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Effect of apocynin, a NADPH oxidase inhibitor, on acute lung inflammation

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ABSTRACT

NADPH-oxidase is an enzyme responsible for reactive oxygen species production (ROS) and inhibition of this enzyme represents an attractive therapeutic target for the treatment of many diseases. The aim of this study was to investigate the effects of apocynin, a NADPH-oxidase inhibitor, in a mouse model of carrageenan-induced pleurisy. Injection of carrageenan into the pleural cavity of mice elicited an acute inflammatory response characterized by: infiltration of neutrophils in lung tissues and subsequent lipid peroxidation, increased production of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) and increased expression of intercellular adhesion molecule (ICAM-1) and platelet-adhesion molecule (P-selectin). Furthermore, carrageenan induced the expression of nuclear factor-κB (NF-κB) inducible nitric oxide synthase (iNOS), nitrotyrosine, poly-ADP-ribosyl polymerase (PARP) as well as induced apoptosis (TUNEL staining, FAS-ligand expression, Bax and Bcl-2 expression) and mitogen-activated protein kinase (MAPK) activation in the lung tissues. Administration of apocynin, 30 min after the challenge with carrageenan, caused a significant reduction of all the parameters of inflammation measured. Thus, based on these findings we propose that NADPH oxidase inhibitor such as apocynin may be useful in the treatment of various inflammatory diseases.

1. Introduction

Carrageenan-induced local inflammation is commonly used to evaluate anti-inflammatory effects of non-steroidal drugs (NSAIDs). Therefore, carrageenan-induced local inflammation (pleurisy) is a useful model to assess the contribution of mediators involved in cellular alterations during the inflammatory process. In particular, the initial phase of acute inflammation (0–1 h) which is not inhibited by NSAIDs such as indomethacin or aspirin, has been attributed to the release of histamine, 5-hydroxytryptamine and bradykinin, followed by a late phase (1–6 h) mainly sustained by prostaglandin release and attributed to the induction of inducible cyclo-oxygenase (COX-2) in the tissue [1]. It appears that the onset of the carrageenan-induced acute inflammation has been linked to neutrophil infiltration and the production of neutrophil-derived free radicals, such as hydrogen peroxide, superoxide and hydroxyl radical, as well as the release of other neutrophil-derived mediators. One of the major sources of superoxide anion ($O_2^{\bullet -}$) is the NADPH-dependent oxidase present in the plasma membrane of phagocytic cells such as PMN leukocytes and macrophages [2]. Although reactive oxygen species (ROS) formation by neutrophils may be a physiological response which is advantageous to the host, the process is certainly also disadvantageous since it may give rise to excessive tissue damage [3]. Furthermore, oxidative stress elicits the activation of the redox-sensitive transcription factors such as nuclear factor-κB (NF-κB) and AP-1, that play a central and crucial role in inducing the expression of inflammatory cytokines and intercellular adhesion molecule ICAM-1 [4,5] and the activation of the redox-sensitive protein kinases such as the mitogen-activated protein kinase (MAPK) superfamily [6]. Therefore, compounds that can interfere with ROS production may be useful tools to prevent tissue destruction. Plants extracts, in this respect, have been tested for their anti-inflammatory activity [7]. Apocynin (4-hydroxy-3-methoxy-acetophenone) is a constituent of the Himalayan herb Picrorhiza kurrooa Royle (Scrophulariaceae) that is well known in traditional Indian medicine (Ayurveda). It is an acetophenone to which a range of biological activities is attributed [8]. It is a prodrug that is converted by peroxidase-mediated oxidation to a dimer, which has been shown to be more efficient than apocynin itself [9]. Apocynin is an inhibitor of the intracellular translocation of two critical cytosolic components of the NADPH-oxidase complex present in the cell membrane [10]. In

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this regard, a possible explanation for the effectiveness of apocynin in the treatment of respiratory diseases might be the fact that apocynin inhibits peroxynitrite ($ONOO^-$) formation [11] that is suggested to induce epithelial damage, mediator release, and consequently hyperresponsiveness [12]. The involvement of NADPH oxidase has been studied in many models of acute lung injury and it has been recognized to contribute importantly [2,13–15]. The present studies, in fact, were designed to evaluate the effects of apocynin in mice model of acute inflammation. In particular, we investigate the effects of apocynin on the lung injury associated with carrageenan induced pleurisy.

2. Materials and methods

2.1. Animals

Male adult CD1 mice (25–30 g, Harlan Nossan, Milan, Italy) were used for all studies. Mice were housed in individual cages (5 for each group) and maintained under 12:12 light–dark cycle at 21 ± 1 °C and 50 ± 5% humidity. The animals were acclimated to their environment for 1 wk and had *ad libitum* access to tap water and standard rodent standard diet. All animal experiments complied with regulations in Italy (D.M. 116192), Europe (O.J. of E.C. L 358/1 12/18/1986) and USA (Animal Welfare Assurance No. A5594-01, Department of Health and Human Services, USA). All behavioral testing was conducted in compliance with the NHI laboratory animal care guidelines and with protocols approved by the Institutional Animal Care and Use Committee (Council directive # 87-848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permission # 92-256 to SC). The study was approved by the University of Messina Review Board for the care of animals.

2.2. Carrageenan-induced pleurisy

Mice were anaesthetized with isoflurane and submitted to a skin incision at the level of the left sixth intercostal space. The underlying muscle was dissected and saline (0.1 ml) or saline containing 2% λ-carrageenan (0.1 ml) was injected into the pleural cavity as previously described [16]. At 4 h after the injection of carrageenan, the animals were sacrificed under CO_2 vapors. The chest was carefully opened and the lung tissues have been collected for the biochemical and histological parameters followed described. Considering that the inflammatory response induced by carrageenan in the pleural space of the mice has a biphasic profile, peaking at 4 and 48 h after pleurisy induction, in this study, we measured the expression of inflammatory mediators and signaling molecules 4 h after injection of carrageenan on the basis of previous studies [17,18].

2.3. Experimental groups

Mice were randomly allocated into the following groups: (i) *CAR + vehicle group*. Mice were subjected to carrageenan-induced pleurisy and received the vehicle for apocynin (10% dimethylsulfoxide (DMSO) (v/v) i.p. bolus 30 min prior and 30 min after carrageenan administration ($N = 10$), (ii) *CAR + apocynin group*. Mice were subjected to carrageenan-induced pleurisy and received apocynin (5 mg/kg 10% DMSO i.p. bolus) 30 min prior and 30 min after carrageenan administration ($N = 10$), (iii) *Sham + saline group*. Identical surgical procedures to the CAR group were performed, except that saline was administered instead of carrageenan ($n = 10$), (iv) *Sham + apocynin group*. Mice received apocynin (5 mg/kg 10% DMSO i.p. bolus) 30 min prior and 30 min after administration of saline ($N = 10$). In this experiment, the dose of apocynin 5 mg/kg i.p. was chosen in agreement with a previous study on cerebral ischemia [19].

2.4. Histological examination

Lung tissues samples were taken 4 h after injection of carrageenan. Lung tissues samples were fixed for 1 week in 10% (w/v) PBS-buffered formaldehyde solution at room temperature, dehydrated using graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, USA). Sections were then deparaffinized with xylene, stained with hematoxylin and eosin. All sections were studied using a Axiovision Ziess (Milan, Italy) microscope. The following morphological criteria were used for scoring: 0, normal lung; grade 1, minimal edema or infiltration of alveolar or bronchiolar walls; grade 3, moderate edema and inflammatory cell infiltration without obvious damage to lung architecture; grade 4, severe inflammatory cell infiltration with obvious damage to lung architecture. All the histological studies were performed in a blinded fashion.

2.5. Immunohistochemical localization of P-selectin, ICAM-1, IL-1β, TNF-α, nitrotyrosine, PAR, Fas ligand, Bax and Bcl-2

At the end of the experiment, the tissues were fixed in 10% (w/v) PBS-buffered formaldehyde and 8 μm sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeabilized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin, respectively. Sections were incubated overnight with anti-P-selectin antibody (Pharmingen, 1:500 in PBS, v/v) (D.B.A. s.r.l., Milan, Italy), anti-ICAM-1 antibody (Pharmingen, 1:500, in PBS, v/v), (D.B.A. s.r.l., Milan, Italy), anti-nitrotyrosine rabbit polyclonal antibody (Upstate, 1:500 in PBS, v/v), (D.B.A. s.r.l., Milan, Italy), anti-PAR antibody (BioMol, 1:200 in PBS, v/v), (D.B.A. s.r.l., Milan, Italy), anti-FAS ligand antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v), (D.B.A. s.r.l., Milan, Italy), anti-TNF-α antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v), (D.B.A. s.r.l., Milan, Italy), anti-IL-1β antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v), (D.B.A. s.r.l., Milan, Italy), anti-Bax antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v) (D.B.A. s.r.l., Milan, Italy), or with anti-Bcl-2 polyclonal antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v) (D.B.A. s.r.l., Milan, Italy). Sections were washed with PBS, and incubated with secondary antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin–biotin peroxidase complex (Vector Laboratories, DBA). In order to confirm that the immunoreaction for the nitrotyrosine was specific, some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity. To verify the binding specificity for IL-1β, TNF-α, PAR, ICAM-1, P-selectin, Fas ligand, Bax and Bcl-2 some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations no positive staining was found in the sections indicating that the immunoreaction was positive in all the experiments carried out. Immunocytochemistry photographs ($n = 5$) were assessed by densitometry. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). All the immunocytochemistry analysis was carried out without knowledge of the treatments.

