Free Radical Research, July 2011; 45(7): 820–834

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Effect of NADPH-oxidase inhibitors in the experimental model of zymosan-induced shock in mice

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(Received date: 25 February 2011; Accepted date: 13 April 2011)

Abstract

The aim of this study was to investigate the effects of NADPH-oxidase inhibitors, in a mouse model of zymosan. Zymosan-induced shock was induced in mice by administration of zymosan (500 mg/kg, i.p.). The pharmacological treatment was the administration of apocynin (5 mg/kg 10% DMSO i.p.) and diphenylene iodonium chloride (DPI) (1 mg/kg i.v.) 1 h and 6 h after zymosan administration. MOF and systemic inflammation in mice was assessed 18 h after administration of zymosan. NADPH-oxidase inhibitors caused a significant reduction of the (1) peritoneal exudate formation, (2) neutrophil infiltration, (3) multiple organ dysfunction syndrome, (4) nitrotyrosine, (5) poly (ADP-ribose) (PAR), (6) cytokine formation, (7) adhesion molecule expression, (8) nuclear factor (NF-κB) expression and (9) apoptosis induced by zymosan. Moreover, NADPH-oxidase inhibitors treatment significantly reduced the systemic toxicity, the loss in body weight and the mortality caused by zymosan. This study has shown that NADPH-oxidase inhibitors attenuate the degree of zymosan-induced non-septic shock in mice.

Keywords: *Inflammation, oxidative stress, apoptosis, cytokines, MOF*

Introduction

Multiple organ dysfunction syndrome (MODS, also known as multiple organ failure (MOF) or multiple organ system failure) is defined as the progressive deterioration of function, which occurs in several organs or systems in patients with septic shock, multiple trauma, severe burns or pancreatitis [1].

Elucidation of the mechanisms involved in the development of MODS will ultimately necessitate the collection of tissue samples and the performance of invasive procedures. These requirements greatly reduce the possibilities for research in human subjects. Therefore, an animal model for MODS is a necessary and valuable tool. In the mid-1980s, the zymosan-induced generalized inflammation (ZIGI) model was introduced. The ZIGI model has been recognized as the one that best resembles human MODS and it has been used widely to study systemic inflammation in relation to organ failure [2].

Zymosan is a substance derived from the cell wall of the yeast *Saccharomyces cerevisiae*. When injected into animals, it induces inflammation by inducing a wide range of inflammatory mediators [3]. In addition, we have reported that zymosan causes—within 18 h—both signs of peritonitis and organ injury. The onset of the inflammatory response caused by zymosan in the peritoneal cavity was associated with systemic hypotension, high peritoneal and plasma levels of nitric oxide (NO), maximal cellular infiltration, exudate formation, cyclooxygenase activity and pro-inflammatory cytokine production [4]. Therefore, we have also discovered that injection of zymosan results in excessive production of reactive oxygen species (ROS) by activated polymorphonuclear leukocytes

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2011 Informa UK, Ltd.
DOI: 10.3109/10715762.2011.581667

PMNs [5] as well as lipid peroxidation in the plasma, intestine and lung.

One of the major sources of superoxide anion ($O_2^{\cdot -}$) is the NADPH-dependent oxidase present in the plasma membrane of phagocytic cells such as PMN leukocytes and macrophages [6]. ROS produced by stimulated PMNs play an important role in host defense against invading micro-organisms. Upon triggering, PMNs start to consume a large amount of oxygen which is converted into ROS, a process which is known as the respiratory or oxidative burst [7]. Although 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 [8]. Therefore, compounds that can interfere with ROS production may be useful tools to prevent tissue destruction.

The structure of NADPH-oxidase is quite complex, consisting of two membrane-bounded elements (gp91-phox or Nox 2 and p22phox), three cytosolic components (p67phox, p47phox and p40phox) and a low-molecular-weight G protein (either rac 2 or rac 1).

Apocynin is an acetophenone to which a range of biological activities is attributed [9]. It is a pro-drug that is converted by peroxidase-mediated oxidation to a dimer, which has been shown to be more efficient than apocynin itself [10]. It has been used as an efficient inhibitor of the complex NADPH-oxidase in many experimental models [11] such as colitis, rheumatoid arthritis and ischemia reperfusion lung injury [12]. Several studies have demonstrated that the generation of ROS by serum-treated zymosan triggered human neutrophils from healthy blood donors was inhibited by apocynin in a dose dependent manner (50% inhibition at ~6 μM) [13]. The mode of action may involve metabolization and inhibition of NADPH assembly by interfering with the intracellular translocation of two cytosolic components p47 phox and p67 phox [14]. The *in vitro* anti-inflammatory effects of apocynin include the neutrophil-mediated oxidative damage, a reduction of polymorphonuclear granulocyte chemotaxis and the inhibition of peroxynitrite ($ONOO^-$) [9]. Moreover, recent data in the literature report the protection achieved by apocynin in various models of zymosan-mediated injury [15,16].

Diphenyleneiodonium chloride (DPI) selectively inhibits flavonoid-containing enzymes by irreversibly binding to the flavin moiety of those enzymes, such as NADPH oxidase, mitochondrial NADH dehydrogenase, NADH oxidase, quinone oxidoreductase, cytochrome P450 reductase and nitric oxide synthase (NOS). DPI has also been shown to suppress the expression of several genes induced by inflammatory cytokines in different cells [17]. We have already demonstrated, in a precedent study, that DPI inhibiting NADPH oxidase had beneficial effects on multiple organ dysfunction syndrome in haemorrhagic shock [18].

Thus, based on these observations, in this study we wished to investigate the potential therapeutic role of NADPH oxidase inhibition, during MOF, an inflammatory process caused by zymosan.

Methods

Animals

Male CD1 mice (20–22 g; Charles River; Milan; Italy) were housed in a controlled environment and provided with standard rodent chow and water. The study was approved by the University of Messina Review Board for the care of animals. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M.116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

Zymosan-induced shock

Mice were randomly allocated into the following groups:

1) *Zymosan + vehicle group*. Mice were treated intraperitoneally (i.p.) with 200 ∝l of zymosan (500 mg/kg, suspended in saline solution, i.p.) and with 200 μl of the vehicle for apocynin 10% dimethylsulphoxide (DMSO) (v/v) i.p, 1 and 6 h after zymosan administration ($n = 20$);
2) *Zymosan + apocynin group*. Identical to the *Zymosan + vehicle group* but were administered 200 μl apocynin (5 mg/kg 10% DMSO i.p) at 1 and 6 h after zymosan ($n = 20$) instead of vehicle;
3) *Zymosan + DPI group*. Identical to the *Zymosan + vehicle group* but were administered 200 μl DPI (1 mg/kg i.v) at 1 and 6 h after zymosan ($n = 20$) instead of vehicle;
4) *Sham + vehicle group*. Identical to the *Zymosan + vehicle group*, except for the administration of 200 μl of saline instead of zymosan ($n = 10$);
5) *Sham + apocynin group*. Identical to *Sham + vehicle group*, except for the administration of 200 μl of apocynin (5 mg/kg in 10% DMSO i.p) 1 and 6 h after saline administration ($n = 10$); and
6) *Sham* + DPI *group*. Identical to *Sham + vehicle group*, except for the administration of 200 μl of DPI (1 mg/kg i.v) 1 and 6 h after saline administration ($n = 10$).

Intraperitoneal (ip) zymosan causes a triphasic inflammatory process leading to MODS. Phase I is an acute systemic inflammatory response. Phase II is the recovery phase. Phase III is characterized by recurrent illness. We chose to evaluate the first phase in which we observe the maximum release of mediators of acute inflammation [19]. Thus, 18 h after administration of zymosan, animals were assessed for shock as described

below. In another set of experiments, animals ($n = 30$ for each group) were randomly divided as described above and monitored for loss of body weight and mortality for 7 days after zymosan or saline administration. The treatment with inhibitors is not repeated during the 7 days of observation.

In this study we chose the dose of 1 mg/kg for DPI treatment based on a chronic study investigating the effect of DPI on alcohol-induced liver injury demonstrating that a dose of 1 mg/kg/day DPI is sufficient to inhibit NADPH oxidase activity in the rat [20] and we chose the dose of 5 mg/kg 10% DMSO for apocynin treatment in agreement with our previous works [21].

