

## Original Contribution

## Sodium salicylate prevents paraquat-induced apoptosis in the rat lung

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**Abstract**

The nonselective contact herbicide, paraquat (PQ), is a strong pneumotoxicant, especially due to its accumulation in the lung through a polyamine uptake system and to its capacity to induce redox cycling, leading to oxidative stress-related damage. In the present study, we aimed to investigate the occurrence of apoptotic events in the lungs of male Wistar rats, 24, 48, and 96 h after PQ exposure (25 mg/kg ip) as well as the putative healing effects provided by sodium salicylate [NaSAL, 200 mg/kg ip] when administered 2 h after PQ. PQ exposure resulted in marked lung apoptosis, in a time-dependent manner, characterized by the “ladder-like” pattern of DNA observed through electrophoresis and by the presence of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL)-positive cells (TPC) as revealed by immunohistochemistry. The two main caspase cascades (the extrinsic receptor-mediated and the intrinsic mitochondria-mediated) and the expressions of p53 and activator protein-1 (AP-1) were also evaluated, to obtain an insight into apoptotic cellular signaling. PQ-exposed rats suffered a time-dependent increase of caspase-3 and caspase-8 and a decrease of caspase-1 activities in lungs compared to the control group. A marked mitochondrial dysfunction evidenced by cytochrome *c* (Cyt *c*) release was also observed as a consequence of PQ exposure. In addition, fluorescence electrophoretic mobility shift assay (fEMSA) revealed a transcriptional induction of the p53 and AP-1 transcription factors in a time-dependent manner as a consequence of PQ exposure. NaSAL treatment resulted in the remission of the observed apoptotic signaling and consequently of lung apoptosis. Taken together, the present results showed that PQ activates several events involved in the apoptotic pathways, which might contribute to its lung toxicodynamics. NaSAL, a recently implemented antidote for PQ intoxications, proved to protect lungs from PQ-induced apoptosis.

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**Keywords:** Paraquat; Lung toxicity; Sodium salicylate; Apoptosis; Rats

**Abbreviations:** AC buffer, cell lysis buffer; AP-1, activator protein-1; Apaf-1, apoptosis-activating factor-1; BC buffer, nuclei lysis buffer; CAT, catalase; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Cyt *c*, cytochrome *c*; DTT, dithiothreitol; dUTP, fluorescein-labeled deoxyuridinetriphosphate; ECL, enhanced chemiluminescence; FADD, Fas-associating protein with death domain; fEMSA, fluorescence electrophoretic mobility shift assay; GPx, glutathione peroxidase; HO<sup>•</sup>, hydroxyl radical; LPO, lipid peroxidation; LPS, lipopolysaccharide; MPO, myeloperoxidase; NaSAL, sodium salicylate; NF-κB, nuclear factor kappa-B; PBS, phosphate-buffered saline; PCD, programmed cell death; PMSF, phenylmethylsulfonyl fluoride; PQ, paraquat; RNase A, ribonuclease A; ROS, reactive oxygen species; SC, specific competitor; SDS, sodium dodecyl sulfate; TdT, terminal deoxynucleotidyl transferase; TBARS, thiobarbituric acid-reactive substances; TNF-α, tumor necrosis factor alpha; TPC, TUNEL-positive cells; TRAIL, TNF-related apoptosis-inducing ligand; TUNEL, deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling; UC, unspecific competitor.

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## Introduction

The main target organ for the toxicity elicited by the herbicide paraquat dichloride (methyl viologen; PQ) is the lung. The mechanisms subjacent to this organ specificity are postulated to be associated with the selective accumulation of PQ in the lung, followed by a sustained redox-cycling effect, leading to oxidative stress-related cell death and inflammation [1,2].

Despite numerous studies concerning PQ-induced toxicity, few of them focus on the apoptotic and on the transcriptional regulatory mechanisms as potential contributory factors for PQ toxicity and the importance of modulating these mechanisms in the treatment of PQ poisonings. PQ-induced apoptosis was first demonstrated in a murine myeloid cell line, mouse 32D cells [3]. Subsequently, the involvement of reactive oxygen species (ROS) in the occurrence of PQ-induced apoptosis was reported, either using the *in vivo* model of the intrahippocampal injection of PQ [4] or using the *in vitro* models of differentiated human neuroblastoma cells (SHSY-5Y) [5] and human lung epithelial cells [6]. Notwithstanding the lack of *in vivo* studies about the putative apoptotic effects of PQ in the lung, it is known that alveolar epithelium undergoes apoptosis in normal tissue remodeling as well as in pathological conditions [7,8]. Our hypothesis is that the selective uptake of PQ by type I and II pneumocytes, and Clara cells [9] may lead to a subsequent induction of apoptosis, which could make the lung cells unable to restore normal tissue architecture and function, leading to irreversible damage.

Apoptosis or programmed cell death (PCD) is an essential process of cell death during embryonic and postnatal tissue remodeling as well as in several pathological conditions [10]. Morphologically, apoptosis is characterized by reduction of cell volume, membrane blebbing, chromatin condensation, nuclear fragmentation, and apoptotic cell body formation [11]. The signaling pathways leading to apoptosis are implemented by a death machinery signaling system whose executionary arm is a family of cysteine proteases, designated caspases (for cysteine aspartic acid-specific proteases) [12–14]. Caspases exist normally as inactive precursors (procaspases) in the cytosolic fraction of the cells. They are cleaved proteolytically at specific amino acid sequences into low molecular weight units (20–23 kDa), when the cell undergoes apoptosis, to form the active enzyme. Two main caspase cascades, the extrinsic receptor mediated and intrinsic mitochondria mediated, have been delineated in mammalian cells [12–14]. The extrinsic pathway for the activation of apoptosis involves the stimulation of death receptors expressed at the cell surface, leading to clustering and formation of a death-inducing signaling complex system, which includes the adapter protein FADD (Fas-associated death domain) and the initiator caspase-8 [15]. Both TNF-related apoptosis-inducing ligand (TRAIL) and tumor necrosis factor (TNF)- $\alpha$  are known to bind to their cell surface receptors, leading to caspase-8 activation. Caspase-8 is a major enzyme activating downstream the effector caspase-3 [16]. The intrinsic pathway involves the release of cytochrome *c* (Cyt *c*) from mitochondria to the cytosolic fraction of the cells at an early

phase of apoptosis [12–14,17]. Cyt *c* [also known as apoptosis-activating factor-2 (Apaf-2)], together with some cytosolic proteins (i.e., Apaf-1) in the presence of dATP [13], recruits and activates the conversion of the latent apoptosis-promoting procaspase-9 to its active form, which then activates caspase-3 [13,17,18]. Thus, both death receptor and mitochondria pathways converge at the level of caspase-3 activation. Caspase-3 is considered to be the central and final apoptotic effector enzyme responsible for many of the biological, morphological, and structural features of apoptosis [12]. Active effector caspases mediate the cleavage of apoptosis regulators, the cleavage of housekeeping proteins, and DNA fragmentation, resulting in morphological features of apoptosis [13,14].

The first caspase to be identified, caspase-1 [interleukin (IL)-1 $\beta$ -converting enzyme], is not a component of cell death machinery, but it indirectly influences the rates of apoptosis through cleavage of IL-1 $\beta$  to its 17-kDa mature form [19]. Rowe et al. [20] hypothesized that caspase-1 might regulate apoptosis of neutrophils. These investigators studied these processes in caspase-1-deficient mice compared with wild-type controls. The results provided evidence for a proapoptotic role of caspase-1 in the lipopolysaccharide (LPS)-unstimulated neutrophils, what is reversed in LPS-treated neutrophils by the antiapoptotic effects mediated by IL-1 $\beta$  cleavage. In addition, using a model of LPS-mediated lung injury, they found that caspase-1-deficient mice show a prolonged inflammatory response [20].

Other actors in the apoptotic story are the transcription factor activator protein-1 (AP-1) and the tumor suppressor protein p53. AP-1 is the designation of the transcriptional complex composed of dimers (homodimeric and heterodimeric) of proteins of the *fos* (c-fos, fos-B, fra1, and fra2) and *jun* oncogene families (c-jun, jun-B, and jun-D) [21]. Much of what is known about the biological function of AP-1 relates to its prominent roles in cell proliferation, differentiation, and transformation and in the induction of apoptosis [22,23]. Its activation occurs in response to a number of diverse stimuli, including oxidative or cellular stress, ultraviolet irradiation, DNA damage, antigen binding by T or B lymphocytes, and exposure to proinflammatory cytokines (e.g., TNF- $\alpha$ , transforming growth factor- $\beta$ , and  $\gamma$ -interferon), overlapping in several instances with the target genes of NF- $\kappa$ B [23]. In addition to increased subunit synthesis, oxidative stress induces AP-1-mediated transcription by enhancing DNA-binding activity as well [24]. With the exception of preexisting c-jun homodimers, induction of AP-1 relies predominantly on novel synthesis of its DNA-binding subunits [25]. The p53 tumor suppressor protein is a 53-kDa transcription factor constitutively expressed at low levels in most cells and tissues [26]. It is presumably the most intensively studied factor of programmed cell death, since its overexpression induces apoptosis [26,27]. Several lines of evidence, supporting a key role for p53 in the control of cell cycle of a range of cell types by controlling the progression through G1-phase, have arisen [26,27].