2.6. MPO activity

MPO activity, an indicator of PMN accumulation, was determined as previously described [20]. At the specified time following injection of carrageenan, lung tissues were obtained and weighed,

each piece homogenized in a solution containing 0.5% (w/v) hexadecyltrimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000 × *g* at 4 °C. An aliquot of the supernatant was then allowed to react with a solution of tetramethylbenzidine (1.6 mM) and 0.1 mM hydrogen peroxide. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide min^{-1} at 37 °C and was expressed in milliunits per gram weight of wet tissue.

2.7. MDA measurement

MDA levels in the lung tissue were determined as an indicator of lipid peroxidation as previously described [21]. Lung tissue collected at the specified time, was homogenized in 1.15% (w/v) KCl solution. A 100 μl aliquot of the homogenate was added to a reaction mixture containing 200 μl of 8.1% (w/v) SDS, 1.5 ml of 20% (v/v) acetic acid (pH 3.5), 1.5 ml of 0.8% (w/v) thiobarbituric acid and 700 μl distilled water. Samples were then boiled for 1 h at 95 °C and centrifuged at 3000 × *g* for 10 min. The absorbance of the supernatant was measured using spectrophotometer at 650 nm.

2.8. Determination of NADPH cytochrome c reductase activity

Portions of lung tissues were homogenized in an isotonic buffer, pH 7.5. Centrifuge sequentially at 1000 g to obtain the post-nuclear supernatants. The NADPH cytochrome c reductase activity was measured utilizing cytochrome c reductase (NADPH) Assay Kit (Catalog Number CY0100, Sigma, Saint Louis) following the manufacturer's instructions. This assay measures the reduction of cytochrome c by NADPH-cytochrome c reductase in the presence of NADPH. The absorption spectrum of cytochrome c changes with its oxidation/reduction state. Upon reduction a sharp absorption peak is observed at 550 nm. The reduction of cytochrome c is monitored by the increase of cytochrome c absorbance at 550 nm.

2.9. Western blot analysis for IκB-α, NF-κB p65, iNOS, Bax, Bcl-2, ERK-2, pERK-1/2, phospho-p38 (Thr180/Tyr182), and phospho-SAPK/JNK (Thr183/Tyr185)

Cytosolic and nuclear extracts were prepared as previously described [22] with slight modifications. Briefly, lung tissues from each mouse were suspended in extraction Buffer A containing 0.2 mM PMSF, 0,15 μM pepstatin A, 20 μM leupeptin, 1 mM sodium orthovanadate, homogenized at the highest setting for 2 min, and centrifuged at 1000 × *g* for 10 min at 4 °C. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, were re-suspended in Buffer B containing 1% Triton X-100, 150 mM NaCl, 10 mM TRIS–HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0,2 mM PMSF, 20 μm leupeptin, 0,2 mM sodium orthovanadate. After centrifugation 30 min at 15,000 × *g* at 4 °C, the supernatants containing the nuclear protein were stored at −80 °C for further analysis. The levels of IκB-α, iNOS, phospho-SAPK/JNK, ERK-2, phospho-ERK1/2, phospho-p38 MAP Kinase, Bax, and Bcl-2 were quantified in cytosolic fraction from spinal cord tissue collected after 24 h after SCI, while NF-κB p65 levels were quantified in nuclear fraction. The filters were blocked with 1× PBS, 5% (w/v) non fat dried milk (PM) for 40 min at room temperature and subsequently probed with specific Abs IκB-α (Santa Cruz Biotechnology, 1:1000), (Biogenerica s.r.l., Catania, Italy) or anti-Bax (1:500; Santa Cruz Biotechnology), (D.B.A. s.r.l., Milan, Italy), or anti-Bcl-2 (1:500; Santa Cruz Biotechnology), (D.B.A. s.r.l., Milan, Italy), or anti-iNOS (1:1000; Transduction, Milan, Italy) (Biogenerica s.r.l., Catania, Italy) or anti-ERK-2, (1:1000 Santa Cruz Biotechnology) (Biogenerica s.r.l., Catania, Italy) or anti-pERK1/2 (1:1000 Santa Cruz Biotechnology) (Biogenerica s.r.l., Catania, Italy) or anti-NF-κB p65 (1:1000; Santa Cruz Biotechnology) (Biogenerica s.r.l., Catania, Italy) or anti-phospho-p38 MAP Kinase (Thr180/Tyr182) (1:1000; Cell Signaling) (Biogenerica s.r.l., Catania, Italy) or anti-phospho-SAPK/JNK (Thr183/Tyr185) (1:1000; Cell Signaling) (Biogenerica s.r.l., Catania, Italy), in 1× PBS, 5% (w/v) non fat dried milk, 0.1% Tween-20 (PMT) at 4 °C, overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000, Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. To ascertain that blots were loaded with equal amounts of proteic lysates, they were also incubated in the presence of the antibody against β-actin protein (1:10,000, Sigma–Aldrich Corp.). The relative expression of the protein bands of IκB-α (∼37 kDa), NF-κB p65 (65 kDa), iNOS (∼130 kDa), Bax (∼23 kDa), Bcl-2 (∼29 kDa), phospho-p38 MAP Kinase (43 kDa), phospho-SAPK/JNK (54 and 46 kDa) was quantified by densitometric scanning of the X-ray films with GS-700 Imaging Densitometer (GS-700, Bio-Rad Laboratories, Milan, Italy) and a computer program (Molecular Analyst, IBM), and standardized for densitometric analysis to β-actin levels. The dual-phosphorylated form of ERK (p-ERK) antibody identified two bands of approximately 44 and 42 kDa (corresponding to p-ERK1 and p-ERK2, respectively). The anti-ERK2 antibody detects total ERK2 (*i.e.* detects both phosphorylated and nonphosphorylated forms of ERK2).

2.10. Terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) assay

TUNEL assay was conducted by using a TUNEL detection kit according to the manufacturer's instructions (Apotag, HRP kit DBA, Milan, Italy). Briefly, sections were incubated with 15 μg/ml proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% H_2O_2 for 5 min at room temperature and then washed with PBS. Sections were immersed in terminal deoxynucleotidyltransferase (TdT) buffer containing deoxynucleotidyl transferase and biotinylated dUTP in TdT buffer, incubated in a humid atmosphere at 37 °C for 90 min, and then washed with PBS. The sections were incubated at room temperature for 30 min with anti-horseradish peroxidase-conjugated antibody, and the signals were visualized with diaminobenzidine. The number of TUNEL positive cells/high-power field was counted in 5–10 fields for each coded slide.

2.11. Materials

Unless otherwise stated, all compounds were obtained from Sigma–Aldrich Company (Milan, Italy). Secondary and nonspecific IgG antibodies for immunohistochemical analysis were from Vector Laboratories Inc.

2.12. Data analysis

All values in the figures and text are expressed as mean ± standard error of the mean (SEM) of *N* observations. For the *in vivo* studies *N* represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days. The results were analyzed by one-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons. A *P*-value of less than 0.05 was considered significant.