Clinical scoring of systemic toxicity

Clinical severity of systemic toxicity in the mice was scored during the experimental period, (7 days) after zymosan or saline injection, on a subjective scale ranging from 0–3; 0 = absence, 1 = mild, 2 = moderate, 3 = serious. The scale was used for each of the toxic signs (conjunctivitis, ruffled fur, diarrhoea and lethargy) observed in the animals. The final score was produced upon totaling each evaluation (maximum value 12). All clinical score measurements were performed by an independent investigator, who had no knowledge of the treatment received by each respective animal.

Assessment of acute peritonitis

Eighteen hours after zymosan or saline injection, a set of animals ($n = 10$ for each group) were anaesthetized with intramuscular injection of ketamine/xylazine in order to evaluate the development of acute inflammation in the peritoneum by exudate collection. The injection of an irritant into the peritoneal cavity induces endothelial permeability [22]. Alterations in endothelial permeability is crucial for macrophage hyper-activation and release of inflammatory mediators that lead to tissue damage [22].

Through an incision in the *linea alba*, 5 ml of phosphate buffered saline (PBS, composition in mM: NaCl 137, KCl 2.7, NaH_2PO_4 1.4, Na_2HPO_4 4.3, pH 7.4) was injected into the abdominal cavity. Washing buffer was removed with a plastic pipette and was transferred into a 10 ml centrifuge tube. The amount of exudate was calculated by subtracting the volume injected (1 ml) from the total volume recovered. Peritoneal exudate was centrifuged at 7000 x g for 10 min at room temperature.

Peritoneal cell exudate collection and differential staining

At 18 h after treatment a set of animals ($n = 10$ for each group) were also anaesthetized with intramuscular injection of ketamine/xylazine in order to collect peritoneal cells. The mice were injected with 5 mL of ice-cold RPMI-1640 medium (Gibco Inc., Grand Island, NY) with 10 U/ml of heparin, into the abdominal cavity. The peritoneal cavities were massaged for 1 min and the lavage fluid was collected. Peritoneal exudate cell (PEC) counts were carried out in a haemocytometer by mixing 100 µL of peritoneal cell exudate and 100 µL of eosin. The PEC was spun in a cytocentrifuge at 600–700 rpm for 5 min onto a slide for the differential count. The slides were carefully removed and allowed to air-dry briefly. PEC cytospins were stained with Wright-Giemsa stain. PEC cytospins were also stained with neutrophil/mast cell-specific chloroacetate esterase staining and macrophage/monocyte-specific alpha naphthyl butyrate esterase stains for the differential count.

Measurement of nitrite/nitrate concentrations

Nitrite/nitrate (NO_2/NO_3) production, an indicator of NO synthesis, was measured in plasma and in the exudate samples collected 18 h after zymosan or saline administration, as previously described [4,23]. Nitrate concentrations were calculated by comparison with OD550 of standard solutions of sodium nitrate prepared in saline solution.

Immunohistochemical localization of nitrotyrosine, PAR, adhesion molecules (ICAM-1, P-Selectin), Bax, Bcl-2, tumor necrosis factor (TNF-α), interleukin (IL-1β) and Fas- ligand

Tyrosine nitration and poly-ADP-ribose-polymerase (PARP) activation were detected, as previously described [24], in pancreas and liver sections using immunohistochemistry.

At 18 h after zymosan or saline injection, tissues were fixed in 10% (w/v) PBS-buffered formalin 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% (v/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 avidin and biotin (Vector Laboratories, Burlingame, CA). The sections were then incubated overnight with 1:1000 dilution of primary anti-nitrotyrosine antibody (Millipore, DBA, Milan, Italy, 1:500 in PBS, v/v), anti-poly (ADP)-ribose (PAR) antibody (Santa Cruz Biotechnology, DBA, Milan, Italy, 1:500 in PBS, v/v), purified hamster anti-mouse ICAM-1 (CD54) (1:500 in PBS, w/v) (DBA, Milan, Italy), purified goat polyclonal antibody directed towards P-selectin which reacts with mice, anti-Bax rabbit polyclonal antibody (1:500 in PBS, v/v), anti-Bcl-2 polyclonal antibody rat

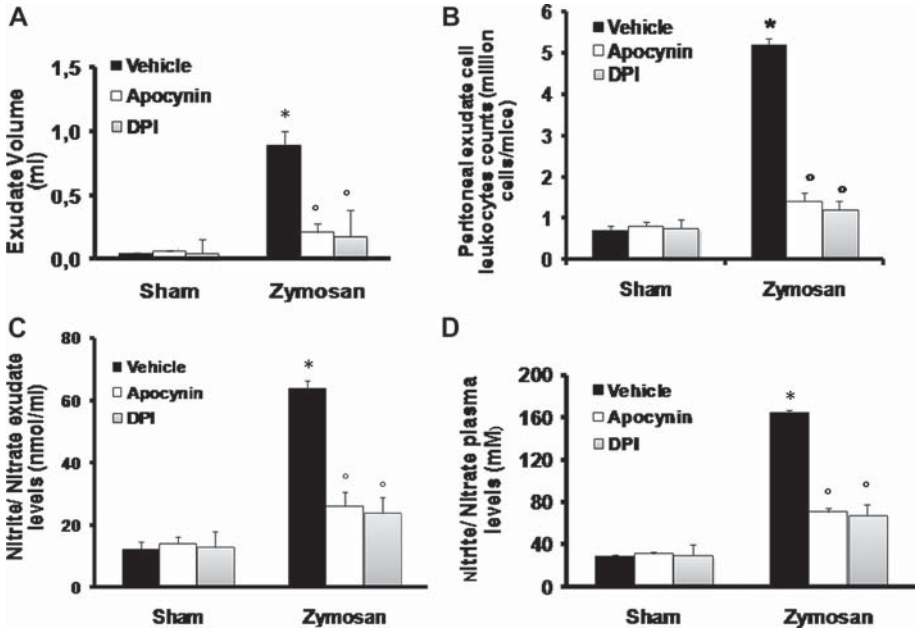

Figure 1. Effect of NADPH oxidase inhibitors on zymosan-induced peritoneal inflammation and on peritoneal exudate and plasma nitrate/nitrite levels. The increase in volume exudate (A) and peritoneal exudate cell leukocyte counts (B) in peritoneal cavity at 18 h after zymosan was reduced by apocynin treatment. Nitrate/nitrite levels were also significantly increased both in peritoneal exudate (C) and in plasma of zymosan-injected mice (D) in comparison to vehicle group (sham group). Apocynin and DPI reduced the zymosan-induced increase of nitrate/nitrite levels in peritoneal exudate and in plasma. Data are means ± SEM of 10 mice for each group. $^{*}p < 0.01$ vs sham, $^{\circ}p < 0.01$ vs zymosan + vehicle.

(1:500 in PBS, v/v), anti-TNF-α antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v), anti-IL-1β antibody (Santa Cruz Biotechnology, 1:500 in PBS, v/v) or anti-Fas Ligand antibody (Abcam, DBA, Milan, Italy, 1:500 in PBS, v/v). Controls included buffer alone or non-specific purified rabbit IgG. Specific labelling was detected with a biotin-conjugated specific secondary anti-IgG and avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA). To verify the binding specificity for nitrotyrosine, PARP, ICAM-1, P-Selectin, Bax, Bcl-2, TNF-α, IL-1β and FasL, some sections were also incubated with primary antibody only (no secondary antibody) or with secondary antibody only (no primary antibody). In these situations, no positive staining was found in the sections, indicating that the immunoreactions were positive in all the experiments carried out. In order to confirm that the immunoreactions for the nitrotyrosine were 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.

Cytokine production

The levels of TNF-α and IL-1β were evaluated in the plasma at 18 h after zymosan or saline administration. The assay was conducted using a colourimetric commercial kit (Calbiochem-Novabiochem, La Jolla, CA). The ELISA has a lower detection limit of 10 pg/ml.