Recently, our group demonstrated that sodium salicylate (NaSAL) constitutes an important and valuable antidote to be used against PQ-induced toxicity, leading to full survival of PQ-

exposed rats. The antidotal effect of NaSAL was mainly a consequence of the effective inhibition of the proinflammatory factor, nuclear factor (NF)- $\kappa$ B, scavenging of ROS, inhibition of myeloperoxidase (MPO), and inhibition of platelet aggregation [28]. Therefore, the same approach was followed here, primarily to determine the ability of PQ to induce apoptotic events in the lungs of Wistar rats and secondly to evaluate if the treatment with NaSAL has also beneficial effects at this level. Apoptosis was assessed by the “ladder-like” pattern of DNA and by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay. Concerning apoptotic cell signaling, the two main caspase cascades were studied through the measurement of the cytosolic Cyt *c* concentrations and of the enzymatic activities of caspases-1, -8, and -3. The expressions of p53 and AP-1 were also evaluated. Overall, this study should lead to a better understanding of the underlying adverse pathways activated by PQ in the respiratory tract and consequently to provide new tools to prevent PQ-induced lung toxicity.

## Materials and methods

### Chemicals and drugs

PQ (1,1'-dimethyl-4,4'-bipyridinium dichloride), NaSAL (2-hydroxybenzoic acid sodium salt), *N*-acetyl-Trp-Glu-His-Asp-*p*-nitroanilide (colorimetric substrate for caspase-1), *N*-acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (colorimetric substrate for caspase-3), *N*-acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide (colorimetric substrate for caspase-8), Chaps (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), ribonuclease A (RNase A), sodium dodecyl sulfate (SDS), proteinase K from *Tritirachium album*, phenol solution (equilibrated with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA), chloroform, Mayer's hematoxylin solution, eosin Y, and the alkaline phosphatase substrate solution (Fast Red TR/Napthol AS-MX) were obtained from Sigma (St. Louis, MO). Anti-cytochrome *c* antibody (556433) was obtained from BD Pharmingen. Mouse monoclonal anti- $\alpha$ -tubulin (clone DM1A) was purchased from Lab Vision Corporation (Fremont, CA). The enhanced chemiluminescence (ECL)-Plus reagent and the entire Western blot reagents were purchased from Amersham Biosciences (Lisbon, Portugal). The saline solution (NaCl 0.9%) and sodium thiopental were obtained from B. Braun (Lisbon, Portugal). The following synthetic oligonucleotides, purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), were used: 5'-Cy5-TAC AGA ACA TGT CTA AGC ATG CTG GGG-3' (p53-FW-Cy5), 5'-TAC AGA ACA TGT CTA AGC ATG CTG GGG-3' (p53-FW), 5'-CCC CAG CAT GCT TAG ACA TGT TCT GTA-3' (p53-FW-R), 5'-Cy5-CGC TTG ATG ACT CAG CCG GAA-3' (AP-1-FW-Cy5), 5'-CGC TTG ATG ACT CAG CCG GAA-3' (AP-1-FW), 5'-TTC CGG CTG AGT CAT CAA CGC-3' (AP-1-R), 5'-GCC TGG GAA AGT CCC CTC AAC T-3' (NF- $\kappa$ B-FW), and 5'-AGT TGA GGG GAC TTT CCC AGG C-3' (NF- $\kappa$ B-R). Cy5 (indodicarbocyanine) is a fluorescence dye attached to the 5' OH end of the oligonucleotide. All the reagents used were of analytical grade.

### Animals

A total of 80 male Wistar rats (aged 8 weeks) were obtained from Charles River S.A. (Barcelona, Spain), with a mean weight of  $249 \pm 23$  g. Animals were kept in standard laboratory conditions (12/12 h light/darkness,  $22 \pm 2^\circ\text{C}$  room temperature, 50–60% humidity) for at least 1 week before starting the experiments. Animals were allowed access to tap water and rat chow ad libitum during this period. Animal experiments were licensed by Portuguese General Directorate of Veterinary Medicine (DGV). Housing and experimental treatment of animals were in accordance to the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research (ILAR 1996). The experiments complied with the current laws of Portugal.

### Experimental protocol

Each animal was individually housed during the experimental period in a polypropylene cage with a stainless-steel net at the top and wood chips at the screen bottom. Tap water and rat chow were given ad libitum during the entire experiment. Treatments in all groups were always conducted between 8:00 and 10:00 AM. The administrations of vehicle (0.9% NaCl), PQ, and NaSAL were all made intraperitoneally (ip) in an injection volume of 0.5 mL/250 g of body weight. The schedule of NaSAL administration (2 h after PQ) was chosen taking into account the estimated average arrival time of the patient to the hospital, after PQ intoxication. The experimental dose of NaSAL was chosen according to literature data of in vivo studies [28]. The PQ-administered dose is known to produce severe lung toxicity and death in rats within a few days [28–30]. Each group was treated as described in Fig. 1. Briefly: (i) control group,  $n=8$ : animals were first administered with 0.9% NaCl. Animals were administered with one more administration of 0.9% NaCl 2 h later and sacrificed 24 h after the second injection. (ii) NaSAL group,  $n=24$ : animals were first administered with 0.9% NaCl. Animals were treated with one administration of NaSAL (200 mg/kg) 2 h later and sacrificed 24 h ( $n=8$ , NaSAL 24 h group), 48 h ( $n=8$ , NaSAL 48 h group), and 96 h ( $n=8$ , NaSAL 96 h group) after the second injection. (iii) PQ group,  $n=24$ : animals were first intoxicated with PQ (25 mg/kg). Animals were administered with one more administration of 0.9% NaCl 2 h later and sacrificed 24 h ( $n=8$ , PQ 24 h group), 48 h ( $n=8$ , PQ 48 h group), and 96 h ( $n=8$ , PQ 96 h group) after the second injection. (iv) PQ+NaSAL group,  $n=24$ : animals were first intoxicated with PQ (25 mg/kg). Two hours later, animals were treated with NaSAL (200 mg/kg) and sacrificed 24 h ( $n=8$ , PQ+NaSAL 24 h group), 48 h ( $n=8$ , PQ+NaSAL 48 h group), and 96 h ( $n=8$ , PQ+NaSAL 96 h group) after the second injection.

### Surgical procedures

Before sacrifice, anesthesia was induced with sodium thiopental (60 mg/kg, ip). In six rats of each group (nonhistological studies), lungs were perfused in situ through the pulmonary

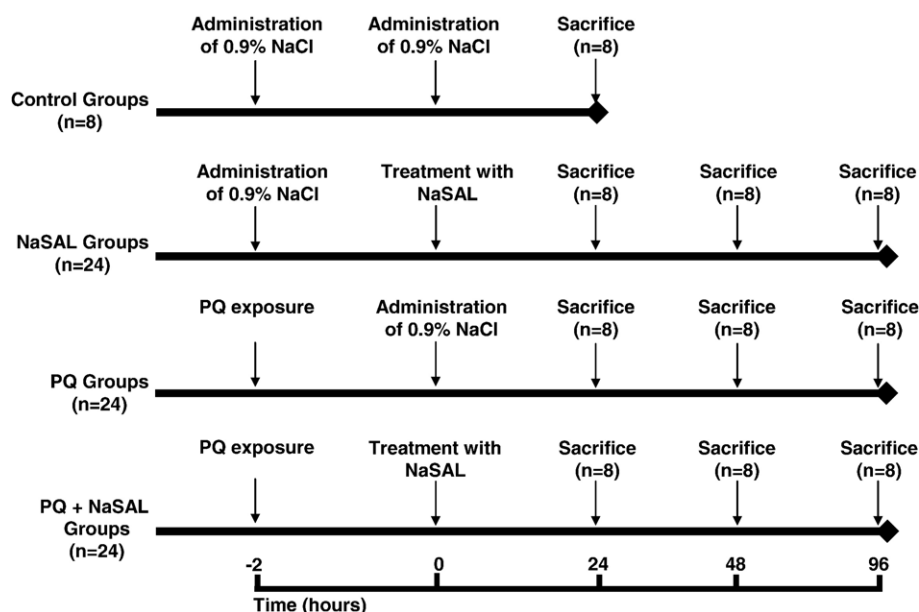


Fig. 1. Schematic representation of the administration protocols for the control, sodium salicylate (NaSAL), paraquat (PQ), and paraquat plus sodium salicylate (PQ + NaSAL) groups.

artery with cold 0.9% NaCl for 3 min at a rate of 10 ml/min to remove most trapped blood volume. Simultaneous with the perfusion initiation, left wall ventricle was cut to avoid cardiovascular volume overload. In the remaining two animals (histological study), the trachea was exposed and intubated. Lungs were inflated by administration of the fixative [4% (v/v) buffered formaldehyde; in situ fixation]. Cubic pieces were then fixed by diffusion for 24 h and subsequently processed for routine paraffin histology. Serial sections (4  $\mu$ m) of the paraffin blocks were cut by a microtome and mounted on slides coated with aminopropyl-triethoxysilane. The slides were dewaxed in xylene and hydrated through graded alcohols finishing in phosphate-buffered saline (10 mM PBS, pH 7.2).