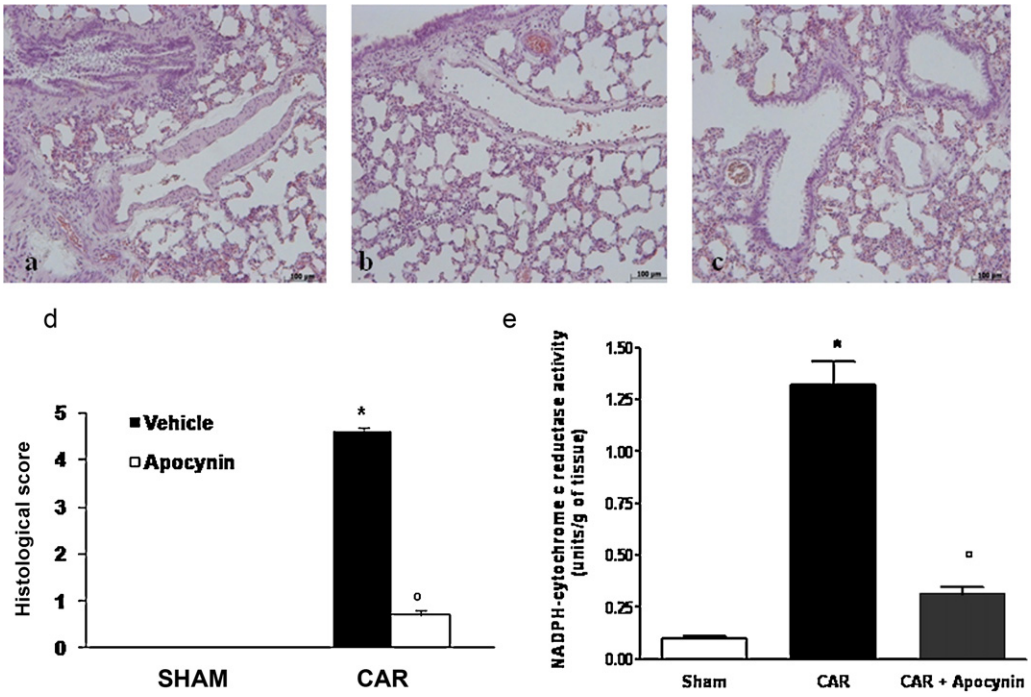

Fig. 1. Effect of apocynin histological alterations of lung after carrageenan-induced injury and on NADPH oxidase activity. Lung section from a sham animals demonstrating the normal architecture on the lung tissue (a). Lung sections taken from carrageenan-treated mice and treated with vehicle demonstrated edema, tissue injury as well as infiltration of the tissue with neutrophils (b). Carrageenan-treated animals treated with apocynin (c) demonstrated reduced lung injury and neutrophil infiltration. The histological score (d) was made by an independent observer. NADPH oxidase activity was significantly elevated at 4 h after carrageenan (CAR) administration in vehicle-treated mice (e), if compared with sham mice (e). Apocynin significantly reduced NADPH oxidase activity in the lung (e). The figure is representative of at least 3 experiments performed on different experimental days. Data are expressed as mean ± s.e.m. from $n = 10$ mice for each group. $^{*}P < 0.01$ versus sham group. $^{\circ}P < 0.01$ versus carrageenan.

3. Results

3.1. Effects of apocynin on carrageenan-induced pleurisy

When compared to lung sections taken from saline-treated animals (sham group Fig. 1a and d), histological examination of lung sections taken from mice treated with carrageenan revealed significant tissue damage and edema (Fig. 1b, see densitometry analysis Fig. 1d), as well as infiltration of neutrophils (PMNs) within the tissues (see Fig. 1b1, see densitometry analysis Fig. 1d). Apocynin (5 mg/kg 10% DMSO) reduced the degree of lung injury (Fig. 1c and d).

3.2. Effect of apocynin on the activation of NADPH-oxidase

In this study we show that activation of NADPH oxidase plays a critical role in the development of carrageenan-induced pleuritis. Indeed and as can be seen in Fig. 1e, the development of carrageenan-induced pleuritis was associated with activation of the NADPH oxidase in lung tissues as measured by cytochrome c reductase (NADPH) activity. The administration of apocynin blocked significantly NADPH oxidase activation in lung tissues (Fig. 1e).

3.3. Effects of apocynin on the expression of adhesion molecules (ICAM-1, P-selectin) and neutrophils infiltration

No positive staining for P-selectin (Fig. 2d and see densitometry analysis Fig. 2g) and for ICAM-1 (Fig. 2a and see densitometry analysis Fig. 2g) was found in lung tissue section from saline-treated mice. At 4 h after carrageenan injection, the staining intensity for ICAM-1 substantially increased along the bronchial epithelium and around the vessels (Fig. 2b see densitometry analysis Fig. 2g). Lung tissue sections obtained from carrageenan-treated mice showed positive staining for P-selectin localized in the bronchial epithelium as well as around the vessels (Fig. 2e and see densitometry analysis Fig. 2g). No positive staining for ICAM-1 or P-selectin was found in the lungs of carrageenan-treated mice that received i.p. injection of apocynin (5 mg/kg 10% DMSO) (Fig. 2c and f). This expression of adhesion molecules appeared to be associated with an influx of leukocytes into the lung tissue, thus we investigated the effect of apocynin on neutrophil infiltration by measurement of MPO activity. MPO activity was significantly elevated at 4 h after carrageenan administration in CAR group (Fig. 2h). Treatment with apocynin attenuated neutrophil infiltration into the lung tissue (Fig. 2h).

3.4. Effects of apocynin on carrageenan-induced nitrotyrosine formation, lipid peroxidation and PARP activation

Immunohistochemical analysis of lung sections obtained from mice treated with carrageenan revealed positive staining for nitrotyrosine (Fig. 3b, see densitometry analysis Fig. 3g). In contrast, no positive staining for nitrotyrosine was found in the lungs of carrageenan-treated mice, which had been treated with apocynin (5 mg/kg) (Fig. 3c, see densitometry analysis Fig. 3g). In addition, at 4 h after carrageenan-induced pleurisy, MDA levels were also measured in the lungs as an indicator of lipid peroxidation. As shown in Fig. 3h, MDA levels were significantly increased in the lungs of carrageenan-treated mice. Lipid peroxidation was significantly attenuated by the intraperitoneal injection of apocynin (Fig. 3h). At the same time point (4 h after carrageenan administration), lung tissue sections were taken in order to determine the immunohistological staining for poly ADP-ribosylated proteins (an indicator of PARP activation). A positive staining for the PAR (Fig. 3e, see densitometry analysis Fig. 3g) was found primarily localized in the inflammatory cells present in the lung tissue from carrageenan-treated mice. Apocynin treatment reduced the degree of PARP activation (Fig. 3f, see densitometry analysis Fig. 3g). Please note that there was no staining for either

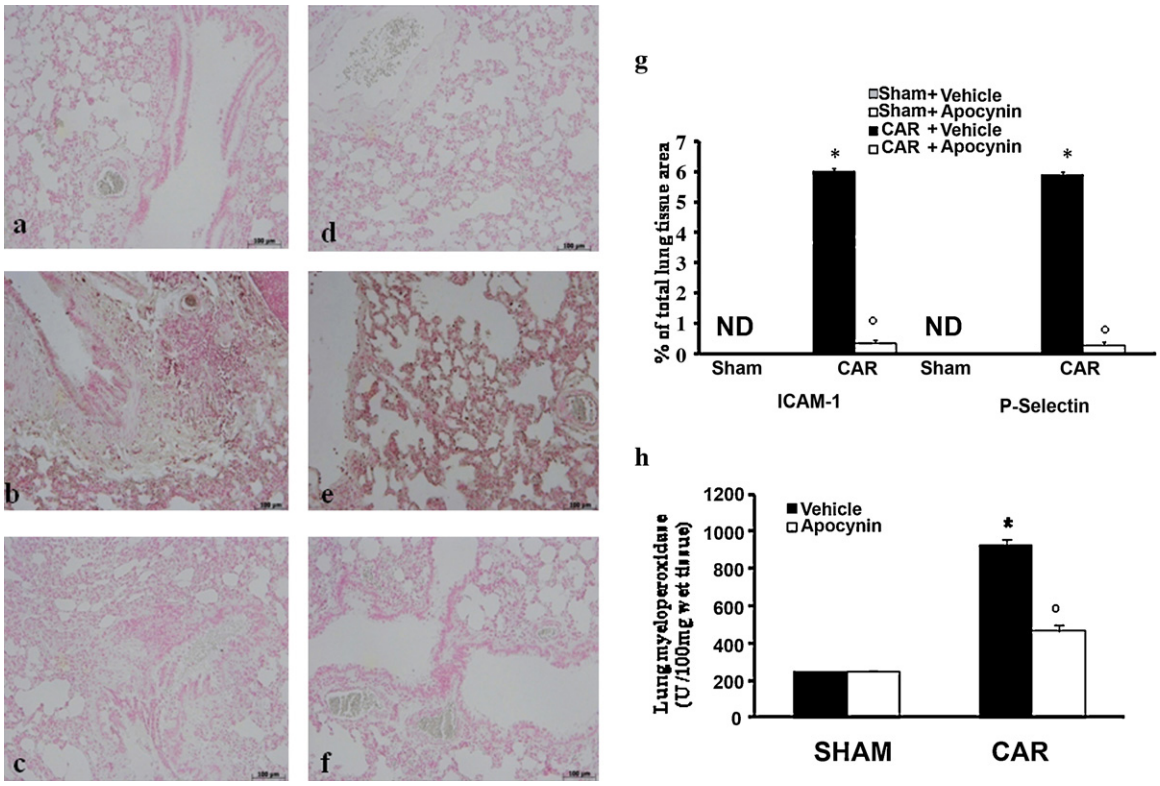

Fig. 2. Effects of apocynin on the expression of ICAM-1, P-selectin and on PMN infiltration. No positive staining for ICAM-1 (a) and for P-selectin (d) was found in lung tissue section from sham-treated mice. At 4 h after carrageenan injection, the staining intensity for ICAM-1 substantially increased along the bronchial epithelium and around the vessels (b). Lung tissue sections obtained from carrageenan-treated mice showed positive staining for P-selectin localized in the bronchial epithelium as well as and around the vessels (e). No positive staining for ICAM-1 or P-selectin was found in the lungs of carrageenan-treated mice that received i.p. injection of apocynin (c and f, respectively). Densitometry analysis (g) of immunocytochemistry photographs for ICAM-1 and for P-selectin from lung tissues was assessed. MPO activity (h) was significantly elevated at 4 h after carrageenan (CAR) administration in vehicle-treated mice, if compared with sham mice. Apocynin significantly reduced MPO activity in the lung (h). The figure is representative of at least 3 experiments performed on different experimental days. Data are expressed as mean ± s.e.m. from $n = 10$ mice for each group. $^{*}P < 0.01$ versus sham group. $^{\circ}P < 0.01$ versus carrageenan.

nitrotyrosine (Fig. 3a, see densitometry analysis Fig. 3g) or PAR (Fig. 3d, see densitometry analysis Fig. 3g) in lung tissues obtained from the sham group of mice.

