ANTIOXIDANTS & REDOX SIGNALING
Volume 6, Number 4, 2004

Forum Original Research Communication

Superoxide-Related Signaling Cascade Mediates Nuclear Factor-κB Activation in Acute Inflammation

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ABSTRACT

The nuclear factor-κB (NF-κB) is a transcription factor that plays a pivotal role in the induction of genes involved in physiological processes, as well as in the response to inflammation. In this study, we used a selective nonpeptidyl superoxide dismutase mimetic, M40403, to investigate the role of superoxide anion in NF-κB activation during acute inflammation in mice. Injection of carrageenan into the pleural cavity of mice induced an acute inflammatory response characterized by fluid accumulation in the pleural cavity that contained a large number of neutrophils, as well as an increased production of tumor necrosis factor-α and interleukin-1β. All parameters of inflammation were attenuated by M40403 (10 mg/kg i.p., 30 min prior to carrageenan administration). These inflammatory events were associated with the activation of NF-κB in the lung. In particular, the appearance of inhibitory protein κB-α (IκB-α) in homogenates of lung tissues was investigated by immunoblot analysis at 4 h after carrageenan administration. IκB-α levels were substantially reduced in the lung tissue from carrageenan-treated mice in comparison with sham-treated mice. Furthermore, to detect NF-κB/DNA binding activity, whole extracts from lung tissue of each mouse were analyzed by electrophoretic mobility-shift assay. The DNA binding activity significantly increased in whole extracts obtained from lung tissues of vehicle-treated mice 4 h after carrageenan administration. Treatment of mice with M40403 caused a significant inhibition of carrageenan-induced IκB-α degradation and NF-κB/DNA binding activity. These data confirm that M40403 exerts a potent antiinflammatory activity and clearly demonstrate that the reduction of the inflammatory process is associated with modification of the activation of signal transduction pathways. *Antioxid. Redox Signal.* 6, 699–704.

INTRODUCTION

THE LOCAL AND SYSTEMIC INFLAMMATORY RESPONSE is also associated with the production of reactive oxygen species (ROS), such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and peroxynitrite (13, 26, 41). Some important proinflammatory roles for O_2^- include endothelial cell damage and increased microvascular permeability (15, 20, 39), formation of chemotactic factors such as leukotriene B_4 (16, 24), recruitment of neutrophils at sites of inflammation (8, 32), lipid peroxidation and oxidation, DNA single-strand damage (14), and formation of peroxynitrite, a potent cytotoxic and proinflammatory molecule (3, 4, 21, 32, 34).

Various studies have clearly demonstrated that nuclear factor κB (NF-κB) plays a central role in the regulation of many genes responsible for the generation of mediators or proteins in inflammation *e.g.,* tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), VCAM-1, ICAM-1, or inducible nitric oxide synthase. Under normal conditions, NF-κB is present within the cytoplasm in an inactive state, bound to its inhibitory protein κB-α (IκB-α). However, an inflammatory stimulation initiates an intracellular signaling cascade, resulting in the

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phosphorylation of IκB-α on serine residues 32 and 36 by IκB kinase. Once liberated from its inhibitory protein, NF-κB translocates to the nucleus, where it orchestrates the transcription of a number of proinflammatory genes (29). Therefore, NF-κB is an attractive candidate to mediate the effects of ROS because the interaction between the inhibitory protein IκB and NF-κB is regulated by protein kinases that contain several redox-sensitive cysteine residues in critical kinase domains (9, 18, 38). We have shown that in models of acute and chronic inflammation, removal of O_2^- with selective superoxide dismutase mimetics such as M404043 inhibits neutrophil infiltration at the site of damage and cytokine release (33). The mechanism by which O_2^-modulates these events is not known, although a likely possibility is the activation of known redox-sensitive transcription factors, including as indicated above NF-κB. In this study, using a well characterized model of acute inflammation (namely, pleurisy), we have evaluated the relationship between O_2^- NF-κB, neutrophil infiltration, and cytokine release. The results of our studies suggest that one mechanism through which O_2^- influences the inflammatory response is the activation of the redox-sensitive transcription factor NF-κB.

MATERIALS AND METHODS

Animals

Male CD mice (weight 14–36 g; Charles River, Milan, Italy) were used in these studies. Animals were kept in a controlled environment and allowed access to rodent chow and water *ad libitum*. 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 current European Union regulations (O.J. of E.C. L 358/1 12/18/1986).

