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Apocynin ameliorates endotoxin-induced acute lung injury in rats

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ABSTRACT

Acute lung injury (ALI) is a serious clinical syndrome with a high rate of mortality. In this study, the effects of apocynin, a NADPH-oxidase (NOX) inhibitor on lipopolysaccharide (LPS)-induced ALI in rats were investigated. Male Sprague–Dawley rats were treated with apocynin (10 mg/kg) intraperitoneally (i.p.) 1 h before LPS injection (10 mg/kg, i.p.). The results revealed that apocynin attenuated LPS-induced ALI as it decreased total protein content, lactate dehydrogenase (LDH) activity and the accumulation of the inflammatory cells in the bronchoalveolar lavage fluid (BALF), In addition, apocynin significantly increased superoxide dismutase (SOD) and reduced glutathione (GSH) activities with significant decrease in the lung malondialdehyde (MDA) content as compared to LPS group in lung tissue and decreased pulmonary artery contraction induced by LPS. It also up-regulated mRNA expression of inhibitory protein kappaB-alpha (NFκBia) and downregulated mRNA expression of Toll-Like receptor 4 (TLR4) and decreased inflammation observed in lung tissues.
Collectively, these results demonstrate the protective effects of apocynin against the LPS-induced ALI in rats through its antioxidant and antiinflammatory effect that may be attributed to the decrease in mRNA expression of TLR4 and increasing that of NFκBia.

1. Introduction

Acute lung injury (ALI) is a frequent complication following sepsis in critically ill patients and is associated with high rates of morbidity and mortality [1]. It is characterized by inflammation, increase in pulmonary vascular permeability, loss of alveolar-capillary barrier in the lungs and flooding of air space with protein-rich pulmonary edema that leads to impaired respiratory function of the lungs [2] in addition to both epithelial and endothelial cell death [3].

Endotoxin or lipopolysaccharide (LPS) is one of the most powerful proinflammatory factors and considered the most important cause of ALI [4]. LPS is a glycolipid that constructs the outer membrane of Gram-negative bacteria and is well-known as an important mediator of sepsis [5]. It can cause experimental ALI *in vivo* that closely resembles ALI in humans [6] as LPS exposure displays major features of microvascular lung injury including lung inflammation, pulmonary edema and leukocyte accumulation in lung tissue in rats and mice [7,8].

Inflammation is the major mechanism through which LPS can induce ALI [9]. LPS activates mainly its membrane Toll-Like receptor 4 (TLR4), in human monocytes, inducing signal transduction pathways leading to activation of various transcription factors such as nuclear factor-kappaB (NF-κB) and activator protein-1 (AP-1) [10]. The degree of inflammatory mediator expression in response to LPS is principally regulated by NF-κB [11]. In the unstimulated cells, NF-κB is constitutively localized as a heterodimer in the cytosol by physical association with the inhibitory protein kappaB-alpha (NFκBia or IKB-α) [12]. LPS activates NF-κB by phosphorylation and proteolytic degradation of IκB-α protein upon which NF-κB migrates to the nucleus which result in activation of a variety of target genes that involved in inflammation [13,14].

Another mechanism of LPS-induced ALI is oxidative stress [15,16]. LPS-induced ALI is associated with an exaggerated production of cell-damaging reactive oxygen species (ROS) by neutrophils sequestered within the lung vasculature and by stimulated macrophages [17]. ROS may play a role in enhancing inflammation, either directly by activation of pro-inflammatory mediators activated by NF-κB transcription or *via* the formation of lipid peroxidation products, through the activation of stress kinases and AP-1 that lead to cell death [13].

Apocynin (4-hydroxy-3-methoxy-acetophenone) is a naturally occurring methoxy-substituted catechol that inhibits the NADPH-oxidase (NOX) which is the major enzyme responsible for generating the initial ROS molecule superoxide in activated leukocytes [18]. Apocynin is a pro-drug that is oxidized by peroxidases in the cell and converted into more active metabolites [19]. Several *in vivo* studies have demonstrated that apocynin can prevent neutrophil oxidative burst and chemotaxis, therefore reduce neutrophil-mediated cell injury [20]. It has been studied as a possible remedy for inflammation-mediated diseases, including asthma, arthritis, and cardiovascular diseases [21]. A previous study reported the protective effect of derivatives of apocynin, but not apocynin itself, on intratracheal LPS-induced ALI in rats through inhibition of NADPH oxidase [22].

Since LPS-induced ALI involves inflammation and oxidative stress, therefore we investigated the protective effect of apocynin in a model of LPS-induced ALI in rats.

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Table 1
Effect of apocynin on LPS-induced changes on total and differential cell count in BALF of rats:

Group	Total cell (cell/lung) $\times 10^6$	Lymphocyte (cell/lung) $\times 10^6$	Neutrophil (cell/lung) $\times 10^6$	Monocyte (cell/lung) $\times 10^6$
Control	0.30 ± 0.05	0.20 ± 0.05	0.07 ± 0.01	0.03 ± 0.01
LPS	2.29 ± 0.26*	1.40 ± 0.36*	0.49 ± 0.01*	0.37 ± 0.04*
Apocynin	0.38 ± 0.06	0.23 ± 0.04	0.08 ± 0.01	0.07 ± 0.01
Apocynin + LPS	0.77 ± 0.17$	0.55 ± 0.13$	0.08 ± 0.03$	0.12 ± 0.06$

Rats were treated with apocynin (10 mg/kg, i.p.) 1 h before LPS injection (10 mg/kg, i.p.) and BALF was collected after 24 h to determine total and differential cell count. Data are expressed as mean ± SEM, n = 6.
*, $Significantly different from control or LPS group respectively, using one-way ANOVA test with Tukey–Kramer multiple comparisons *post hoc* test at $p < 0.05$.
LPS: lipopolysaccharide; ANOVA: analysis of variance.