3.5. Effects of apocynin on the expression of TNF-α and IL-1β induced by carrageenan

In this study we have evaluated the TNF-α and IL-1β expression in the lung tissues by immunohistochemical detection. Tissue sections obtained from vehicle-treated animals at 4 h after carrageenan injection demonstrate positive staining for TNF-α mainly localized in the infiltrated inflammatory cells, pneumocytes as well as in vascular wall (Fig. 4b, see densitometry analysis Fig. 4g). In contrast, no staining for TNF-α was found in the lungs of carrageenan-treated mice that had been treated with apocynin (Fig. 4c, see densitometry analysis Fig. 4g). Similarly, at 4 h after carrageenan injection, positive staining for IL-1β mainly localized in the infiltrated inflammatory cells was observed in lung tissue sections obtained from vehicle-treated animals (Fig. 4e, see densitometry analysis Fig. 4g). Apocynin treatment reduced the degree of IL-1β expression (Fig. 4f, see densitometry analysis Fig. 4g). Please note that there was no staining for either TNF-α (Fig. 4a, see densitometry analysis Fig. 4g) or IL-1β (Fig. 4d, see densitometry analysis Fig. 4g) in lung tissues obtained from the sham group of mice.

3.6. Effect of apocynin on IκB-α degradation and NF-κB p65 activation

We evaluated IκB-α degradation and nuclear NF-κB p65 expression by Western blot analysis to investigate the cellular mechanisms whereby treatment with apocynin attenuates the development of acute lung injury. Basal expression of IκB-α was detected in lung samples from sham-treated animals, whereas IκB-α levels were substantially reduced in lung tissues obtained from vehicle-treated animals at 4 h after carrageenan injection (Fig. 5a, see densitometry analysis Fig. 5a1). Apocynin (5 mg/kg 10% DMSO) treatment prevented carrageenan-induced IκB-α degradation (Fig. 5a, see densitometry analysis Fig. 5a1). Moreover, NF-κB p65 levels in the lung nuclear fractions were also significantly increased at 4 h after carrageenan injection compared to the sham-treated mice (Fig. 5b, see densitometry analysis Fig. 5b1). Apocynin treatment significantly reduced the levels of NF-κB p65, as shown in Fig. 5b (see densitometry analysis Fig. 5b1).

3.7. Effects of apocynin on carrageenan-induced iNOS expression

A significant increase in iNOS expression 4 h after carrageenan injection, as assayed by Western blot analysis, was also detected in lungs obtained from mice subjected to carrageenan-induced pleurisy (Fig. 5c). Apocynin (5 mg/kg 10% DMSO) treatment significantly attenuated this iNOS expression (Fig. 5c).

3.8. Apocynin modulates expression of Fas ligand after carrageenan injection

Immunohistological staining for Fas ligand in the lung was also determined at 4 h after carrageenan injection. Lung sections from sham-treated mice did not stain for Fas ligand (Fig. 6a, see densitometry analysis Fig. 6g), whereas lung sections obtained from carrageenan-treated mice exhibited positive staining for Fas

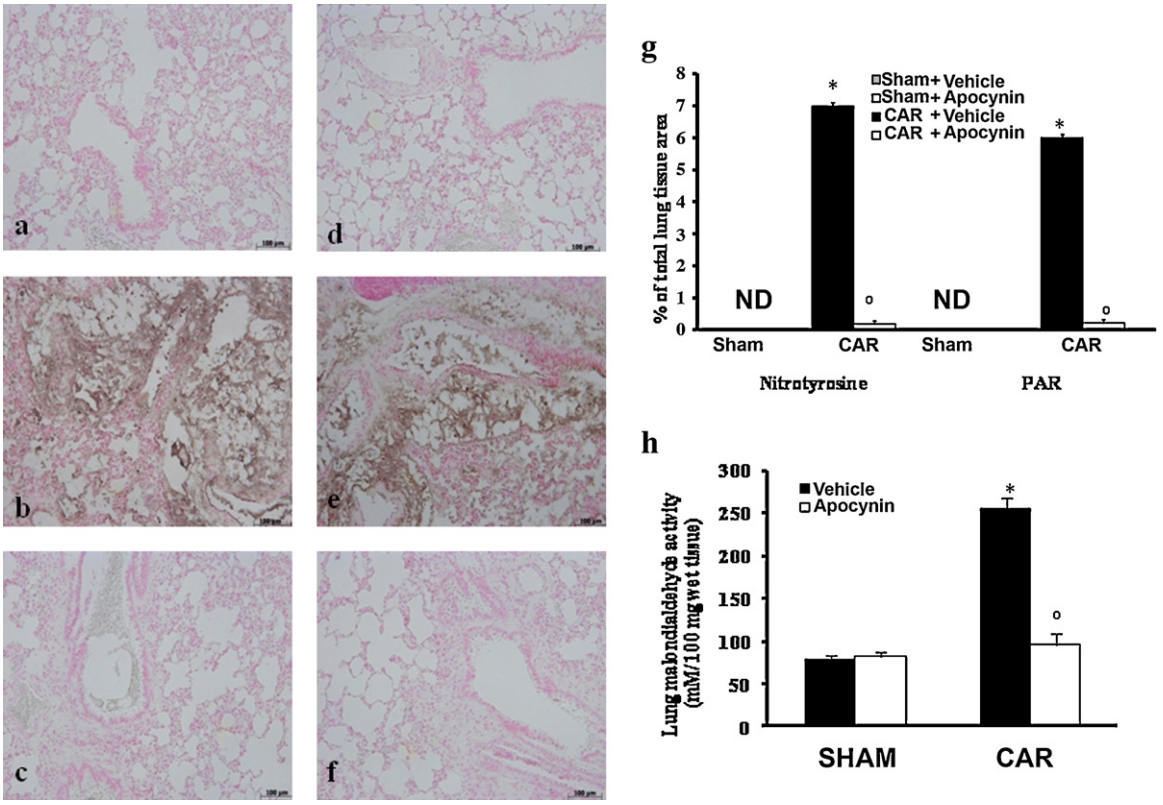

Fig. 3. Effect of apocynin carrageenan-induced nitrotyrosine formation and lipid peroxidation and PARP activation. No positive staining for nitrotyrosine (a) and for PAR (d) was found in lung tissue section from sham-treated mice. In tissue sections obtained from carrageenan-treated mice positive stainings for nitrotyrosine (b) and for PAR (e) were mainly localized on inflammatory cells. Apocynin treatment reduced the degree of positive staining for nitrotyrosine (c) and for PAR (f) in the lung tissues. Densitometry analysis (g) of immunocytochemistry photographs for nitrotyrosine and for PAR from lung tissues was assessed. In addition, a significant increase of MDA levels (h) were observed in the lung collected at 4 h from carrageenan-treated mice when compared with sham-treated mice. MDA levels (h) were significantly attenuated by the intraperitoneal injection of apocynin. The figure is representative of at least 3 experiments performed on different experimental days. Data are expressed as mean ± s.e.m. from $n = 10$ mice for each group. $^{*}P < 0.01$ versus sham group. $^{\circ}P < 0.01$ versus carrageenan.

ligand (Fig. 6b, see densitometry analysis Fig. 6g) primarily localized in the inflammatory cells present in the lung tissue. Apocynin (5 mg/kg 10% DMSO) treatment reduced the degree of positive staining for Fas ligand in the lung tissues (Fig. 6c, see densitometry analysis Fig. 6g).

3.9. Effects of apocynin on apoptosis in lung tissues after carrageenan-induced pleurisy

To investigate whether acute lung inflammation is associated with apoptotic cell death we measured TUNEL-like staining in lung tissues. At 4 h after carrageenan administration, lung tissues demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments (Fig. 6e, see Fig. 6h). In contrast, no apoptotic cells or fragments were observed in the tissues obtained from carrageenan-mice treated with apocynin (Fig. 6f, see Fig. 6h). Similarly, no apoptotic cells were observed in lungs of sham-treated mice (Fig. 6d, see Fig. 6h).