#### Tissue processing for nonhistological studies

Lungs were removed, cleaned of all major cartilaginous tissues of the conducting airways, pat-dried with gauze, and processed as follows: right lungs (except the posterior and the postcaval lobe) were homogenized (Ultra-Turrax homogenizer) in 2.5 ml of an ice-cold isotonic buffer (300 mM sucrose, 10 mM Hepes, 2 mM EGTA, pH 7.4) followed by addition of 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5  $\mu$ g/ml of each proteases inhibitor (pepstatin A, leupeptin, and aprotinin). Cytosolic fractions were prepared essentially as described by Atlante et al. [31] with slight modifications. Homogenates were centrifuged (600g, 4°C, for 10 min) to remove the nuclei and unbroken cells. The resulting supernatants were then centrifuged (9500g, 4°C, for 10 min). Supernatants (cytosolic fraction) were recovered and stored (–80°C) until processed for Cyt *c* quantification. DNA was extracted from the posterior and postcaval lobe according to the standard method described by Shimelis et al. [32] with slight modifications. Briefly, lobes were homogenized (Ultra-Turrax

homogenizer) in 2.5 ml of a lysis buffer (Tris-HCl 100 mM, pH 8, EDTA 50 mM, 0.5% SDS). The homogenates were first incubated with RNase A (200  $\mu$ g/ml, 2 h, 37°C) and subsequently with proteinase K (200  $\mu$ g/ml, 2 h, 37°C). DNA was extracted twice with buffered phenol and with 1:1 mixture of buffered phenol:chloroform. For each extraction step, a mixture by inversion (5 min) followed by centrifugation (2000g, for 10 min) to separate the phases was performed. The superior aqueous phase was recovered and DNA was precipitated by adding 0.1 vol of 3 M sodium acetate, pH 5.2, and 3 vol of ice-cold ethanol. The tubes were inverted for 5 min and the DNA was pelleted by centrifugation (13,000g, 4°C, for 30 min). Thereafter, DNA pellet was washed twice with 70% ethanol (4°C) to remove salt, air-dried overnight at 4°C (the Eppendorfs were left open but covered with aluminum foil during this procedure), and then redissolved in 0.5 ml of TE buffer (Tris-HCl 10 mM, 1 mM EDTA, pH 7.6). Left lungs were used for preparation of cytoplasmic and nuclear extracts. Briefly, left lungs were homogenized (Ultra-Turrax homogenizer) in a AC buffer (cell lysis buffer: 1 g of tissue/3 ml) containing 10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2% igepal, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.25 mM PMSF and incubated on ice for 15 min. After a brief vortexing, the lysates were centrifuged (850g, 4°C for 10 min). The supernatants (cytoplasmic extracts) were saved and the pellets were resuspended (washing step) in 500  $\mu$ l of AC buffer and incubated for 15 min on ice and then centrifuged (14,000g, 4°C for 30 s). The supernatants (cytoplasmic extracts) were added to those obtained in the previous step, divided into aliquots, and stored at –80°C for posterior determination of caspases-1, -8, and -3 activities. The pellets were resuspended in 500  $\mu$ l of BC buffer (nuclei lysis buffer) containing 20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2% igepal, 0.5 mM EDTA, 20% glycerol, 1 mM DTT, 0.25 mM PMSF, aprotinin (5  $\mu$ g/ml),



pepsatin (5 µg/ml), and leupeptin (5 µg/ml) and incubated on ice for 30 min. After a brief vortexing, the lysates were centrifuged (14,000g, 4°C, for 10 min). Supernatants (nuclear extracts) were collected, divided into aliquots, and stored at –80°C for semiquantification of p53 and AP-1 by fluorescent electrophoretic mobility shift assay (fEMSA).

#### *Oligonucleotides and DNA annealing*

Double-stranded fluorescent targets were prepared by mixing equimolar amounts of the two complementary single-stranded oligonucleotides (p53-FW-Cy5 or p53-FW with p53-R, AP-1-FW-Cy5 or AP-1-FW with AP-1-R, and NF-κB-FW with NF-κB-R) as described previously [28].

#### *Protein quantification*

Protein quantification was performed accordingly to the method of Lowry et al. [33], using bovine serum albumin as standard.

#### *Semiquantification of transcriptional activation of lung nuclear proteins by fluorescent electrophoretic mobility shift assay*

The p53- and AP-1-binding assays and respective analysis were performed according to a previously reported method based on the binding of the transcription factors to their specific DNA recognition sequences [28]. Specificity of the DNA–protein complex was confirmed by the addition of a 50-fold excess of either unlabeled specific competitor (SC, specific probe without the Cy5 label) or unlabeled nonspecific competitor (UC, which was the NF-κB unlabeled oligonucleotide, either for AP-1 or p53).

#### *Quantification of caspase activities*

The enzymatic activities of caspases-1, -8, and -3 in lung tissues were evaluated using the commercially available caspase-1, -8, and -3 colorimetric substrates. Briefly, samples were adequately diluted (100 µg protein/well) in buffer (25 mM Hepes, pH 7.4, 0.1 M NaCl, 10% saccharose, supplemented with 10 mM DTT). Triplicate samples were incubated for 90 min in the dark at 37°C with 40 µM of each specific substrate. The cleavage of the substrate peptide by the respective caspases releases the chromophore *p*-nitroanilide, which is quantified spectrophotometrically at 405 nm. The enzymatic activities of caspases-1, -8, and -3 in lung tissue homogenates were expressed as absorbance units ( $U_{\text{abs}}$ )/100 µg protein.

#### *Measurement of cytochrome *c* translocation*

The levels of Cyt *c* in the cytosolic fraction (50 µg protein) were analyzed by Western blot on 12% SDS-polyacrylamide gel under constant current (14–15 mA) according to the conventional methods partially modified by Fuentes et al. [34]. Briefly, separated proteins were electrotransferred (250 mA for 60 min) to PVDF (polyvinylidene difluoride) membranes using a Mini

Trans-Blot Cell apparatus (Bio-Rad). The blots were blocked with 10% nonfat dried milk in TTBS (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.2% Tween 20) at 4°C overnight and then incubated with primary antibody (diluted 1:1000 in TTBS+5% nonfat dried milk) for 1 to 2 h at room temperature. After washing (two times 5 min with TTBS), membranes were incubated (60 min at room temperature) with peroxidase-conjugated secondary antibodies (1:5000 in TTBS with 10% nonfat dried milk). After washing (2×5 min and 1×10 min), bound antibodies were visualized by chemiluminescence using the ECL-Plus reagent.

#### *DNA fragmentation analysis by electrophoresis*

DNA concentrations were evaluated by ultraviolet spectrophotometry at 260 nm. Ten micrograms of DNA aliquots were electrophoresed on 2% agarose gel at 70±2 V for 2 h and stained with 0.8 µg/ml of ethidium bromide. For visualization of apoptotic alterations, DNA bands were observed on a transilluminator and recorded in photographs.