Western blot analysis for IκB-α, NF-κB p65

Liver and pancreas tissues were homogenized in cold lysis buffer A (HEPES 10 mM pH = 7.9; KCl 10 mM; EDTA 0.1 mM; EGTA 0.1 mM; DTT 1 mM; PMSF 0.5 mM; Trypsin inhibitor 15 μg/ml; PepstatinA 3 μg/ml; Leupeptin 2 μg/ml; Benzamidina 40 μM). Homogenates were centrifuged at 12 000 g for 3 min at 4°C and the supernatant (cytosol + membrane extract) was collected to evaluate contents of IkB-α, and β-actin. The pellet was resuspended in buffer C (HEPES 20 mM; $MgCl_2$ 1.5 mM; NaCl 0.4 mM; EDTA 1 mM; EGTA 1 mM; DTT 1 mM; PMSF 0.5 μg/ml; Leupeptin 2 μg/ml; Benzamidina 40 μM; NONIDET P40 1%; Glicerolo 20%) and centrifuged at 12 000 g for 12 min at 4°C and the supernatant (nuclear extract) was collected to evaluate the content of NF-kB p65. Protein concentration in the homogenate was determined by Bio-Rad Protein Assay (BioRad, Richmond CA) and 50 μg of cytosol and nuclear extract from each sample was analysed. Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred on a PVDF membrane (Hybond-P Nitrocellulose, Amsherman Biosciences, Biogenerica S.R.L, Catania (CT), Sicily, Italy). The membrane was blocked with 0.1% TBS-Tween containing 5% non-fat milk for 1 h at room temperature. After the blocking, the membranes were incubated with the relative primary antibody overnight at 4°C; anti-IkB-α diluted 1:1000 (Santa Cruz Biotechnology), anti-NFkB p65 diluted 1:250 (Santa Cruz

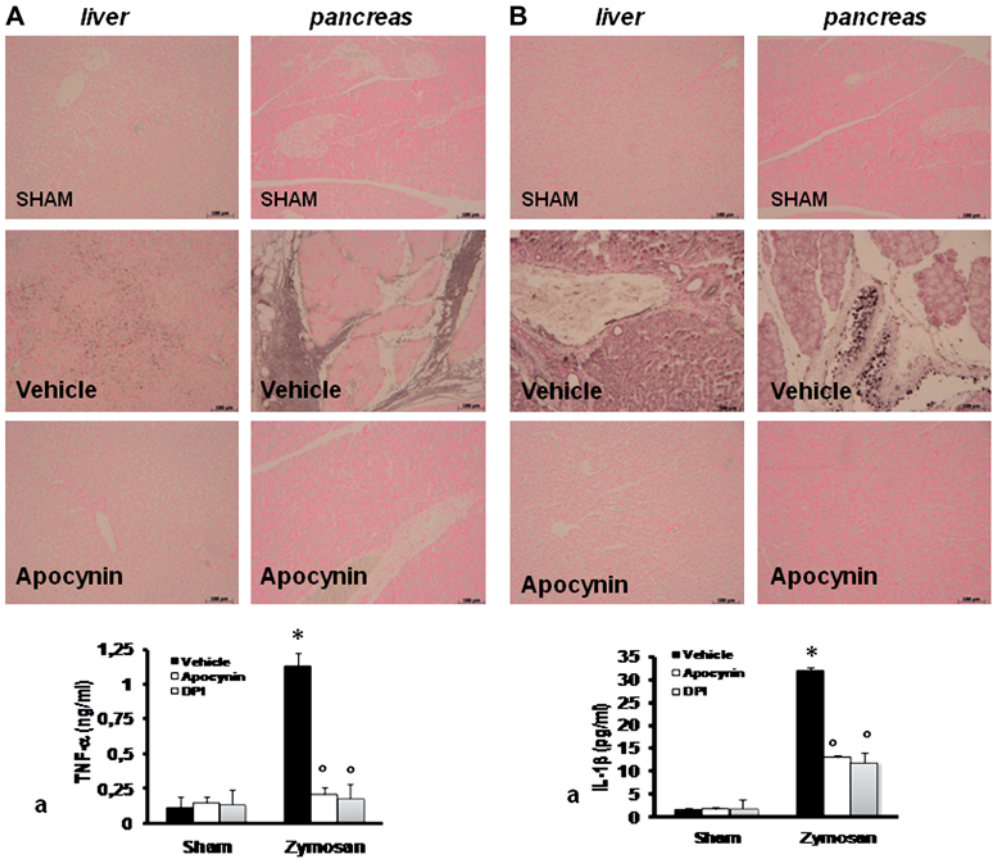

Figure 2. Effect of NADPH oxidase inhibitors on TNF-α and IL-1β production. No positive staining for TNF-α and IL-1β was found in the pancreas as well as in the liver (A and B) from sham-treated mice. On the contrary, 18 h following zymosan injection, positive TNF-α and IL-1β staining was found in the pancreas and liver (A and B). There was no detectable immunostaining in the pancreas and liver (A and B) of zymosan-injected mice when mice were treated with apocynin. In addition, the plasma increase of TNF-α (Aa) and IL-1β (Ba) at 18 h after zymosan was reduced by apocynin and DPI treatment. Figures are representative of at least three experiments performed on different experimental days. Data are means ± SEM of 10 mice for each group. $^{*}p < 0.01$ vs sham, $^{\circ}p < 0.01$ vs zymosan + vehicle.

Biotechnology) and anti-β-actin 1:5000 (Santa Cruz Biotechnology). After the incubation, the membranes were washed three times for 10 min with 0.1% TBS Tween and were then incubated for 1 h with peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Jackson Immuno Research Laboratories, DBA, Milan, Italy) diluted 1:2000, the membranes were then washed three times for 10 min and protein bands were detected with SuperSignal West Pico Chemioluminescent (PIERCE, DBA, Milan, Italy). Densitometric analysis was performed with a quantitative imaging system (ImageJ, DBA, Milan, Italy).

Quantification of organ function and injury

Blood samples were taken at 18 h after zymosan or saline injection and centrifuged (1610 × *g* for 3 min at room temperature) to separate plasma. Levels of amylase, lipase, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubine and alkaline phosphatase were measured by a veterinary clinical laboratory using standard laboratory techniques. For the evaluation of acid base balance and blood gas analysis (indicator of lung injury), arterial blood levels of pH, PaO_2 and $PaCO_2$ and bicarbonate ion (HCO_3^-) were determined by a pH/blood gases analyser as previously described [25].

Light microscopy

Pancreas and liver samples were taken 18 h after zymosan or saline injection. The tissue slices were fixed in Dietric solution [14.25% (v/v) ethanol, 1.85% (w/v) formaldehyde, 1% (v/v) acetic acid] for 1 week at room temperature, dehydrated by graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Sections (thickness 7 μm) were deparaffinized with xylene, stained with haematoxylin and eosin and observed in a Dialux 22 Leitz microscope.

Materials

Unless stated otherwise, all reagents and compounds were obtained from Sigma Chemical Company (Milan, Italy). Apocynin was obtained from Calbiochem-Novabiochem (Milan, Italy). DPI was obtained from Santa Cruz Biotechnology (Milan, Italy).