3.10. Western blot analysis and immunohistochemistry for Bax and Bcl-2

The presence of Bax in lung homogenates was investigated by Western blot 4 h after carrageenan administration. No Bax expression was detected in lung tissues obtained from sham-treated animals (Fig. 7a). Bax levels were substantially increased in the lung tissues from carrageenan-treated mice (Fig. 7a). On the contrary, apocynin (5 mg/kg 10% DMSO) treatment prevented the carrageenan-induced Bax expression (Fig. 7a). To detect Bcl-2 expression, whole extracts from lung tissues of mice were also analyzed by Western blot analysis. A basal level of Bcl-2 expression was detected in lung tissues from sham-treated mice (Fig. 7b). At 4 h after carrageenan administration, Bcl-2 expression was significantly reduced (Fig. 7b). Treatment of mice with apocynin (5 mg/kg 10% DMSO) significantly attenuated carrageenan-induced inhibition of Bcl-2 expression (Fig. 7b). Lung samples were also collected 4 h after carrageenan administration in order to determine the immunohistological staining for Bax and Bcl-2. Lung tissues taken from sham-treated mice did not stain for Bax (Fig. 8a, see densitometry analysis Fig. 8g) whereas lung sections obtained from carrageenan-treated mice exhibited positive staining for Bax (Fig. 8b, see densitometry analysis Fig. 8g). Apocynin (5 mg/kg 10% DMSO) treatment reduced the degree of positive staining for Bax in the lung of mice subjected to carrageenan-induced pleurisy (Fig. 8c, see densitometry analysis Fig. 8g). In addition, lung sections from sham-treated mice demonstrated positive staining for Bcl-2 (Fig. 8d, see densitometry analysis Fig. 8g) whereas in carrageenan-treated mice Bcl-2 staining was significantly reduced (Fig. 8e, see densitometry analysis Fig. 8g). Apocynin (5 mg/kg 10% DMSO) treatment significantly attenuated the loss of positive staining for Bcl-2 in mice subjected to carrageenan-induced pleurisy (Fig. 8f, see densitometry analysis Fig. 8g).

3.11. Apocynin modulates the activation of MAPK pathways

To investigate the cellular mechanisms by which treatment with apocynin may attenuate the development of acute lung inflammation, we also evaluated the phosphorylation of ERK1/2

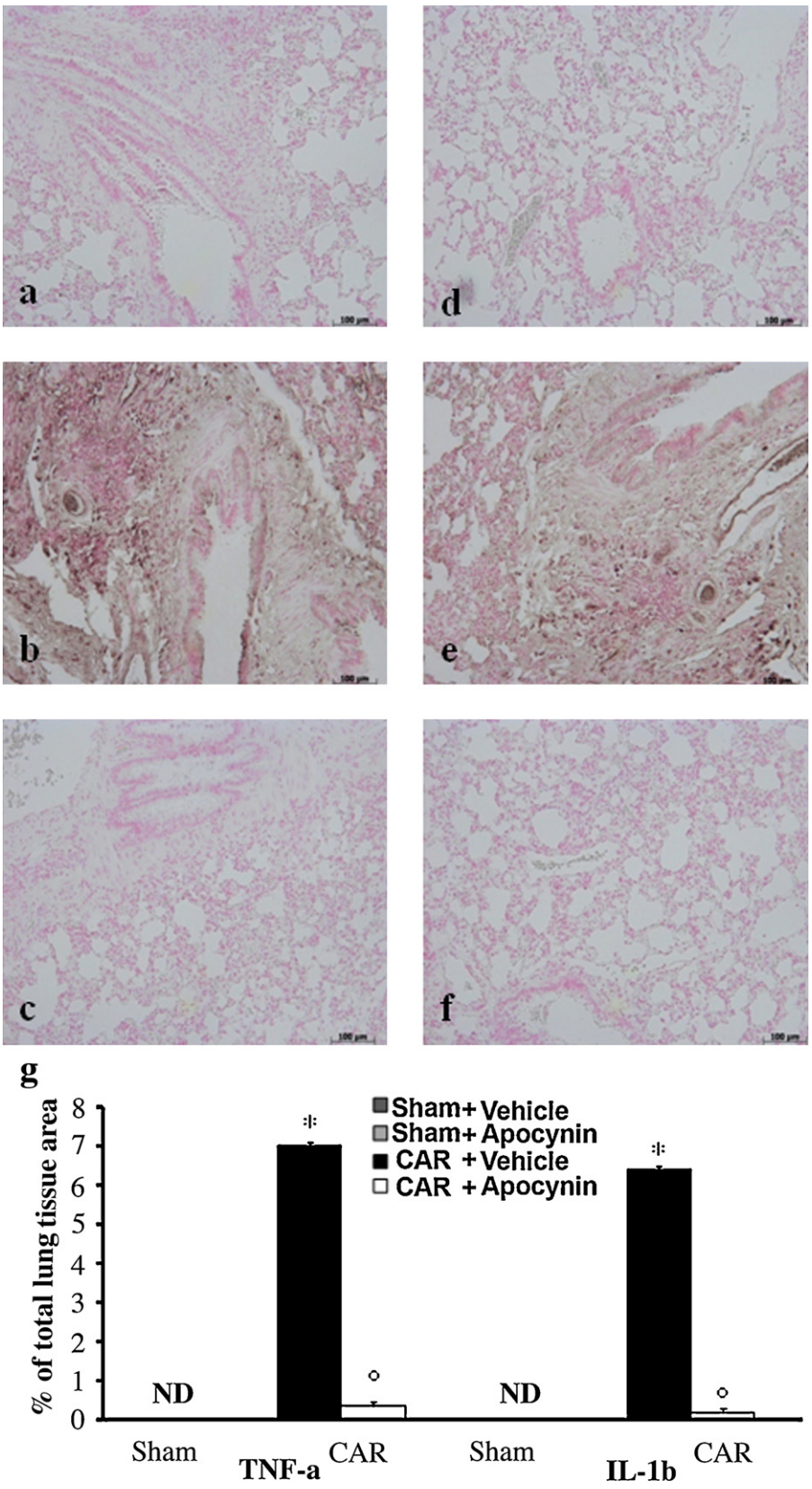

Fig. 4. Effect of apocynin on carrageenan-induced pro-inflammatory cytokine release in the lung. No positive staining for TNF-α (a) and for IL-1β (d) was found in lung tissue section from sham-treated mice. In tissue sections obtained from carrageenan-treated mice positive staining for TNF-α (b) and for IL-1β (e) were mainly localized in inflammatory cells. Apocynin treatment reduced the degree of positive staining for TNF-α (c) and for IL-1β (f) in the lung tissues. Densitometry analysis (g) of immunocytochemistry photographs for TNF-α and for IL-1β from lung tissues was assessed. The figure is representative of at least 3 experiments performed on different experimental days. Data are expressed as mean ± s.e.m. from $n = 10$ mice for each group. $^{*}P < 0.01$ versus sham group. $^{\circ}P < 0.01$ versus carrageenan.