#### *DNA fragmentation analysis by deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate (dUTP) nick end-labeling assay*

DNA strand breaks of the lung tissue were analyzed qualitatively and semiquantitatively, by fluorescence and light microscopy, using a TUNEL assay commercial kit (In SituCell Death Detection Kit; Roche Molecular Biochemicals, Germany), with slight modifications introduced by Correia-da-Silva et al. [35]. Briefly, deparaffinized sections were pretreated with proteinase K (20 µg/ml) in 0.05 M Tris/HCl, pH 7.6 (30 min at 37°C), to break up membranes and free DNA, and then washed in PBS solution. The sections were dried and incubated (1 h, in a humidified chamber, at 37°C) with a reaction mixture containing terminal deoxynucleotidyl transferase and fluorescein-labeled deoxyuridinetriphosphate. Fluorescence photos were taken at this point. In light microscopy, incorporated fluorescein was detected by an anti-fluorescein-antibody conjugated with alkaline phosphatase. The slides were washed with PBS and incubated for 25 min in alkaline phosphatase substrate solution. The reaction was stopped with tap water and slides were counterstained with Mayer's hematoxylin solution (diluted 1:2), and mounted in aqueous medium (Aquatex; Merck, Darmstadt, Germany). TUNEL-positive cells were identified by the presence of red reactivity. Negative controls were prepared without TdT enzyme and sections previously treated with DNase I (100 U; Roche Molecular Biochemicals) were used as positive controls. TUNEL-positive cells (TPC; green points at fluorescence microscopy) were counted/field (magnification 100×) using 5 slides/group and results recorded in a blinded fashion by an experienced histologist.

#### *Statistical analysis*

Results are expressed as mean±SE (standard error). Statistical comparison between groups was estimated using the

nonparametric method of Kruskal-Wallis followed by Dunn's test. In all cases, *P* values lower than 0.05 were considered statistically significant.

## Results

### DNA laddering analysis

Apoptosis can be measured by visualizing the fragmentation of nuclear DNA resulting in the appearance of incrementally sized low-molecular-weight DNA bands on ethidium bromide-stained agarose gels (DNA ladder). In our work, electrophoretic analysis of DNA extracted from whole lung tissue of rats exhibited marked DNA fragmentation in the PQ 48 and 96 h groups (Fig. 2). An increase of smear in the lung samples of the PQ 24 h group was evident, which might be indicative of an increase of DNA fragmentation. In contrast, in the control, NaSAL 24, 48, and 96 h, and in the PQ 24 h groups no DNA laddering was detected. The posttreatment with NaSAL completely prevented PQ-induced DNA fragmentation.

### TUNEL analysis

The TUNEL assay detects nuclear DNA fragmentation by labeling free 3'-OH terminals with dUTP by TdT catalysis (Figs. 3A, 3B, and 3C). TUNEL analysis from whole lung tissue of control and only NaSAL-treated groups revealed very few TPC. Animals from PQ groups exhibited a time-dependent marked DNA fragmentation. The posttreatment with NaSAL of the PQ-exposed animals significantly reduced PQ-induced DNA fragmentation. The green fluorescent points in Fig. 3A correspond mainly to type I and II cell nuclei as suggested by

immunohistochemistry analysis (Fig. 3B). In Fig. 3C, the semiquantification of the TPC obtained at the fluorescence TUNEL assay clearly shows the apoptotic effect of PQ and the healing provided by NaSAL posttreatment.

### Enzymatic activities of the caspases-1, -8, and -3

The enzymatic activities of the caspases-1, -8, and -3 in lungs are presented in Fig. 4. Animals from the PQ group exhibited a significant rise of the activities of both caspase-8 and caspase-3 in lung tissue 24, 48, and 96 h post-PQ exposure, compared with animals from control and NaSAL groups. On the other hand, a statistically significant decrease was observed in the activity of caspase-1 in the lungs of the PQ 24, 48, and 96 h groups, compared to control and NaSAL groups. The post-treatment with NaSAL of rats exposed to PQ completely reverted the PQ-mediated effects to all studied caspases toward values similar to those obtained for NaSAL groups. Also, noteworthy was the increase of caspase-1 in the NaSAL 24 and 48 h groups, caspase-8 in the NaSAL 24, 48, and 96 h groups, and caspase-3 in the NaSAL 24 and 48 h groups compared to the respective control groups.

### Determination of cytochrome *c* concentrations

Cyt *c* is an important apoptogenic factor in the intrinsic apoptotic pathway [13]. We observed a significant increase of cytosolic Cyt *c* concentration as a consequence of PQ exposure, with the maximal induction observed 48–96 h later in comparison to control and NaSAL groups (Fig. 5), suggesting that the intrinsic pathway is involved in PQ apoptosis. Statistically significant decreases in cytosolic Cyt *c* concentrations were observed in animals of NaSAL 24, 48, and 96 h groups compared to control group. Of note was the significant decrease of cytosolic Cyt *c* of PQ+NaSAL groups in comparison not only to PQ-exposed but also to control groups.

### Activation of AP-1

fEMSA was performed to study the effects of PQ in the rat lung expression of AP-1 and p53. As shown in Fig. 6A, PQ induced a significant time-dependent activation of AP-1 in rat lungs (Lanes 2–4) compared to control (Lane 1) and NaSAL groups (Lanes 8–10). The AP-1-binding activity appeared to result from the formation of a single complex or complexes of very similar mobility. Only barely detectable expression levels of AP-1 were observed in whole lung nuclear extracts from the control group. Noteworthy, was also the significant reduction of AP-1 lung activation in the NaSAL 24, 48, and 96 h groups relative to control, the signal being completely absent in these groups. Concerning the PQ+NaSAL 24, 48, and 96 groups (Lanes 11, 12, and 13, respectively), NaSAL treatment resulted in a significant reduction of PQ-induced AP-1 activation, the AP-1 expression being similar to that of the control group. The specificity of the DNA–protein complex was confirmed in the PQ 96 h group by the persistence of the bands in the competition experiment with a 50-fold molar excess of the UC (Lane 6) and