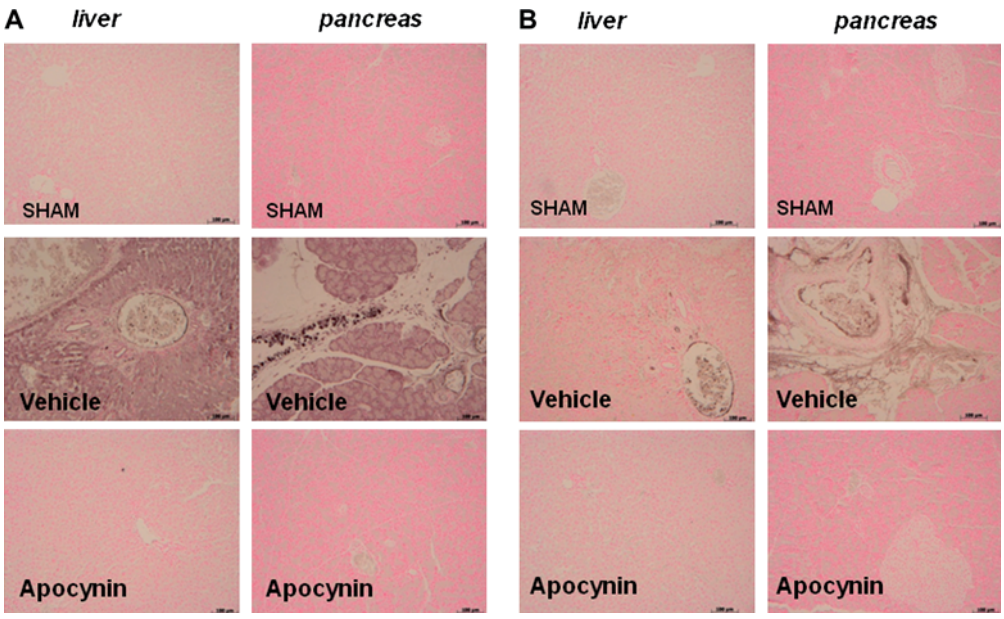

Figure 3. Effect of NADPH oxidase inhibitors on immunohistochemical localization of ICAM-1 and P-Selectin in pancreas and liver. Eighteen hours following zymosan injection, a positive ICAM-1 (A) and P-Selectin (B) staining was found in pancreas and liver. There was no detectable immunostaining for ICAM-1 and P-Selectin in pancreas and liver (A and B) of zymosan-injected mice when mice were treated with apocynin. No positive staining for ICAM-1 and P-selectin was found in the pancreas as well as in the liver (A and B) from sham-treated mice. Figures are representative of at least three experiments performed on different experimental days.

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 (histological or immunohistochemistry colouration) performed on different experimental days on the tissue sections collected from all animals in each group. The results were analysed by one-way ANOVA followed by a Bonferroni's *post-hoc* test for multiple comparisons. A p-value of less than 0.05 was considered significant. Statistical analysis for survival data

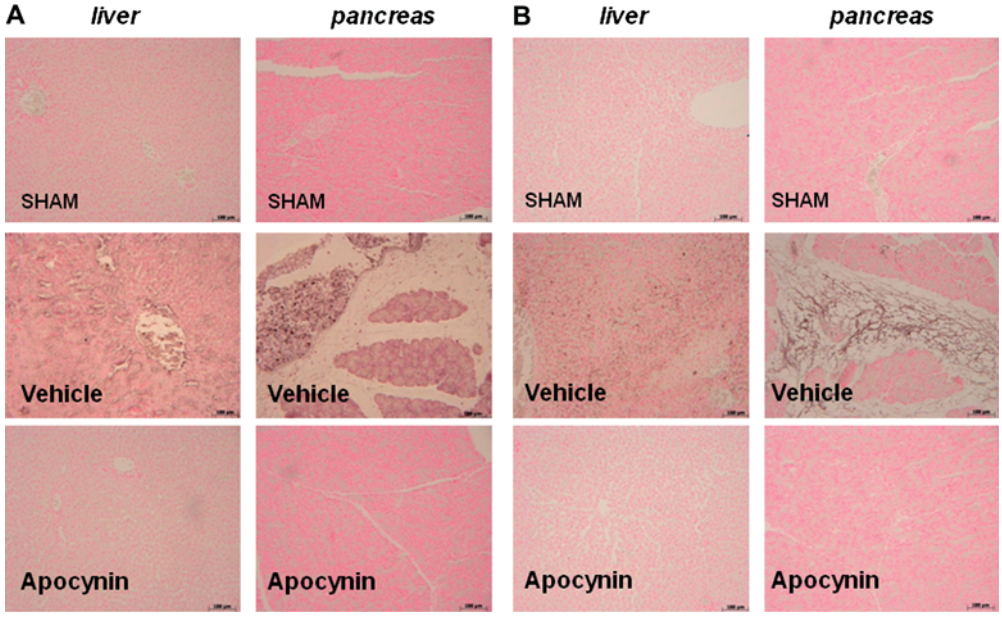

Figure 4. Effect of NADPH oxidase inhibitors on immunohistochemical localization of nitrotyrosine and PAR in pancreas and liver. Eighteen hours following zymosan injection, a positive nitrotyrosine (A) and PAR (B) staining was found in the pancreas and liver. There was no detectable immunostaining for nitrotyrosine and PAR (A and B, respectively) in the pancreas and liver of zymosan-injected mice when mice were treated with apocynin. No positive staining for nitrotyrosine and PAR (A and B, respectively) was found in the pancreas as well as in the liver from sham-treated mice. Figure is representative of at least three experiments performed on different experimental days.

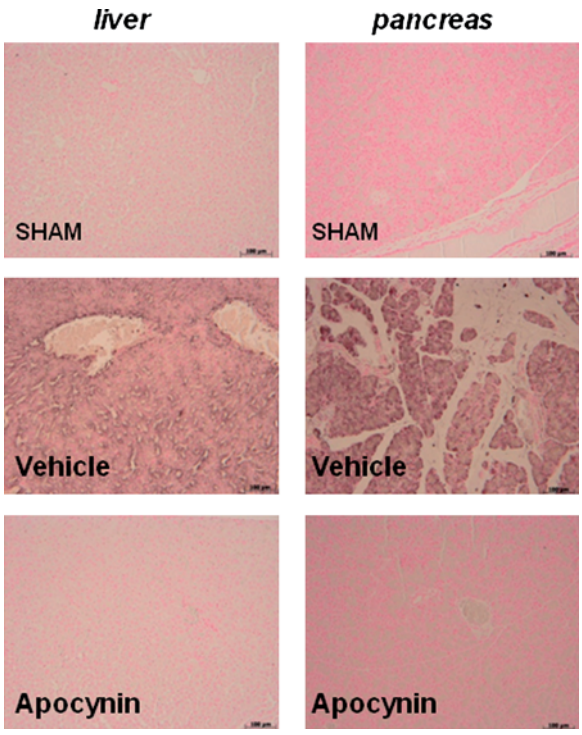

Figure 5. Effect of NADPH oxidase inhibitors on Fas-ligand expression. Eighteen hours following zymosan injection, positive FasL staining was found in pancreas and liver. There was no detectable immunostaining for FasL in pancreas and liver of zymosan-injected mice when mice were treated with apocynin. No positive staining for FasL was found in the pancreas as well as in the liver from sham-treated mice. Figures are representative of at least three experiments performed on different experimental days.

was calculated by Kaplan-Meier survival analysis. The Mann-Whitney U-test (two-tailed, independent) was used to compare medians between the body weight and the clinical score. For such analyses, $p < 0.05$ was considered significant.

Results

Effect of NADPH oxidase inhibitors on inflammatory response in the peritoneal cavity

All mice, at 18 h after zymosan administration, developed acute peritonitis with a significant production of turbid exudate (Figure 1A). PEC counts were done in the mice following intraperitoneal injection of zymosan or saline solution in order to determine whether there were any quantitative changes in peritoneal infiltrates. Zymosan injection was associated with an increase in PEC counts at 18 h in mice compared to the saline controls (Figure 1B). Since there was a quantitative increase in PECs following zymosan injection, cytospin preparations were done of the PEC for a differential estimation of the types of cells present. Wright-Giemsa stained slides of all controls appeared to contain mostly mononuclear cells including resident macrophages and lymphocytes and very few polymorphonuclear neutrophils, as previously demonstrated [26]. All the cells appeared healthy and intact. At 18 h after zymosan administration, almost all cells appeared lysed and, because of excessive phagocytosis by the leukocytes, the neutrophils could not be differentiated from macrophages. Since the cells appeared lysed and the nucleus could not be differentiated, cell staining for specific esterases for neutrophil and macrophages was done in order to attempt differentiation between cell populations in the zymosan injected animals. In agreement with previous observations [26], we confirmed the presence of 90% mononuclear cells in the peritoneal cavity along with 10% PMNs in all the sham-treated animals. In contrast, the zymosan-treated samples could not be differentiated due to excessive phagocytosis and lysis of cells. Exudate formation and the degree of PEC count were significantly reduced in mice treated with apocynin and DPI (Figures 1A and B).

