope by counting at least 300 cells. For qRT-PCR analysis, lungs without performance of lung lavage were collected and stored at −80 °C.

Determination of NO production

The production of NO was estimated indirectly as the accumulation of nitrites, the metabolic end-product of NO reactions, in the medium or BALF using the Griess reagent as described previously (Pekarova et al. 2009a). Briefly, 150 μl of culture supernatant or BALF was added to 150 μl of Griess reagent and incubated for 20 min, and the absorbance was measured at 546 nm on a SLT Rainbow spectrophotometer (Tecan, Männedorf, Switzerland).