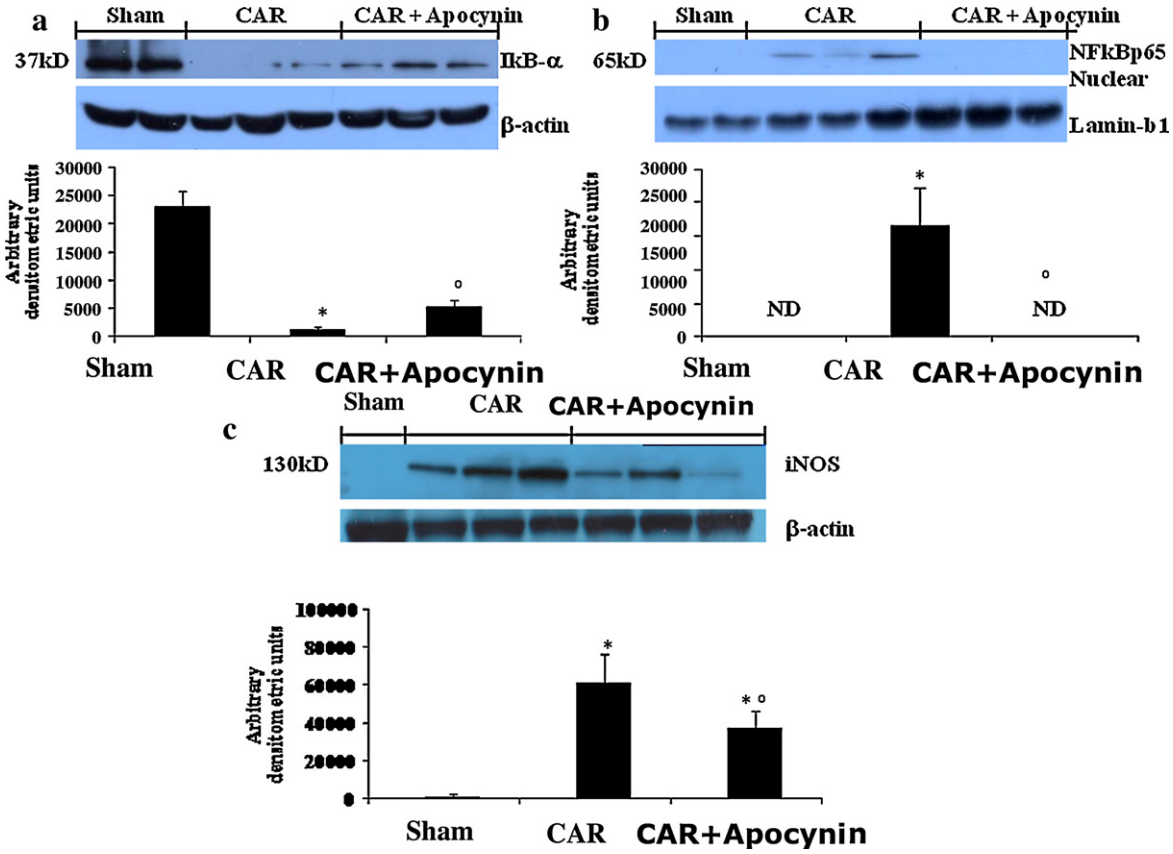

Fig. 5. Western blots of IκB-α, nuclear NF-κBp65 and iNOS expression. Basal expression of IκB-α (a) and no expression of NF- κB p65 (b) and iNOS (c) was detected in lung samples from sham-treated animals whereas reduced IκB-α levels and increased NF-κB p65 and iNOS levels were detected in lung tissues obtained from vehicle-treated animals at 4 h after carrageenan injection (a, b, c, respectively). Apocynin treatment prevented carrageenan-induced IκB-α degradation (a) and reduced NF-κB p65 (b) and iNOS expression (c). A representative blot of lysates obtained from 5 animals per group is shown and densitometry analysis of all animals is reported. The results in panels (a)–(c) are expressed as mean ± s.e.m. from $n = 5/6$ lung tissues for each group. $^{*}P < 0.01$ versus sham group. $^{\circ}P < 0.01$ versus carrageenan.

which results in expression of pro-inflammatory genes mediating the inflammatory responses characteristic of acute lung injury and phosphorylation of phosphoSAPK/JNK. The activation of MAPK pathways in particular the phosphorylation of ERK1/2 expression was investigated by Western blot in lung tissues homogenates at 4 h after carrageenan injection. A significant increase in pERK1/2 levels was observed in carrageenan-treated mice (Fig. 9a). The treatment of mice with apocynin significantly reduced the level of pERK1/2 (Fig. 9a). In addition, at 4 h after carrageenan injection, the expression of phosphoSAPK/JNK in lung tissues homogenates was investigated by Western blot. A significant increase in phosphoSAPK/JNK (Fig. 9b) levels was observed in the lung tissues from carrageenan-treated mice. On the contrary, apocynin treatment prevented the carrageenan-induced (Fig. 9b) expression of these kinases. Moreover, we evaluated the phospho-p38 expression by western Blot analysis to further investigate the cellular mechanisms by which treatment with apocynin may attenuate the development of acute lung injury. Carrageenan injection caused a significant increase in the phospho-p38 expression at 4 h after carrageenan administration (Fig. 9c). The treatment with apocynin significantly reduced the p38 expression (Fig. 9c).

4. Discussion

Oxidative stress describes an imbalance between ROS synthesis and antioxidants. Apocynin is an efficient inhibitor of NADPH oxidase, a functional enzyme that generates ROS during the inflammatory process not as a byproduct, but rather as the primary function of the enzyme system [23]. It is assumed that apocynin is activated by H_2O_2 and MPO to form an apocynin radical, which then oxidizes thiols in the NADPH-oxidase. Indeed, thiols are critical for the function of p47phox, and thiol oxidizing agents have been shown to block NADPH-oxidase activation [9,24]. This study provides the first evidence that apocynin attenuates the degree of acute inflammation in the mouse. What, then, is the mechanism by which apocynin reduces acute inflammation? One consequence of increased oxidative stress is the activation and inactivation of redox-sensitive proteins [25]. Previous studies showed that the expression of activated ERK1/2 and p38 MAPK may play a key role in production of inflammatory cytokines and free radicals, such as nitric oxide (NO) [26]. Various protein kinases including protein kinase C (PKC) and MAPKs are also involved in NADPH oxidase phosphorylation and activation. The main step is phosphorylation of its cytosolic protein p47phox [27] that contains at least six potential serine phosphorylation targets for PKC and two MAPK phosphorylation sites [28,29] and the migration of the cytosolic components to the cell membrane so that the complete oxidase can be assembled [30]. Apocynin prevents the translocation of p47phox to Nox2 in leukocytes, monocytes, and endothelial cells [9,31]. In the present study, we have observed an increase of phosphorylated MAPKs (ERK, p38, and JNK) in the lung tissues at 4 h after carrageenan which is significantly reduced by the treatment with apocynin. Therefore, apocynin could alter NADPH oxidase activity through the inhibition of MAPK induced-p47phox phosphorylation and subsequent translocation; in this regard we have also evaluated the activation of the NADPH oxidase in lung tissues as measured by cytochrome c reductase (NADPH) activity.

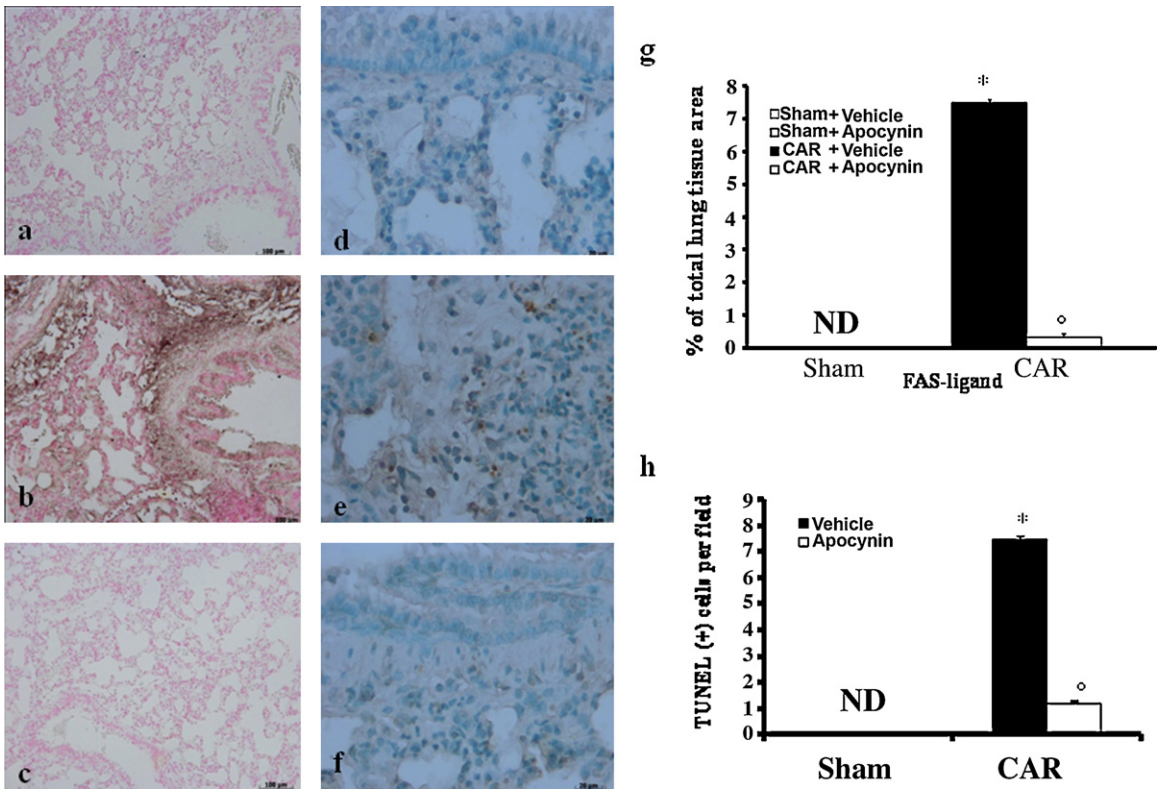

Fig. 6. Effect of apocynin on carrageenan-induced Fas ligand expression and on apoptosis. No positive staining for FAS-ligand (a) as well as no apoptotic cells (d) was found in lung tissue section from sham-treated mice. In tissue sections obtained from carrageenan-treated mice showed positive stainings for FAS-ligand (b) mainly localized in inflammatory cells as well as a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments. (e) Apocynin treatment reduced the degree of positive staining for FAS-ligand (c) as well as the presence of apoptotic cells or fragments (f) in the lung tissues. Densitometry analysis (g) of immunocytochemistry photographs for FAS-ligand from lung tissues was assessed. The number of TUNEL positive cells/high-power field was counted in 5–10 fields for each coded slide (h). The figure is representative of at least 3 experiments performed on different experimental days. Data are expressed as mean ± s.e.m. from $n = 10$ mice for each group. $^{*}P < 0.01$ versus sham group. $^{\circ}P < 0.01$ versus carrageenan. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