Effect of NADPH oxidase inhibitors on NO formation

The biochemical and inflammatory changes observed in the peritoneal cavity of zymosan-treated mice were associated with a significant increase in peritoneal exudate (Figure 1C) and plasma NO_2/NO_3 levels (Figure 1D). Both the increase in exudate and plasma NO_2/NO_3 levels were significantly reduced in mice treated with apocynin and DPI (Figures 1C and D).

Effect of NADPH oxidase inhibitors on cytokine production

The levels of TNF-α and IL-1β were significantly elevated in the plasma of zymosan-injected mice (Figures 2Aa and Ba). In contrast, the levels of these cytokines were significantly lower in the plasma of zymosan-injected mice treated with apocynin and DPI (Figures 2Aa and Ba). Circulating levels of these cytokines were not increased in sham-treated mice (Figures 2Aa and Ba). In addition, no positive staining for TNF-α and IL-1β were observed in pancreatic (Figures 2A and B, respectively) or liver (Figures 2A and B, respectively) tissues obtained from sham-treated mice. In contrast, at 18 h after zymosan injection, a substantial increase in TNF-α and IL-1β expression was found in the pancreas (Figures 2A and B, respectively) as well as in the liver (Figures 2A and B). The positive staining for TNF-α and IL-1β were significantly attenuated in the pancreas (Figures 2A and B, respectively) and liver (Figures 2A and B, respectively) in mice with peritonitis treated with apocynin.

Effect of NADPH oxidase inhibitors on ICAM and P-selectin expression

At 18 h after zymosan injection, expression of the adhesion molecules ICAM-1 and P-selectin were

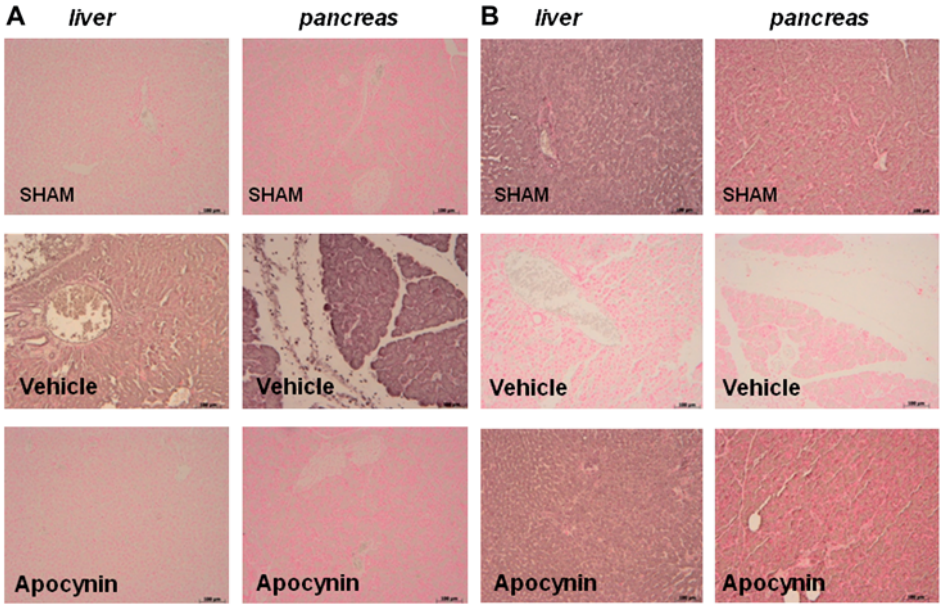

Figure 6. Effect of NADPH oxidase inhibitors on Bax and Bcl-2 expression. Eighteen hours following zymosan injection, positive Bax (A) staining was found in pancreas and liver. No positive staining for Bax (A) was detected in pancreas and liver of zymosan-treated mice when mice were treated with apocynin. No positive staining for Bax (A) was found in the pancreas as well as in the liver from sham-treated mice. At 18 h after zymosan administration, no positive staining for Bcl-2 (B) was observed in pancreas and liver from zymosan-injected mice. On the contrary, positive staining for Bcl-2 (B) was observed in pancreas and liver from mice treated with apocynin and from sham-treated mice. Figures are representative of at least experiments performed on different experimental days.

evaluated to assess neutrophil infiltration. In zymosan-injected mice, an increase of immunohistochemical staining for ICAM-1 (Figure 3A) and P-selectin (Figure 3B) was demonstrated on the surface of endothelial cells in the inflamed pancreas (Figures 3A and B, respectively) and inflamed liver (Figures 3A and B, respectively), while the immunostainings for ICAM-1 and P-selectin were markedly reduced in pancreatic (Figures 3A and B, respectively) and liver (Figures 3A and B, respectively) tissues obtained from mice that were treated with apocynin. No staining for either ICAM-1 or P-selectin was found in tissue sections obtained from sham-treated mice (Figures 3A and B, respectively).

Effect of NADPH oxidase inhibitors on nitrosative stress and PARP activation

At 18 h following the i.p. injection of zymosan, sections of the pancreas and liver were analysed for the

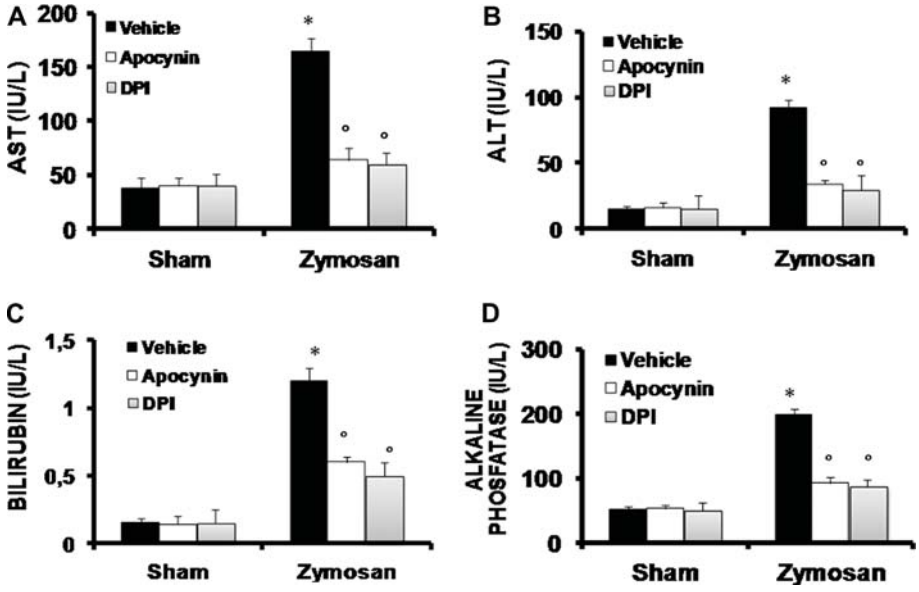

Figure 7. Effect of NADPH oxidase inhibitors on liver injury. Administration of zymosan resulted in significantly increased levels of plasma AST (A), ALT (B), bilirubin (C) and alkaline phosphatase (D). Apocynin and DPI treatment significantly reduced all these parameters in zymosan injected mice. Data are means ± SEM of 10 mice for each group. $^{*}p < 0.01$ vs sham, $^{\circ}p < 0.01$ vs zymosan + vehicle.