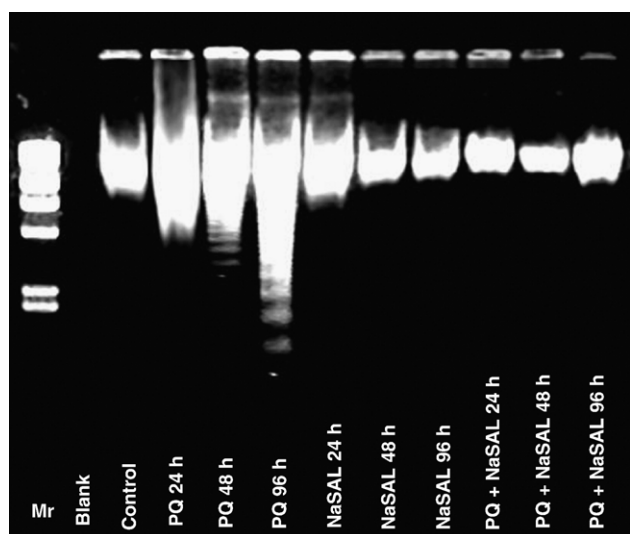
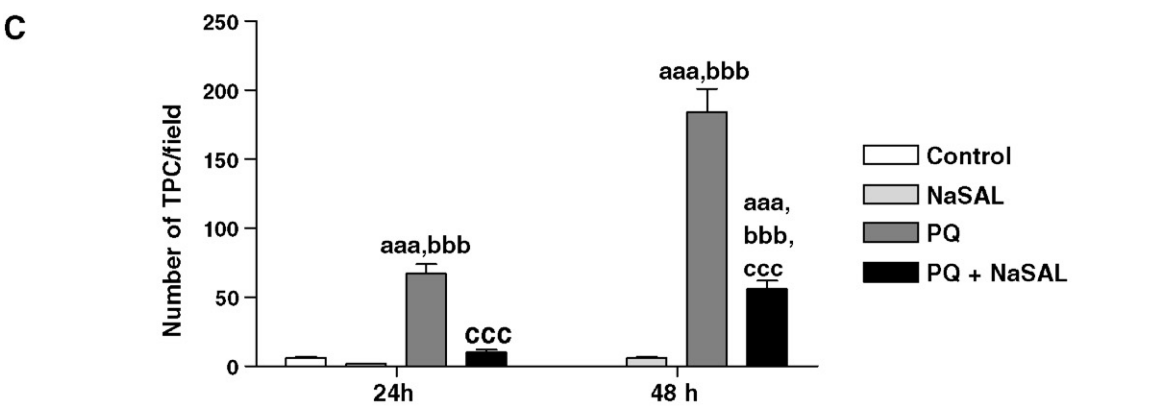
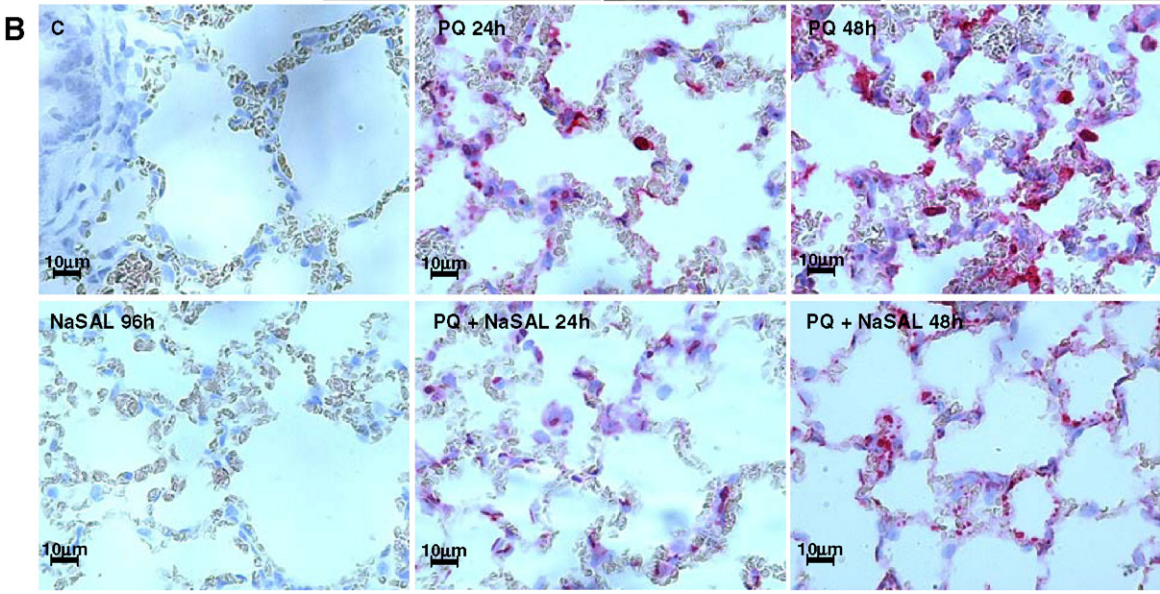
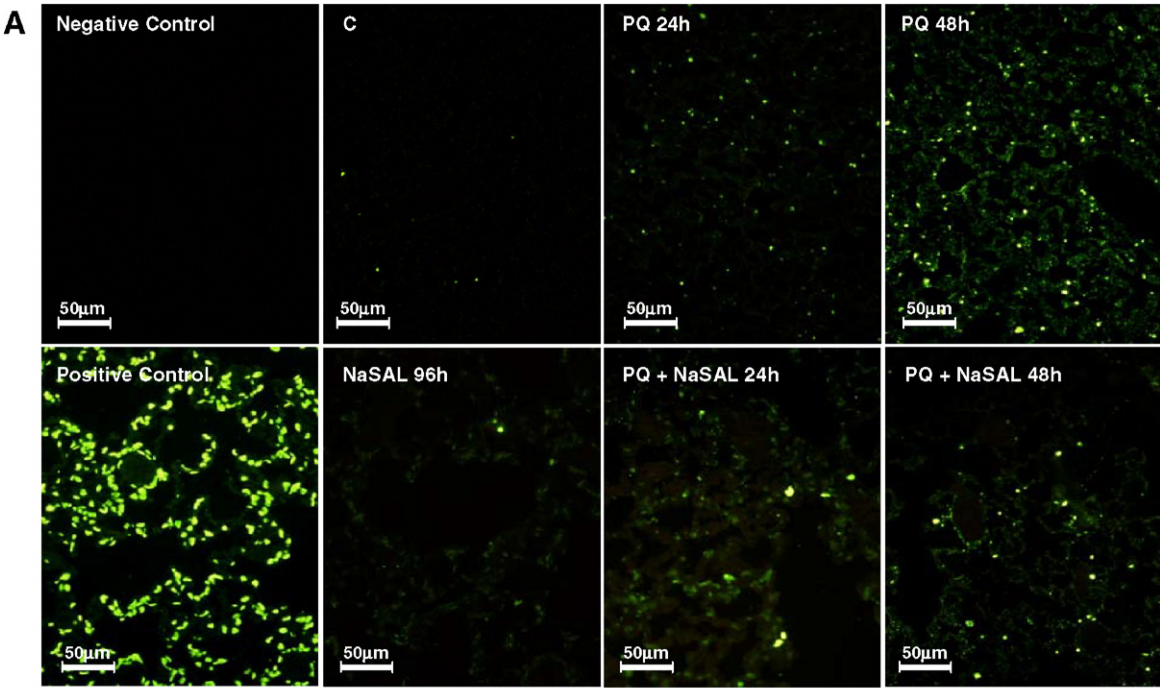


Fig. 2. Representative electrophoretic analysis of the DNA ladder formation in whole lungs of rats from control, sodium salicylate (NaSAL), paraquat (PQ), and paraquat plus sodium salicylate (PQ+NaSAL) groups at three different sample times. Mr indicates the lane of the molecular weight marker. These experiments were repeated using six different lung homogenates with comparable results.





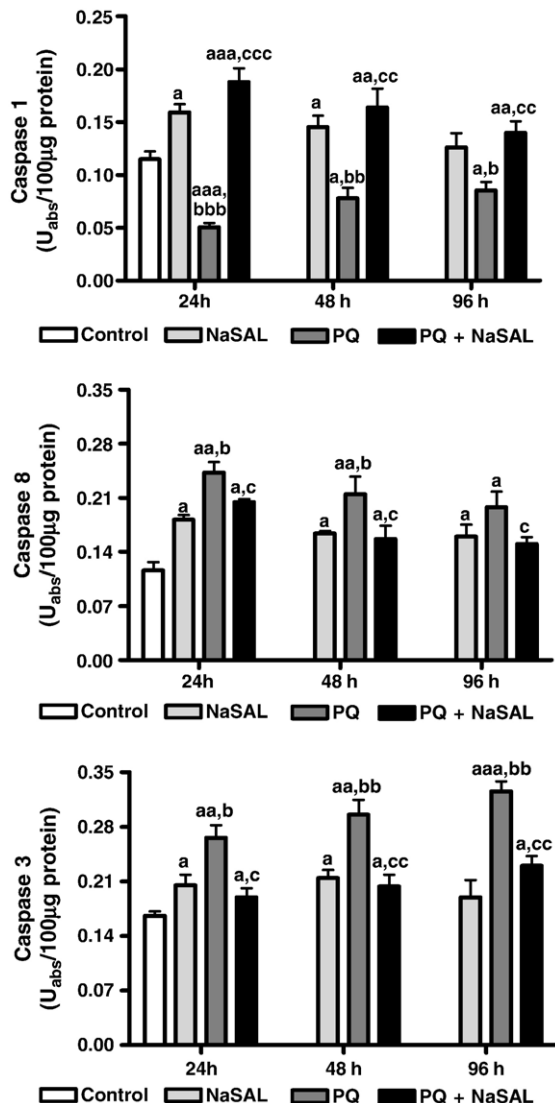


Fig. 4. Activity of the caspases-1, -8, and -3 in the control, sodium salicylate (NaSAL), paraquat (PQ), and paraquat plus sodium salicylate (PQ+NaSAL) groups at three different sampled times. Data are expressed as absorbance units ( $U_{abs}/100 \mu\text{g protein}$ ). Values are given as mean  $\pm$  SE ( $n=6$ ). <sup>a</sup> $P<0.05$ , <sup>aa</sup> $P<0.01$ , and <sup>aaa</sup> $P<0.001$  versus control group, <sup>b</sup> $P<0.05$ , <sup>bb</sup> $P<0.01$ , and <sup>bbb</sup> $P<0.001$  versus NaSAL group, <sup>c</sup> $P<0.05$  and <sup>cc</sup> $P<0.01$  versus PQ group.

by its disappearance in the competition experiment with a 50-fold molar excess of the SC (Lane 7).

#### Activation of p53

Among the molecular markers of apoptosis, the tumor suppressor gene, p53, is known to be a member of the DNA

damage-response pathway. It has been demonstrated that p53 protein is redox-modulated and therefore responsive of oxidative stress [26]. In this study, it was shown that the mechanism underlying PQ-triggered apoptosis implies a dramatic induction of the transcription factor p53 in rat lungs in a time-dependent manner (Lanes 2–4) compared to control (Lane 1) and only NaSAL-treated groups (Lanes 8–10) (Fig. 6B). Lungs from the control group showed basal levels of p53 protein. NaSAL itself caused minor inductions of p53 expression, mainly in NaSAL 24 and 48 h groups. NaSAL, administered 2 h after exposing rats to PQ, attenuated the p53 expression increase in comparison to the PQ-only exposed groups (Lanes 11, 12, and 13, respectively). The specificity of the DNA–protein complex was confirmed in the PQ 96 h group by the persistence of the bands in the competition experiment with a 50-fold molar excess of the UC (Lane 6) and by its disappearance in the competition experiment with a 50-fold molar excess of the SC (Lane 7).