The administration of apocynin blocked significantly lung NADPH oxidase activation. Recent study also showed that the mechanism of action of apocynin involved the inhibition of the NADPH-oxidase-dependent superoxide production, the reduction of the intracellular GSH/GSSG ratio and prevention of the activation of the nuclear transcription factor NF-κB, which is an important mediator of inflammation [32,33]. NF-κB is normally sequestered in the cytoplasm, bound to regulatory proteins IκBs. In response to a wide range of stimuli including oxidative stress, infection, hypoxia, extracellular signals, and inflammation, IκB is phosphorylated by the enzyme IκB kinase [25]. The net result is the release of the NF-κB dimer, which is then free to translocate into the nucleus. We report here that carrageenan administration caused a significant increase in the nuclear translocation of the subunit p65 in the lung tissues at 4 h after carrageenan administration, whereas apocynin treatment significantly reduced the NF-κB translocation and inhibited the IκB-α degradation. Moreover, various experimental evidence have clearly suggested that NF-κB plays a central role in the regulation of many genes responsible for the generation of mediators or proteins in acute lung inflammation associated with carrageenan administration [17] such us TNF-α, IL-1β, iNOS and COX-2. In that regard, several studies demonstrated that apocynin significantly inhibited the expression of TNF-α and IL-1β levels which are potent triggers involved in leukocyte migration [34,35] and that suppression of NADPH oxidase activity by apocynin leads to attenuation of ROS-mediated signal transduction and iNOS expression in LPS + IFNγ-stimulated microvascular endothelial cells [36]. Our study also demonstrates that apocynin attenuates the TNF-α and IL-1β production and iNOS expression in the lung of carrageenan-treated mice. For many years, much attention has been paid to the effects of NO in respiratory diseases [37] but recently, the focus has been shifted toward RNS in general, and to peroxynitrite in particular [38,39]. In this regard, it has been shown that apocynin is capable of inhibiting peroxynitrite [11] in murine macrophages. To probe the pathological contributions of $ONOO^{-}$ to acute lung injury we have used the appearance of nitrotyrosine staining in the inflamed tissue. We have observed here that the immunoassaying of nitrotyrosine is reduced in the lung of carrageenan-treated mice and treated with apocynin. In addition, the increased levels of MDA, which is the products of lipid peroxidation, by carrageenan administration were significantly reduced in the apocynin-treated animals probably in part dependent on the observed reduction of neutrophils infiltration (MPO) into the lung. Yang et al., have also demonstrated that WT mice treated with apocynin and p47phox−/− mice displayed significantly reduced pulmonary dysfunction and injury (vascular permeability, edema, MPO, and MDA) in ischemia–reperfusion (IR) injury after lung transplantation [40]. During inflammation initiation, circulating leukocytes must first be able to adhere selectively and efficiently to vascular endothelium. This process is facilitated by induction of vascular cell adhesion molecules on the inflamed endothelium, such as vascular cell adhesion molecule VCAM-1, ICAM-1, E-selectin [41,42]. In the present study, we report that acute inflammation in the mice results in the expression of ICAM-1 and P-selectin and increase of MPO activity. We found that treatment of mice with apocynin attenuated the expression of P-selectin as well as the up-regulation of ICAM-1 and MPO activity. In that regard, several studies have also showed that apocynin could decrease the long-lasting E-selectin expression on endothelial cells subjected to anoxia/reoxygenation [43] and that

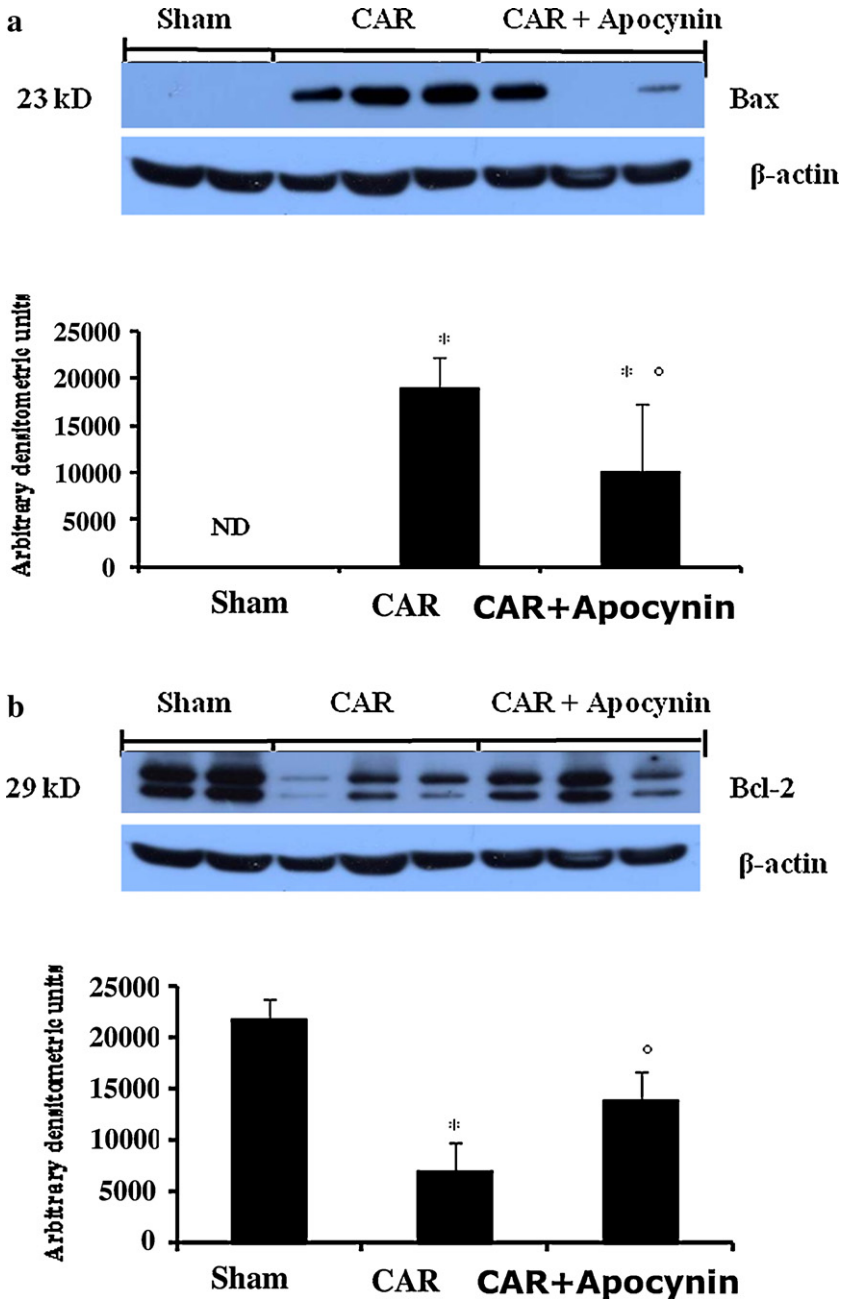

Fig. 7. Effect of apocynin on carrageenan-induced Bax and Bcl-2 expression in the lung. Representative Western blots showing no Bax expression and a basal level of Bcl-2 expression in lung tissues obtained from sham-treated animals (a and b). Bax levels were increased in the lung tissues from carrageenan-treated mice (a) while Bcl-2 expression was significantly reduced (b). Apocynin treatment prevented the carrageenan-induced Bax expression (a) and significantly attenuated carrageenan-induced inhibition of Bcl-2 expression (b). A representative blot of lysates obtained from 5 animals per group is shown and densitometry analysis of all animals is reported. The results in panels (a1) and (b1) are expressed as mean ± s.e.m. from $n = 5/6$ lung tissues for each group. $^{*}P < 0.01$ versus sham group. $^{\circ}P < 0.01$ versus carrageenan.

ICAM-1 upregulation was less significant in the gp91 KO and apocynin-treated mice after middle cerebral artery occlusion [44]. Various studies have demonstrated that PARP activation after single DNA strand breakage induced by ROS plays an important role in the process of acute lung injury [45]. In this study we confirm the increase in PAR formation in the lung tissues from carrageenan-treated mice as well as that apocynin treatment attenuates PARP activation. Generation of ROS have been implicated in induction of cell death and inflammation in the lung tissues after carrageenan injection [46,47]. Furthermore, cell death induced by ROS depends on Fas ligand expression mediated by redox sensitive activation of NF-κB [48]. Fas ligand plays a central role in apoptosis induced by a variety of chemical and physical insults [49]. Recently it has been point out that Fas ligand signaling plays a central role in acute inflammation (e.g. acute lung injury) [16,50]. We confirm here that the carrageenan-induced pleurisy leads to a substantial activation of Fas ligand in the lung tissues that was significantly reduced in lungs from mice treated with apocynin. In addition, we demonstrated here that the treatment with apocynin lowers Bax expression in treated group when compared with lung sections obtained from carrageenan-treated mice, while on the contrary, increases Bcl-2 expression. Moreover, we have also demonstrated that treatment with apocynin attenuates the degree of apoptosis, measured by TUNEL detection kit, in the lung at 4 h after carrageenan administration. Recent studies have also shown that inhibition of NADPH oxidase by apocynin, reduces cardiomyocyte apoptosis in response to angio-