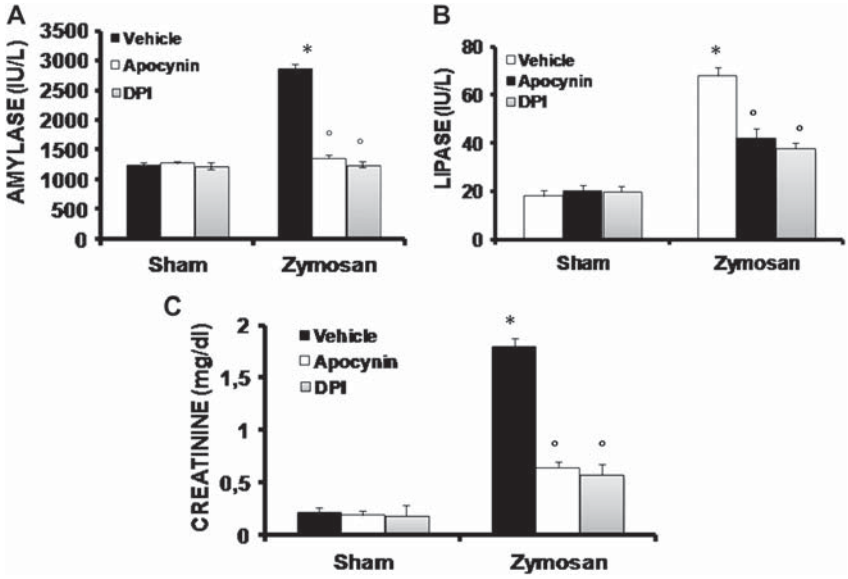

Figure 8. Effect of NADPH oxidase inhibitors on pancreatic and renal injury. Administration of zymosan resulted in significantly increased levels of plasma amylase (A), lipase (B) and creatinine (C). Apocynin and DPI treatment significantly decreases all these parameters in zymosan injected mice. Data are means ± SEM of 10 mice for each group. $^{*}p < 0.01$ vs sham, $^{\circ}p < 0.01$ vs zymosan + vehicle.

presence of nitrotyrosine. Immunohistochemical analysis, using a specific anti-nitrotyrosine antibody, revealed a positive staining in the pancreas and liver (Figure 4A) from zymosan-treated mice. A marked reduction in nitrotyrosine staining was found in the pancreas and liver (Figure 4A) of the zymosan-challenged mice, which were treated with apocynin. Immunohistochemical analysis of pancreas and liver (Figure 4B) sections obtained from mice challenged with zymosan also revealed positive staining for PAR. In contrast, staining for PAR was absent in sections of pancreas and liver (Figure 4B) from apocynin-treated zymosan-challenged mice. There was no staining for either nitrotyrosine or PAR in sections of the pancreas (Figures 4A and B, respectively) or liver (Figures 4A and B, respectively) from sham-treated mice.

Effect of NADPH oxidase inhibitors on FasL, Bax and Bcl-2

Immunohistological staining for the Fas Ligand in the pancreas and liver was determined 18 h after zymosan-induced injury. Tissue sections from the sham-operated mice did not stain for the Fas Ligand (Figure 5), whereas sections obtained from the zymosan-challenged mice exhibited positive staining for the Fas Ligand, in the pancreas and liver (Figure 5). Treatment with apocynin reduced the degree of

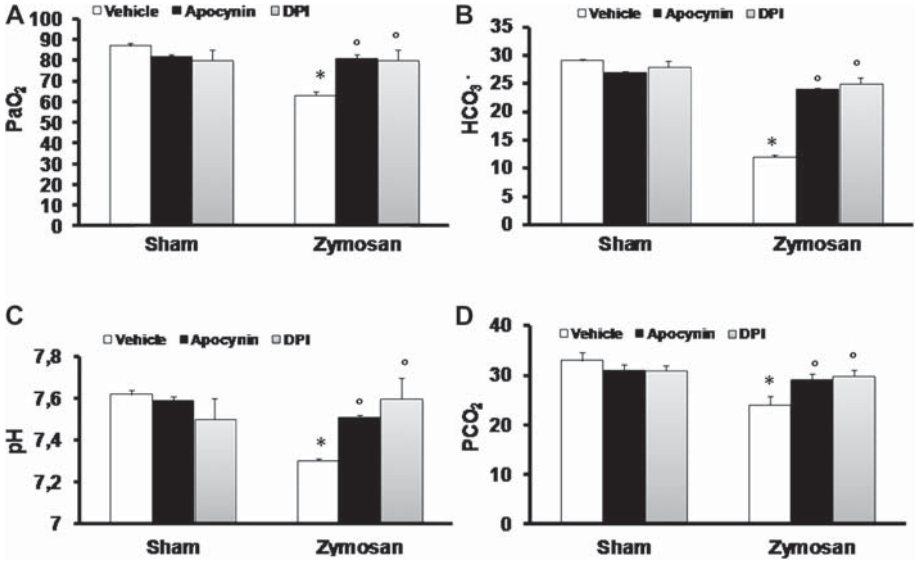

Figure 9. Effect of NADPH oxidase inhibitors on lung injury. Zymosan injection resulted in a significant fall in the arterial levels of PaO_2 (A), PCO_2 (B), pH (C) and HCO_3^- (D). Administration of apocynin and DPI prevents the lung dysfunctions. Data are means ± SEM of 10 mice for each group. $^{*}p < 0.01$ vs sham, $^{\circ}p < 0.01$ vs zymosan + vehicle.

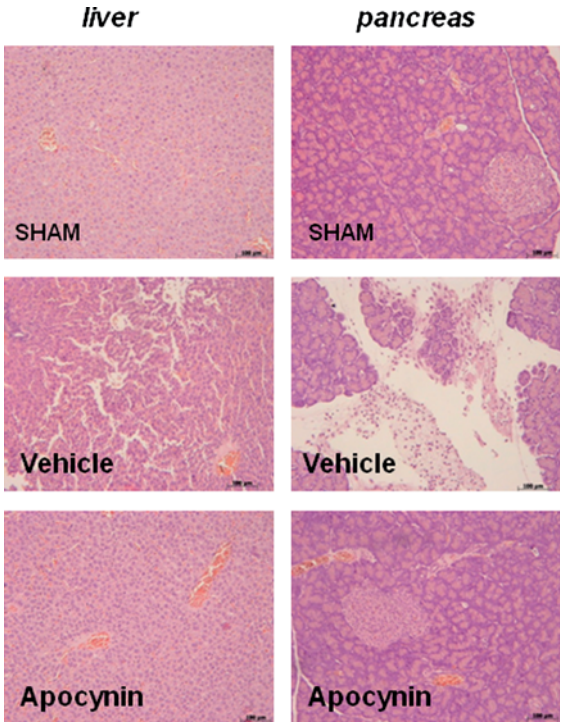

Figure 10. Effect of NADPH oxidase inhibitors on histological evaluation. Pancreas and liver sections from zymosan-injected mice revealed morphological alterations and inflammatory cell infiltration. Pancreas and liver from zymosan-injected mice treated with apocynin demonstrated reduced morphological alterations and inflammatory cell infiltration. No morphological alteration and inflammatory cell infiltration was found in the pancreas as well as in the liver from sham-treated mice. Figures are representative of at least three experiments performed on different experimental days.

positive staining for the Fas Ligand in the pancreas and liver (Figure 5).

To determine the immunohistological staining for Bax (Figure 6A) and Bcl-2 (Figure 6B), samples of pancreas and liver were also collected 18 h after zymosan injection. Tissues taken from sham-treated mice did not stain for Bax (Figure 6A), whereas pancreatic and liver (Figure 6A) sections obtained from zymosan-injected mice exhibited positive staining for Bax. Apocynin treatment reduced the degree of positive staining for Bax in the pancreas and liver (Figure 6A) of mice subjected to zymosan-induced injury.

In addition, pancreas and liver sections from sham-treated mice demonstrated positive staining for Bcl-2 (Figure 6B), whereas in zymosan-injected mice Bcl-2 staining was significantly reduced (Figure 6B). Apocynin treatment significantly attenuated the loss of positive staining for Bcl-2 in pancreatic and liver (Figure 6Bc) samples of mice subjected to zymosan-induced injury.

Effect of NADPH oxidase inhibitors on multiple organ dysfunction syndrome caused by zymosan

Hepatocellular injury. In sham-operated mice, administration of saline did not result in any significant alterations in the plasma levels of AST (Figure 7A), ALT (Figure 7B), bilirubin (Figure 7C) or alkaline phosphatase (Figure 7D). When compared with sham-treated mice, mice challenged with zymosan had significantly higher plasma concentrations of ALT, AST, bilirubin and alkaline phosphatase. These findings all are consistent with the development of hepatocellular injury. Treatment with apocynin and DPI decreased the liver injury caused by zymosan.