#### Discussion

The present work was undertaken to determine the ability of PQ to induce apoptotic events in Wistar rat lungs and the putative protective effect of NaSAL treatment. It was clearly shown that PQ exposure results in marked lung apoptosis, in a time-dependent manner, characterized by the ladder-like pattern of DNA observed through electrophoresis and by the presence of TPC as revealed by immunohistochemistry. Considering the apoptotic cellular signaling, the two main caspase cascades were studied through the measurement of the cytosolic concentrations of Cyt *c*, and the enzymatic activities of caspases-1, -8, and -3, since the activation of the key executioner, caspase-3, does not provide sufficient information to firmly identify the intrinsic or extrinsic nature of the induced apoptotic pathways [12]. PQ-exposed rats suffered a time-dependent increase of caspase-3 and caspase-8 and a decrease of caspase-1 activities in the lung. Also observed was a marked mitochondrial dysfunction, evidenced by Cyt *c* release, and a transcriptional activation of the p53 and AP-1 transcription factors, in a time-dependent manner as a consequence of PQ exposure. Overall, this work led to a better understanding of the mechanisms induced by the PQ in the respiratory tract, showing that PQ induces several events involved in the apoptotic pathways, which might trigger its lung toxicity. In addition, NaSAL, a recently implemented antidote for PQ intoxications, proved to protect lungs from PQ-induced apoptotic-related toxicity.

The observed marked increase of caspase-8 activity in lungs of the PQ 24, 48, and 96 h groups (Fig. 4) suggests an activation of apoptosis as a consequence of PQ exposure via the extrinsic

Fig. 3. (A) Representative fluorescence photomicrographs of lungs, previously prepared by the TUNEL assay, from control (C), sodium salicylate (NaSAL), paraquat (PQ), and paraquat plus sodium salicylate (PQ+NaSAL) groups at two different sample times. Negative and positive controls are shown in order to certify the performance of method. Samples were analyzed in a drop of PBS under a fluorescence microscope using an excitation wavelength in the range of 450–490 nm and detection in the range of 515–565 nm (green). It is possible to observe a time-dependent increase of green points' density in PQ and PQ plus NaSAL groups, which is more propounded in PQ groups. These green points correspond in B (light photomicrographs of lungs prepared by TUNEL assay) to the red-stained nucleus. The green fluorescent points correspond mainly to type I and II cell nuclei as suggested by immunohistochemistry analysis. (C) TUNEL-positive cells (TPC; green points at fluorescence microscopy) counted/field (magnification 100 $\times$ ). Values are given as mean  $\pm$  SE ( $n=5$  slides/group). <sup>aaa</sup> $P<0.001$  versus control group, <sup>bbb</sup> $P<0.001$  versus NaSAL group, <sup>ccc</sup> $P<0.001$  versus PQ group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



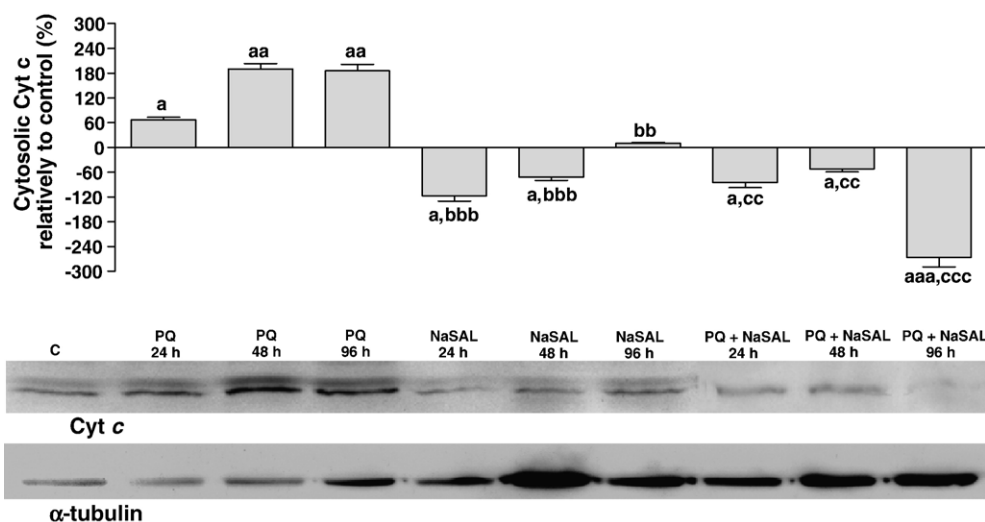


Fig. 5. Immunoblot analysis of the cytochrome *c* (Cyt *c*) release from rat lung mitochondria into the cytosol of the control (C), sodium salicylate (NaSAL), paraquat (PQ), and paraquat plus sodium salicylate (PQ+NaSAL) groups. Blot is representative of six independent experiments.  $\alpha$ -Tubulin Western blot is included as a loading protein control. Values are given as mean  $\pm$  SE ( $n=6$ ). <sup>a</sup> $P<0.05$ , <sup>aa</sup> $P<0.01$ , and <sup>aaa</sup> $P<0.001$  versus control group, <sup>bb</sup> $P<0.01$  and <sup>bbb</sup> $P<0.001$  versus PQ group, <sup>cc</sup> $P<0.01$  and <sup>ccc</sup> $P<0.001$  versus NaSAL group.

receptor-mediated pathway. The increase of caspase-8 activity could be the result of an up-regulation of TNF- $\alpha$  mRNA expression in lung, as already observed by Ishida et al. [36] in PQ-exposed mice. Also, according to data reported in the literature, the increase of caspase-8 activity observed in NaSAL 24, 48, and 96 h groups could be explained by its ability to induce apoptosis via death receptors, mainly through TRAIL [37]. NaSAL-elicited protection of lungs against PQ-induced apoptosis may be partially due to the blockade of the extrinsic pathway. By its own, NaSAL has been shown to blunt the increase in TNF- $\alpha$  mRNA and to reduce the serum TNF- $\alpha$  protein level in mice [38]. Corroborating our results, Guthmann et al. showed that eliminating the TNF- $\alpha$  effect in vivo by anti-TNF- $\alpha$  antibodies prevented the proapoptotic sensitization of type II cells and consequently the activity of caspase-8 and caspase-3 [39]. In our study, PQ caused as well a significant release of Cyt *c* into the cytosolic fraction of the lung cells, thereby indicating the potential activation of the mitochondrial intrinsic pathway (Fig. 5). Since, in mitochondria, Cyt *c* resides as soluble protein in the intermembrane space, functioning as an electron carrier in oxidative phosphorylation, the significant release of Cyt *c* might be the result of mitochondrial dysfunction and reduction of mitochondrial transmembrane potential as a consequence of PQ toxicity [31]. On the other hand, lung samples of animals from NaSAL 24, 48, and 96 h groups exhibited a significant decrease of Cyt *c* release. In agreement with these results, a decrease of Cyt *c* release was observed in animals from PQ+NaSAL 24, 48, and 96 h groups compared to controls and to PQ-only exposed animals. We previously showed that the majority of pneumocytes exhibited mitochondrial swelling as being the most frequent histopathological alteration of PQ exposure [28–30], which gives credit to the involvement of this organelle in PQ cytotoxicity. By a different mechanism, the results of McCarthy et al. also showed the role of mitochondrial impairment in PQ toxicity, largely as a

consequence of ROS generation [5]. These authors showed that pretreatment with the antioxidant coenzyme Q10, before PQ exposure, was able to inhibit ROS generation from isolated mitochondria as well as the collapse of mitochondrial membrane potential. In accordance with the Cyt *c* results, we previously demonstrated that the occurrence of the above referred mitochondrial alterations was drastically attenuated in the PQ+NaSAL 24, 48, and 96 h groups, compared to the PQ-only exposed animals [28]. To confirm the involvement of caspase-3 in PQ-induced apoptosis, we determined its lung activity. As observed in Fig. 4, lung caspase-3 activity increased significantly 24, 48, and 96 h after PQ exposure compared to controls, thereby suggesting the activation of the apoptotic machinery. Although to a lower extent, NaSAL itself also induced caspase-3 activation, which is in accordance with previously reported results [40]. Noteworthy is the reduced activity of caspase-3 in PQ+NaSAL 24, 48, and 96 h groups compared to PQ-only exposed groups. There was a positive correlation among the decrease of enzymatic activity of the initiator caspase-8 and the decrease of Cyt *c* release in PQ+NaSAL 24, 48, and 96 h groups, and the respective decrease of effector caspase-3 activity. Taken together, it may be considered that PQ exposure activates caspase-3 activity as a consequence of the activation of the active initiator caspase-8 and via Cyt *c* release. Thus, it is legitimate to speculate that the strong protection (which ended up in full survival) conferred by NaSAL to PQ-intoxicated rats [28], besides inhibition of NF- $\kappa$ B activation, MPO activity and platelet aggregation, and scavenging of ROS, also results from the blockade of the intrinsic and extrinsic apoptotic pathways.