2. Materials and methods

2.1. Drugs and chemicals

Lipopolysacchride (LPS, *Escherichia coli* serotype 0111:B4), apocynin (Acetovanillone; purity ≥98%), 1,1′, 3,3′-tetramethoxypropane, acetylcholine hydrochloride (ACh), phenylephrine hydrochloride (PE), sodium nitroprusside (SNP), pentobarbital sodium, Ellman's reagent [5,5′-dithio-bis(2-nitrobenzoic acid)], pyrogallol, reduced glutathione, thiobarbituric acid (TBA), Tris (hydroxymethyl) aminomethane, and trichloroacetic acid (TCA) were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Experimental animals

Male Sprague–Dawley rats, with average age of 6–8 weeks were purchased from "Egyptian Organization for Biological Products and Vaccines", Giza, Egypt.

This study protocol was approved by the "Research Ethics Committee" of Faculty of Pharmacy, Mansoura University, Egypt in accordance with "The Principles of Laboratory Animal Care" (NIH publication No. 85-23, revised 1985).

2.3. Experimental protocol

The rats were allocated into 4 groups each consists of 6 rats. Group (1): Control group; rats receiving normal saline i.p. Group (2): LPS group: rats receiving LPS (10 mg/kg, i.p.) [23,24]. Group (3): Apocynin group: Rats receiving apocynin (10 mg/kg, i.p.) [25–27]}. Group (4): (Apocynin + LPS group): rats receiving LPS (10 mg/kg i.p.) 1 h after apocynin (10 mg/kg, i.p.) injection.

Normal saline was used for dissolving LPS, 95% ethyl alcohol was used for dissolving apocynin then diluted with saline so that the final volume of the organic vehicle is negligible and not affecting the parameters measured.

Rats were anesthetized with pentobarbital sodium (40 mg/kg intravenously) after 24 h, then bronchoalveolar lavage fluid (BALF), pulmonary arterial (PA) rings were isolated for *in-vitro* vascular reactivity and lung tissue was used for quantitative RT-PCR.

Another set of experimental animals that undergo the same experimental protocol ($n = 4$) was served for measuring the oxidative biomarkers and histopathological examination. Samples were analyzed freshly on the same day.

2.3.1. BALF preparation

BALF preparation was carried out by cannulating the trachea and infusing the lung 3 times with 6 ml sterile of 0.9% saline, with 50–70% fractions recovered of the initial volume of saline. BALF fractions were spun using cooling centrifuge (Sigma D-37520, Germany) at 2000 *g* for 10 min and 4 °C.

The cell-free supernatant was used to measure total protein content and lactate dehydrogenase (LDH) activity. The cell pellets of BALF fractions were pooled and resuspended with 500 μl of sterile saline and aliquots of the cell suspensions were used to determine total and differential cell count.

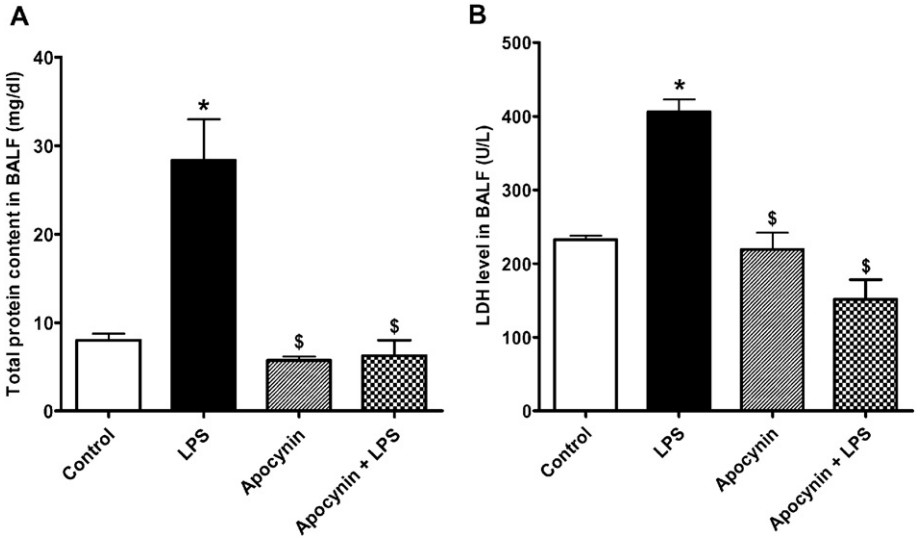

Fig. 1. Effect of apocynin on LPS-induced changes in total protein content and LDH activity in BALF of rats. Rats were treated with apocynin (10 mg/kg, i.p.) 1 h before LPS injection (10 mg/kg, i.p.) and BALF was collected after 24 h to determine total protein content (A) and LDH (B). Data are expressed as mean ± SEM, n = 6. *, $Significantly different from control or LPS group respectively, using one-way ANOVA test with Tukey–Kramer multiple comparisons *post hoc* test at $p < 0.05$. LPS: lipopolysaccharide; LDH: lactate dehydrogenase; BALF: Bronchoalveolar lavage fluid: ANOVA: analysis of variance.