Pancreatic injury. In sham-operated mice, administration of saline did not result in any significant alterations in the plasma levels of lipase and amylase (Figures 8A and B). When compared with sham-operated mice,

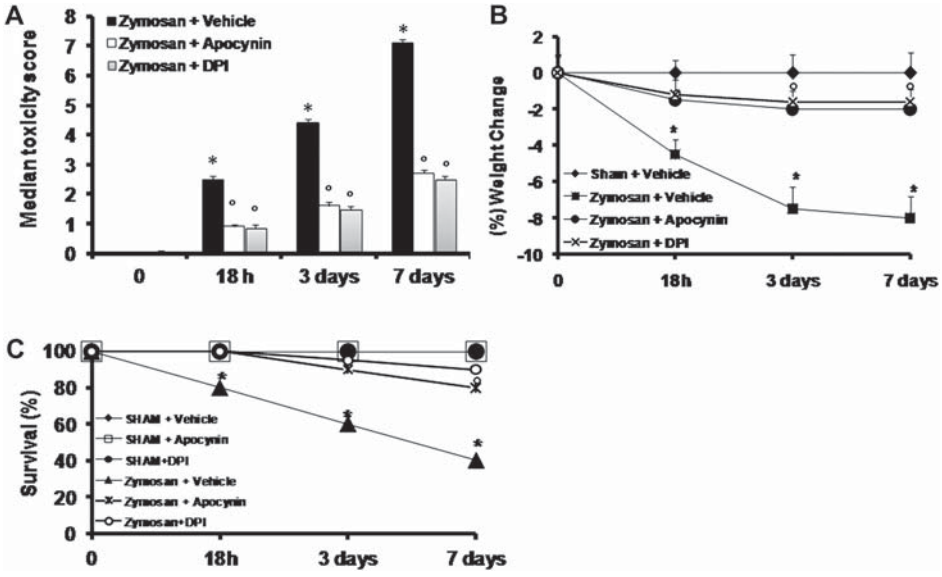

Figure 11. Effect of NADPH oxidase inhibitors on toxicity score (A), body weight change (B) and mortality (C). Data are means ± SEM of 10 mice for each group. $^{*}p < 0.01$ vs sham, $^{\circ}p < 0.01$ vs zymosan + vehicle.

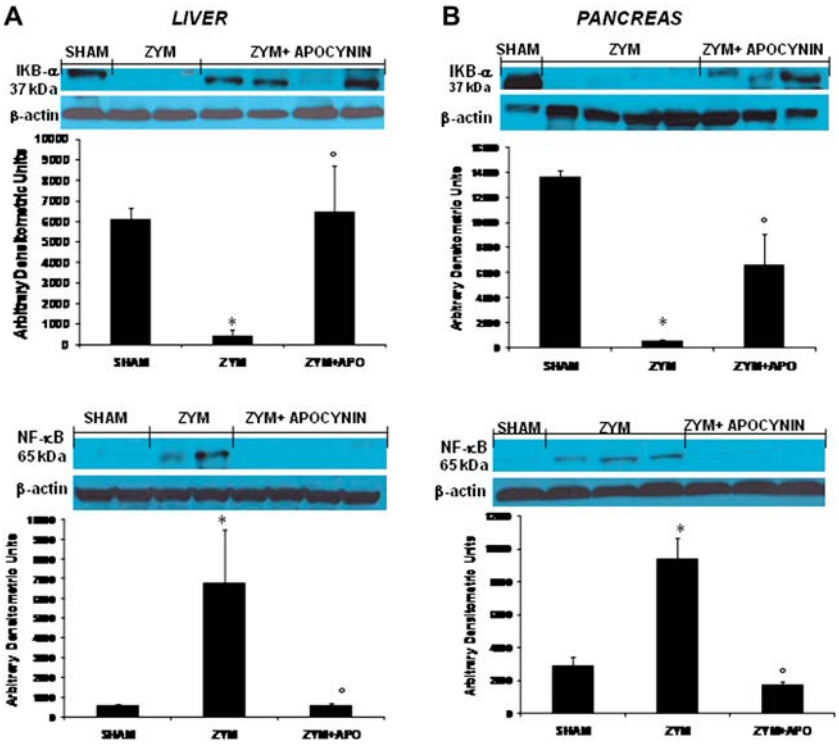

Figure 12. Effect of NADPH oxidase inhibitors on IkB-*α* degradation and NF-kB p-65 activation. By Western Blot analysis, a basal level of IkB-*α* was detected in the liver and pancreas tissues (A and B) from sham-operated animals, whereas in zymosan-induced mice IkB-*α* levels were substantially reduced (A and B). Treatment with NADPH oxidase inhibitors significantly increases the levels of IkB-*α*, after zymosan injection (A and B). Moreover, at 18 h following zymosan-treatment, the levels of NF-kB p-65 sub-unit protein in the nuclear fractions of the liver and pancreas tissues were also significantly increased compared to the sham-operated mice (A and B). The levels of NF-kB p-65 protein were significantly reduced in the nuclear fractions of the lung tissues from animals that had received NADPH oxidase inhibitors (A and B). *β*-actin (A and B) was used as internal control. A representative blot of lysates obtained from five animals per group is shown and densitometry analysis of all animals is reported. The results in (A) and (B) are expressed as mean ± SEM from $n = 5/6$ liver and pancreas tissues for each group. $^{*}p < 0.01$ vs sham, $^{\circ}p < 0.01$ vs zymosan + vehicle.

injection of zymosan significantly increased plasma levels of lipase and amylase; these findings are consistent with the development of pancreatic injury (Figures 8A and B). Treatment with apocynin and DPI decreased the pancreatic injury caused by zymosan (Figures 8A and B).

Renal dysfunction. In sham-operated mice, administration of saline did not result in any significant alteration in the plasma level of creatinine (Figure 8C). When compared with sham-operated mice, zymosan injection in mice resulted in a significant increase in the plasma creatinine concentration, a finding that is indicative of the development of renal dysfunction. Treatment with apocynin and DPI decreased the renal dysfunction caused by zymosan (Figure 8C).

Effects on the lung injury. In sham mice, administration of saline did not result in any significant alterations in the PaO_2, PCO_2, HCO_3^- and pH arterial blood levels (Figure 9). When compared with sham mice, zymosan administration resulted in a significant fall in the arterial levels of PaO_2, PCO_2, HCO_3^- and pH, demonstrating the development of lung dysfunction (Figure 9). The treatment with apocynin and DPI significantly reduced the lung injury caused by zymosan (Figure 9).

Effect of NADPH oxidase inhibitors on pancreas and liver injury (histological evaluation) caused by zymosan

At 18 h after zymosan administration, histological examination of pancreas and liver (Figure 10) sections revealed marked pathological changes. In the pancreas and liver, there was extravasation of red cells and neutrophils and oedema (Figure 10). The treatment with apocynin resulted in substantial reduction in the extent of histological damage in the pancreas and liver (Figure 10). No histological alteration was observed in the pancreas or liver (Figure 10) from sham-treated mice.

Effect of NADPH oxidase inhibitors on zymosan-induced body weight loss and mortality

Injection of zymosan caused severe illness in the mice, which was characterized by systemic toxicity and significant loss of body weight (Figures 11A and B). At the end of the observation period (7 days), 60% of zymosan-treated mice were dead (Figure 11C).

Treatment with apocynin and DPI reduced the development of systemic toxicity (Figure 11A), the loss in body weight (Figure 11B) and mortality (Figure 11C) caused by zymosan. Apocynin and DPI treatment did not cause significant changes in these parameters in sham mice.

Effect of NADPH oxidase inhibitors on IκB-α degradation and NF-κB p65 activation

To investigate the inflammatory cellular mechanisms by which treatment with NADPH oxidase inhibitors may attenuate the development of zymosan-induced injury, we evaluated IκB-α degradation and nuclear NF-κB p65 translocation by Western Blot analysis.

A basal level of IκB-α was detected in the liver (Figure 12A, see densitometry analysis) and pancreas (Figure 12B, see densitometry analysis) tissues of sham-animals, whereas in zymosan-injected mice, IκB-α levels were substantially reduced in the liver (Figure 12A, see densitometry analysis) and pancreas (Figure 12B). NADPH oxidase inhibitors prevented zymosan-induced IκB-α degradation, with IκB-α levels observed in these animals similar to those of the sham group (Figures 12A and B, see densitometry analysis). In addition, zymosan injection caused a significant increase in NF-kB p65 levels in the nuclear fractions from liver (Figure 12A, see densitometry analysis) and pancreas (Figure 12B, see densitometry analysis) tissues, compared to the sham-treated mice. NADPH oxidase inhibitors treatment significantly reduced the levels of NF-kB p65 in the liver (Figure 12A, see densitometry analysis) and pancreas (Figure 12B, see densitometry analysis) tissues.