Caspase-1 activity was diminished in rats from PQ 24, 48, and 96 h groups compared to control or NaSAL-only treated animals (Fig. 4). Since Tomita et al. [41] observed an increase of IL-1 $\beta$  in lung tissue of rats exposed to PQ and its involvement in pulmonary inflammation, we hypothesize that the decrease of

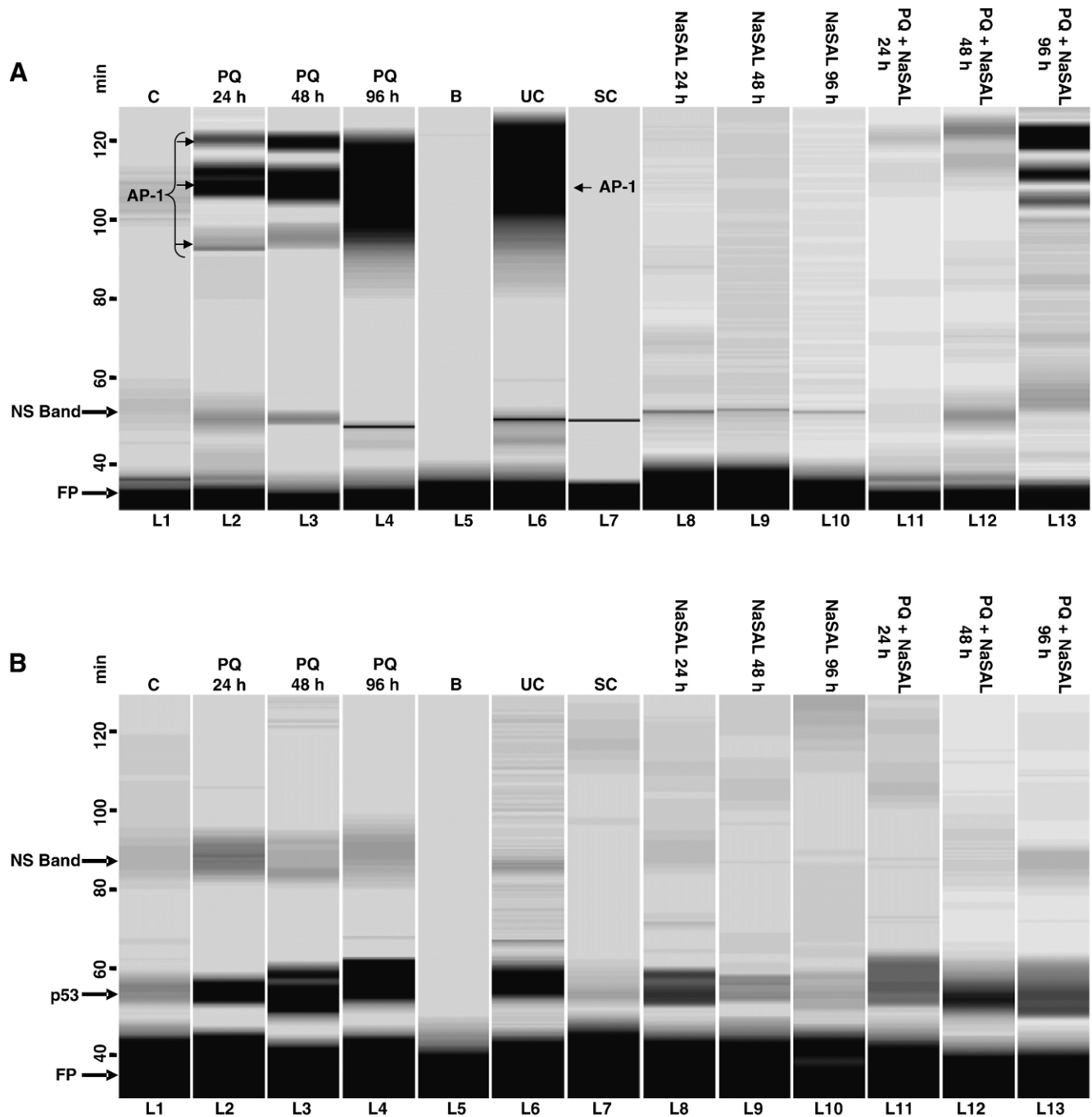


Fig. 6. Representative fEMSA gel views of activator protein-1 (AP-1) (A) and p53 (B) activation induced by PQ in lungs at 24, 48, and 96 h. Nuclear extracts from the different groups were prepared and subjected to fEMSA as described under Materials and Methods. Lane 1, control group; Lane 2, PQ 24 h; Lane 3, PQ 48 h; Lane 4, PQ 96 h; Lane 5, blank; Lane 6, competition experiment with a 50-fold molar excess of a nonspecific competitor (UC) compared to specific probe (SP); Lane 7, competition experiment with a 50-fold molar excess of a specific competitor (SC, unlabeled specific probe) compared to SP; Lanes 8, 9, and 10, sodium salicylate (NaSAL) 24, 48, and 96 h groups, respectively; Lanes 11, 12, and 13, PQ+NaSAL 24, 48, and 96 h groups, respectively. The positions of specific AP-1/DNA-binding complexes (bands 1–3) and p53/DNA-binding complexes are indicated in A and B, respectively. NS band represents nonspecific binding. The localization of the free probe (FP) is also indicated. The results presented in A and B are representative of six independent experiments.

caspase-1 activity observed in rats of PQ 24, 48, and 96 h groups could be the consequence of antiapoptotic IL-1 $\beta$  effects. These data are of particular relevance to neutrophils, because they express caspase-1 [42] and exhibit delayed apoptosis following exposure to exogenous IL-1 $\beta$  [43] or to LPS, the latter effect being in part mediated via autocrine production of

IL-1 $\beta$  [42]. This process is also important for the normal resolution of inflammation in tissues, because it leads to recognition and clearance of the apoptotic neutrophils by macrophages [44]. Inhibition of caspase-1 activity as the consequence of PQ exposure might thus imply higher recruitment and permanence of inflammatory cells, namely neutrophils, which will not be

removed from lungs by apoptosis. One of the main and interesting results concerning the inclusion of NaSAL in the therapy of rats intoxicated by PQ was the increase of caspase-1 activity compared to PQ-only exposed and control groups. We have previously shown, both by assessing the MPO activity and by histopathological studies, the widespread infiltration of neutrophils and macrophages in the lungs of PQ-only exposed rats [28–30] as well as its reduction in PQ+NaSAL groups [28]. Considering the involvement of caspase-1 in neutrophil apoptosis, our results suggest that the increase of caspase-1 activity is a possible explanation for the decrease of neutrophil lung infiltration in PQ+NaSAL groups [28].

In most cells, the morphological and biochemical features of apoptosis seem to be associated with the cleavage of genomic DNA into large fragments and later into oligonucleosomal fragments by a nuclease which is activated exclusively in apoptosis [45]. With reference to our electrophoretic results (Fig. 2), we only observed DNA fragmentation in PQ 48 and 96 h groups (a characteristic ladder-like pattern of DNA), although an increase of smear was already observed in the PQ 24 group. Using TUNEL assay techniques, it was also possible to observe a marked increase of apoptosis of lung cells as a consequence of PQ exposure, in a time-dependent manner (Figs. 3A, 3B, and 3C), as well as its remission by NaSAL. The reason why the activation of caspase-8 (24, 48, and 96 h), caspase-3 (24 and 48 h), and caspase-1 (24 and 48 h) in only NaSAL-treated groups did not induce apoptosis of lung cells visualized by DNA laddering and/or TUNEL assay is not clear. Although it is a widely accepted concept that activation of caspase-3 marks the “point of no return” in the pathway to apoptotic death of mammalian cells, our results could not corroborate that. Noteworthy, Guthmann et al. [39] did not find apoptosis, either in lung tissue or in freshly isolated type II cells in response to sublethal hyperoxia despite the significant activation of caspases-8 and -3. They concluded that the increase of caspases-8 and -3, in response to sublethal hyperoxia, did not mark the point of no return. The same conclusion could be ascertained considering our results. This interpretation of our findings is also strongly corroborated by Perfettini and Kroemer [46], who postulated that caspase activation is not synonymous of apoptotic demise. In addition, it could not be excluded that the extent of caspase-3 activation, 1.23- and 1.29-fold in the NaSAL 24 and 48 h groups, respectively, in comparison with control group, is not high enough to induce apoptosis, although a 1.62-, 1.78-, and 1.96-fold increase of caspase-3 in the PQ 24, 48, and 96 h groups, respectively, in comparison with control group, results in apoptosis in lung cells. Thus, we hypothesize that caspase-3 activation is then followed by apoptosis, when its activation occurs via strong mitochondrial damage resulting in Cyt *c* release in PQ-induced pulmonary ROS toxicity. This concept is supported by recent findings showing that mitochondrial Cyt *c* release is a key event in hyperoxia-induced lung injury [47].