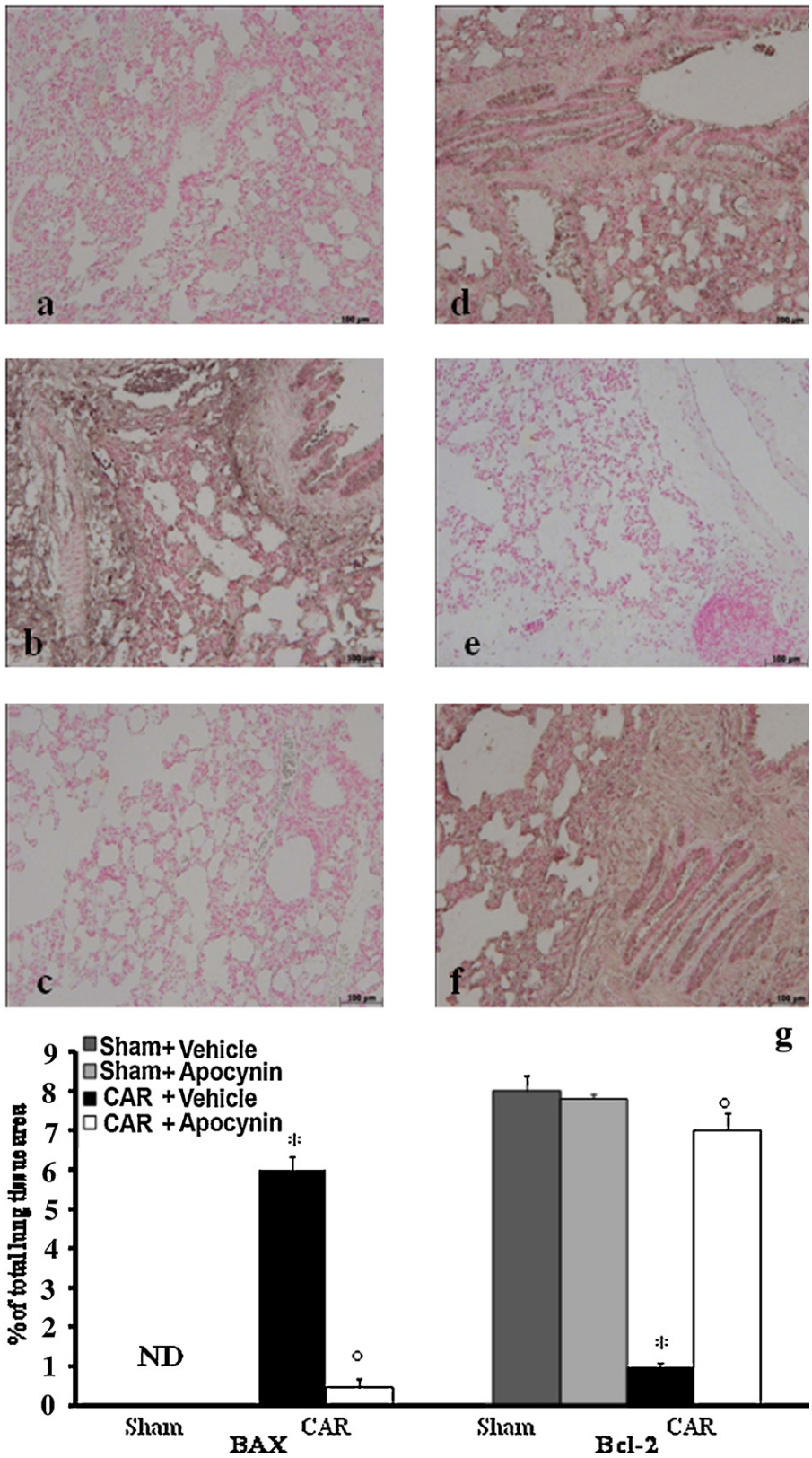

Fig. 8. Immunohistochemical localization of Bax and Bcl-2 in the lung. No positive staining for Bax (a) was found in lung tissue section from sham-treated mice. Tissue sections obtained from carrageenan-treated mice showed positive stainings for BAX (b) mainly localized in inflammatory cells. On the contrary, positive staining for Bcl-2 was observed in the lung tissues from sham-treated mice (d) while the staining was significantly reduced in carrageenan-treated mice (e). Apocynin treatment reduced the degree of positive staining for Bax in the lung tissues (c) as well as attenuated the loss of positive staining for Bcl-2 in the lung tissues (f). Densitometry analysis (g) of immunocytochemistry photographs for Bax and for Bcl-2 from lung tissues was assessed. The figure is representative of at least 3 experiments performed on different experimental days. Data are expressed as mean ± s.e.m. from $n = 10$ mice for each group. $^{*}P < 0.01$ versus sham group. $^{\circ}P < 0.01$ versus carrageenan.

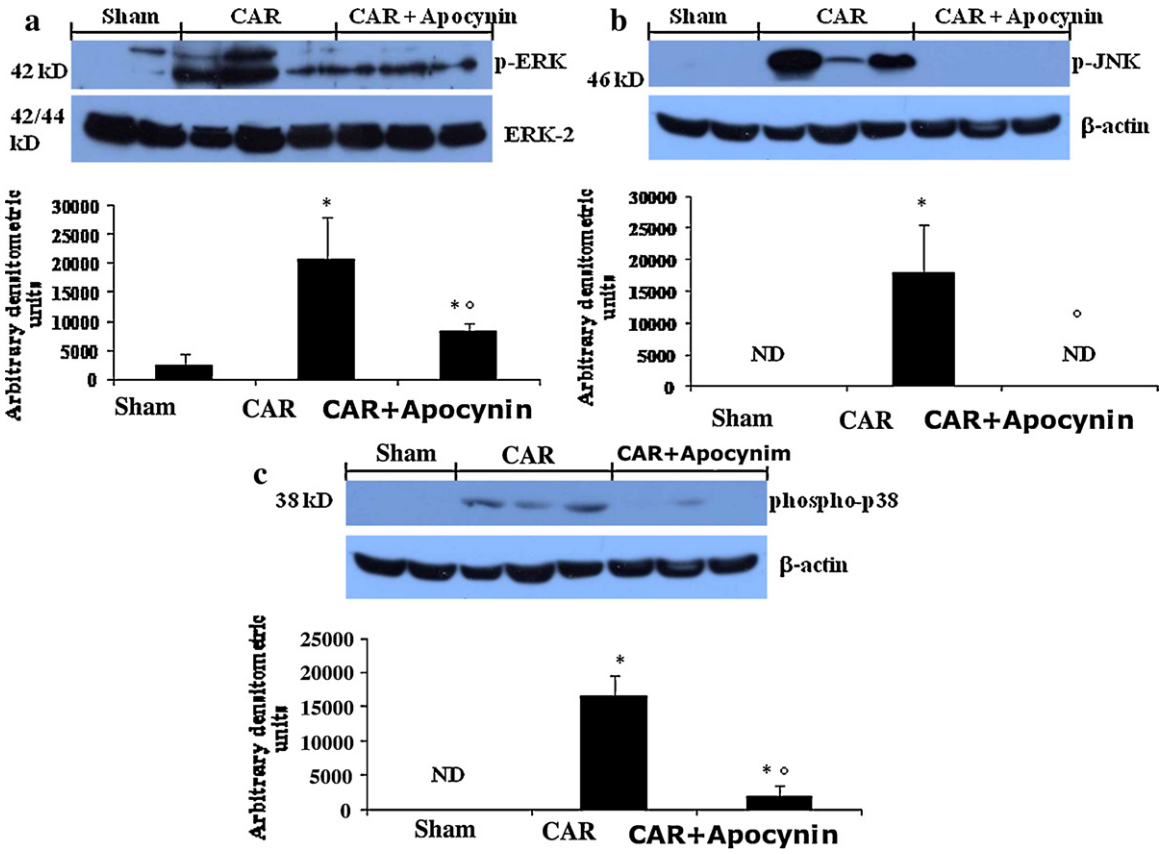

Fig. 9. Effect of apocynin treatment on MAPK pathways activation. A significant increase in pERK1/2 (a), phosphoSAPK/JNK (b) and phospho-p38 levels (c) were observed in carrageenan-treated mice in comparison to sham-treated animals (a–c) and apocynin treated mice (a–c). A representative blot of lysates obtained from 5 animals per group is shown and densitometry analysis of all animals is reported. The results in panels (a)–(c) are expressed as mean ± s.e.m. from $n = 5/6$ lung tissues for each group. $^{*}P < 0.01$ versus sham group. $^{\circ}P < 0.01$ versus carrageenan.

tensin II [51]. This means that the apocynin by inhibiting NF-κB prevents the loss of the anti-apoptotic way and reduced the pro-apoptotic pathway activation with a mechanism still to discover.

Thus, in this study, we propose the following cycle: inflammation → MAPK expression → NADPH oxidase activation → reactive oxygen species production → more MAPK and NF-κB expression → PARP-mediated endothelial injury → PMN infiltration → cytokines release → lung damage. Inhibition of NADPH oxidase by apocynin would intercept this cycle at the early stage. The confirmation of this proposed feedback cycle, however, requires further investigation. Taken together, the results of the present study enhance our understanding of the role of the NADPH oxidase dependent ROS generation in the pathophysiology of acute inflammation implying that inhibitors of the activity NADPH oxidase such as apocynin may be useful in the therapy of inflammation.

Acknowledgments

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