Discussion

Oxidative stress describes an imbalance between ROS synthesis and antioxidants. The normal production of oxidants is counteracted by several anti-oxidative mechanisms in the body [14]. In pathologic conditions, in which excessive production of ROS outstrips endogenous antioxidant defense, oxidative stress may irreversibly modify biologic macromolecules such as DNA, protein, carbohydrates and lipids.

We believe that the management of oxidative stress is an important strategy in managing the development and progression of many diseases and we believe that NADPH oxidase inhibitors have the potential to manage oxidative stress. In this study, we wanted to demonstrate a possible therapeutic involvement of NADPH oxidase inhibitors in an acute inflammatory disease such as zymosan-induced multiple organ failure. We demonstrated a beneficial role of apocynin and DPI as their treatment decreased the development of acute peritonitis, organ dysfunction and injury, which was associated with a severe illness, a survival of ~60% and characterized by systemic toxicity and significant loss of body weight.

One consequence of increased oxidative stress is the activation and inactivation of redox-sensitive proteins [27]. Several studies showed that the mechanism of action of apocynin involved the inhibition of the NADPH-oxidase-dependent superoxide production, the reduction of the intracellular (reduced glutathione/oxidized glutathione (GSH/GSSG)) ratio and prevention of the activation of the nuclear transcription factor NF-κB, which is an important mediator of inflammation [28]. 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. The net result is the release of the NF-κB dimer, which is then free to translocate into the nucleus. By inhibiting the activation of NF-κB, the production of joint destructive inflammatory mediators under its control such as TNF-α, IL-1β, cyclooxygenase COX-2 and inducible nitric oxide synthase iNOS may be reduced as well. 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 [29] in arthritic animals. A recent study also reported that DPI prevents liver injury, most likely by inhibiting free radical formation via Kupffer cell NADPH oxidase, preventing NF-κB activation and inflammatory cytokine production in alcohol-induced liver injury [20].

There is evidence that the pro-inflammatory cytokines TNF-α and IL-1β help to propagate the extension of a local or systemic inflammatory process [30]. We confirm here that zymosan-induced shock leads to a substantial increase in the levels of both TNF-α and IL-1β in the plasma after 18 h. In the present study, we found that treatment of mice with apocynin and DPI attenuated the production of TNF-α and IL-1β during zymosan-induced shock. These data are confirmed by immunohistochemical localization of these cytokines. Indeed, the assessment of pancreatic and liver tissue sections have revealed a higher presence of TNF-α and IL-1β in samples obtained from zymosan-injected mice, while apocynin treatment exhibited a fall in the immunohistochemical levels.

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 moleculeVCAM-1, ICAM-1 and E-selectin [31]. Recent studies showed that apocynin could decrease the long-lasting E-selectin expression on endothelial cells subjected to anoxia/reoxygenation [32] and that apocynin prevents the increased vascular permeability caused by ischemia and reperfusion in isolated

sheep lungs [12]. In this study, we observed that, 18 h after administration, zymosan induced the expression of P-selectin in the endothelium of small vessels and up-regulated the surface expression of ICAM-1 and P-Selectin on endothelial cells in the pancreas and liver. In contrast, there was significantly less expression of P-selectin and ICAM-1 in the pancreas and liver obtained from mice treated with apocynin.

iNOS and its product NO are also involved in many inflammatory diseases. Moreover, in zymosan-induced shock and inflammation the role of NO has been demonstrated [33]. However, NO reacts with superoxide to form peroxynitrite; by inhibiting the formation of superoxide with apocynin and hence reducing the amount of peroxynitrite, the inflammation-mediated reduction of cartilage proteoglycan synthesis might be restored in mice suffering from zymosan-induced acute arthritis [9].

We show here that NO levels, evaluated as NO_2/NO_3, assessed in exudate and plasma, were increased at 18 h after zymosan-injection, while apocynin decreased the levels of NO.

Nitrotyrosine formation, along with its detection by immunostaining, was initially proposed as a relatively specific marker for the detection of the endogenous formation 'footprint' of peroxynitrite and an increased nitrotyrosine staining is considered as an indication of increased nitrosative stress [34]. Thus, by immunohistochemical localization, we have seen an increase in nitrotyrosine staining in samples of pancreas and liver obtained from zymosan-induced injured mice, while an improvement was due to apocynin administration.

There is a large amount of evidence that the production of ROS at the site of inflammation contributes to multiple organ failure [35]. A novel pathway of inflammation, governed by the nuclear enzyme PARP, has been proposed in relation to hydroxyl radical- and peroxynitrite-induced DNA single strand breakage. This pathway plays an important role in various forms of inflammation as well as in zymosan-induced shock [33]. We demonstrated here that apocynin attenuates the increase in PARP activity in the pancreas and liver from zymosan-injected mice. Thus, we propose that the anti-inflammatory effects of apocynin may be at least in part due to the prevention of the activation of PARP. Oxidative stress is considered to be an important condition to promote cell death in response to a variety of signals and pathophysiological situations [36]. Furthermore, cell death induced by ROS depends on Fas-ligand expression mediated by redox sensitive activation of NF-κB [37]. Fas-ligand plays a central role in apoptosis induced by a variety of chemical and physical insults [38]. In this study, we have clearly shown the degree of cell death, assessed by immunohistochemical localization of FasL; we found that zymosan-injection causes an increase of FasL expression in tissue sections of pancreas and liver; on the other hand, apocynin treatment decreases this expression. In addition, recent studies also demonstrated that DPI treatment resulted in a reduction of oxidative DNA damage, caspase-3 activation, decreased caspase-3 activity and diminished prevalence of apoptosis in ethanol-exposed mouse embryos [39].

Therefore, in the present study, we have identified pro-apoptotic transcriptional changes, including up-regulation of pro-apoptotic Bax and down-regulation of anti-apoptotic Bcl-2, using immunohistochemical staining. In particular, we demonstrated that the treatment with apocynin lowers the signal for Bax in the treated group when compared with pancreas and liver sections obtained from zymosan-injected mice, while, on the contrary, the signal is much more express for Bcl-2 in the apocynin-treated mice than in zymosan-injected mice. 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.

To further confirm these data, we found that NADPH oxidase inhibitors not only prevent lung dysfunction and reduce zymosan-induced loss of blood PaO_2, PCO_2, HCO_3^- and pH levels, but also diminish other blood parameters, such as the levels of AST, ALT, bilirubin and alkaline phosphatase that are altered after the onset of zymosan-induced MOF. Recent findings also confirm that inhibitors of NADPH oxidase (such as DPI or apocynin) are able to reduce the liver and intestinal injury caused by a model of haemorrhagic shock without causing any apparent side-effects [18]. Furthermore, high concentrations of lipase, amylase and creatinine, indicating the degree of MODS, are all reduced by apocynin and DPI, reducing the pathophysiology of MOF. A histological resolution of organ damage to administration of apocynin was highlighted in pancreas and liver by haematoxylin-eosin staining too. Indeed, the degree of histoarchitectural modifications in these tissues decreased significantly after treatment with apocynin.

The multiple organ failure, replicated here through zymosan-injection, is a disease with severe implications, involving several mechanisms not yet fully known. However, our results clearly suggest that NADPH inhibitors such us apocynin and DPI be used successfully as a therapeutic agent in the treatment of conditions associated with inflammation such us haemorrhagic shock [18] and multiple organ system dysfunction. This hypothesis warrants further investigation in the future by using more specific and potent inhibitors of NADPH oxidase, which are currently not commercially available.

Acknowledgements

The authors would like to thank Carmelo La Spada for his excellent technical assistance during this study, Mrs Caterina Cutrona for secretarial assistance and

Miss Valentina Malvagni for editorial assistance with the manuscript.

Declaration of interest

The authors report no conflicts of interest. This study was supported by a grant from IRCCS Centro Neurolesi 'Bonino-Pulejo'.

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This paper was first published online on Early Online on 2 June 2011.

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