By using fEMSA, we demonstrated that PQ exposure led to an increase of AP-1 DNA binding in lungs (Fig. 6A). Similar results were also demonstrated in vitro by Chen and Sun [48] and Li and Sun [49] using PC12 cells, and by Zhou et al. [50] in

skeletal muscle cells. These results are, to some extent, similar to the effects observed for NF- $\kappa$ B [28], although with slower kinetics, which is consistent with the mode of activation requiring de novo synthesis of fos and jun subunits [25]. In accordance with data reported in the literature, where NaSAL proved to inhibit the transactivation of AP-1 [51], we also observed that NaSAL itself reduces AP-1 activation. In agreement, NaSAL, given 2 h after PQ, inhibited the increase of AP-1 DNA-binding activity induced by PQ. Since the promoter regions of many inflammatory cytokines and chemokines (e.g., TNF- $\alpha$  and IL-1 $\beta$ ) contain AP-1-binding sites [52], the inhibition of AP-1 activation by NaSAL contributes to protect lungs against oxidative stress-induced inflammation. Moreover, AP-1 is involved in the regulation of antioxidant enzymes by the presence of AP-1-response elements in the promoter regions of genes encoding glutathione peroxidase (GPx) and catalase (CAT) [53]. We previously observed that NaSAL attenuates the increase of CAT and GPx activities near to the levels of control and NaSAL groups and that it was possible to establish a relationship between NF- $\kappa$ B expression and CAT and GPx activities [28]. The same correlation could be ascertained for AP-1. Nevertheless, the question of whether or not AP-1 activation plays an essential role on PQ-induced apoptosis still needs to be addressed.

Besides AP-1, PQ also induced an increase of p53 expression in lungs (Fig. 6B). In accordance with our results, it was previously demonstrated that ROS play several distinct roles in the p53 pathway, such as being important activators of p53 expression through their capacity to induce DNA strand breaks, and also by regulating the DNA binding of p53 [26], since p53 protein contains a DNA-binding domain structure that depends on the binding of zinc to critical redox-sensitive cysteines [54]. In agreement with AP-1 discussion, GPx activity is also transcriptionally activated by p53 [55]. Our findings are corroborated by the results of previous studies designed to investigate the role of p53 in the progression of PQ-induced apoptosis [56]. These authors used two cell lines, wild-type p53-expressing human lung epithelial-like cell line (L132) and a p53-deficient human promyelocytic leukemia cell line (U937), and explored the linkage among p53, DNA damage, and apoptosis. Following PQ exposure of L132 cells, the percentage of S-phase cells decreased significantly and the expression of p53 protein increased, suggesting that entry into S-phase from G1-phase was blocked. U937 cells showed complete resistance to PQ. Those results suggested that PQ-induced DNA damage caused G1 arrest and apoptosis only in L132 cells, and that p53 protein accumulation was required for the induction of apoptosis by PQ. In addition, TNF- $\alpha$ , which is induced by PQ (see above) has also been described as enhancing p53 mRNA expression, through the induction of the NF- $\kappa$ B [57]. On the other hand, the inclusion of NaSAL in the therapeutic regime of PQ-intoxicated rats decreased PQ-induced p53 expression. This might be the result of salicylates being important hydroxyl radical (HO $\cdot$ ) scavengers [58]. Our hypothesis is that HO $\cdot$  works as a messenger for the activation of this tumor suppressor protein. Nevertheless, the slight increase of p53 expression observed in NaSAL 24 and 48 h groups



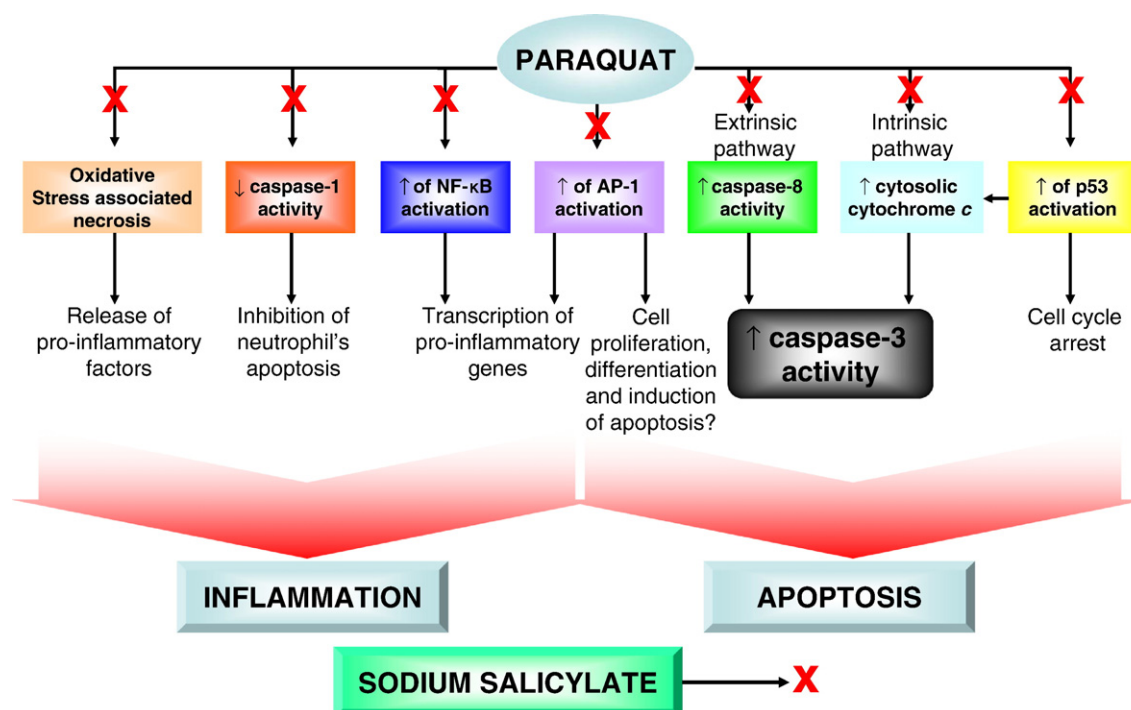


Fig. 7. Schematic illustration overview of the multiple toxic acute signals induced by paraquat in the lungs and the prevention obtained by sodium salicylate treatment. AP-1, activator protein-1; NF-κB, nuclear factor kappa-B; p53, tumor suppressor protein.

compared to control should not be dismissed. It might be the result of the NF-κB inhibition [28], since NF-κB inhibition has been correlated to the enhancement of p53 expression [59,60].

In conclusion, our results demonstrate that PQ causes apoptosis by Cyt *c* release, increase of caspase-3 and -8 activity, decrease of caspase-1 activity, and increase of p53 and AP-1 expression, resulting in DNA fragmentation (Fig. 7). Treatment with NaSAL of PQ-intoxicated rats blocked to some extent these events, with the consequent abolition of DNA fragmentation. In view of our results, it is plausible to conclude that a high-dose therapy with NaSAL, starting as soon as possible after PQ intoxication, may constitute a promising treatment of PQ poisonings, not only as the consequence of the effective inhibition of proinflammatory factors such as NF-κB, scavenging ROS, inhibition of MPO, and inhibition of platelet aggregation [28], but also by its potential beneficial effects at the apoptotic pathways (Fig. 7). Despite the relevance of each beneficial effect, it seems logical that this results from a multiprotective action of NaSAL. This is important, since NaSAL was previously shown to protect lungs of PQ-challenged rats, as confirmed by an amelioration of practically all toxicological parameters, which ended up in the achievement of full survival of the tested animals [28]. The present data reinforce the potential use of this interesting molecule in the protection against PQ-induced lung damage.

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