Experimental groups

Mice were randomly allocated into the following groups: (a) *Carrageenan + vehicle group*. Mice were subjected to carrageenan-induced pleurisy and received the vehicle for M40403 [26 m*M* sodium bicarbonate buffer (pH 8.1–8.3)] 30 min prior to carrageenan ($n = 10$), (b) *M40403 group*. Same as the Carrageenan + vehicle group, but mice were administered M40403 (10 mg/kg i.p. bolus) 30 min prior to carrageenan ($n = 10$), (c) *Sham + saline group.* Sham-operated group in which mice were subjected to the identical surgical procedures as the carrageenan group, except that saline was administered instead of carrageenan ($n = 10$); (d) *Sham + M40403 group.* Identical to Sham + saline group except for the administration of M40403 (10 mg/kg i.p. bolus) 30 min prior to identical surgical procedures ($n = 10$).

Carrageenan-induced pleurisy

Mice were anesthetized with isoflurane and submitted to a skin incision at the level of the left sixth intercostal space. The underlying muscle was dissected, and saline (0.2 ml) or saline containing 1% λ-carrageenan (0.2 ml) was injected into the pleural cavity. The skin incision was closed with a suture, and the animals were allowed to recover. At 4 h after the injection of carrageenan, the animals were killed by inhalation of CO_2. The chest was carefully opened and the pleural cavity rinsed with 2 ml of saline solution containing heparin (5 U/ml) and indomethacin (10 μg/ml). The exudates and washing solution were removed by aspiration and the total volume measured. Any exudate that was contaminated with blood was discarded. The amount of exudates was calculated by subtracting the volume injected (2 ml) from the total volume recovered. The leukocytes in the exudates were suspended in phosphate-buffered saline (PBS) and counted with an optical microscope in a Burker's chamber after vital trypan blue staining.

Preparation of cytosolic fractions and nuclear extracts

The animals were killed after the experimental procedure by inhalation of CO_2, and the lung tissue was removed, immediately frozen in liquid nitrogen, and stored at −80°C until protein extraction.

All extraction procedures were performed on ice with ice-cold reagents. Tissues were washed twice in PBS (ICN Biomedicals, Milan, Italy), and cytosolic extracts were prepared by homogenizing the tissues in an extraction buffer [10 m*M* HEPES (pH 7.9), 10 m*M* KCl, 0.1 m*M* EDTA, 0.1 m*M* EGTA, 1 m*M* DL-dithiothreitol (DTT), 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF), 15 μg/ml soybean trypsin inhibitor, 3 μg/ml pepstatin A, 2 μg/ml leupeptin, 40 μ*M* benzamidine (all from Sigma, Milan, Italy)] and incubating on ice for 15 min. After centrifugation at 13,000 *g* at 4°C for 5 min, the supernatant containing the cytosolic fraction was collected, and protein concentration was determined by the Bio-Rad protein assay kit (Bio-Rad, Milan, Italy); then it was aliquoted and stored at −80°C. The nuclear pellet was resuspended in a high-salt extraction buffer [20 m*M* HEPES (pH 7.9), 420 m*M* NaCl, 1.5 m*M* $MgCl_2$, 0.1 m*M* EDTA, 0.1 m*M* EGTA, 1% (vol/vol) Nonidet P-40, 25% (vol/vol) glycerol, 1 m*M* DTT, 0.5 m*M* PMSF, 15 μg/ml soybean trypsin inhibitor, 3 μg/ml pepstatin A, 2 μg/ml leupeptin, 40 μ*M* benzamidine] and incubated under continuous shaking at 4°C for 30 min. The nuclear extract was then centifuged for 15 min at 13,000 *g*. The supernatant was collected, protein concentration was determined by the Bio-Rad protein assay kit, and then it was aliquoted and stored at −80°C.

Western blot analysis of IκB-α

Immunoblotting analysis of IκB-α proteins was performed on cytosolic fractions. In brief, protein concentration was determined, and equivalent amounts (50 μg) for each sample were mixed with gel loading buffer [50 m*M* Tris, 8% (wt/vol) sodium dodecyl sulfate, 10% (wt/vol) glycerol, 10% (vol/vol) 2-mercaptoethanol, 0.008% (wt/vol) bromophenol blue] in a ratio of 1:1, boiled for 3 min, and electrophoresed on a 12% (wt/vol) discontinuous polyacrylamide gel. The proteins were transferred onto nitrocellulose membranes, saturated by incubation for 3 h at room temperature with 10% (wt/vol) nonfat dry milk in PBS 0.1% Triton X-100, and then incubated with anti-IκB-α antibody (1:500) (sc-371, Santa

Cruz Biotechnology, Milan, Italy) overnight at 4°C. The membranes were washed three times with 0.5% Triton X-100 in PBS and then incubated with anti-rabbit immunoglobulins coupled to peroxidase (1:10,000) (Amersham). The immunocomplexes were visualized by the ECL chemiluminescence method (Amersham, Milan, Italy). Subsequently, the relative expression of the proteins was quantified by densitometry scanning of the x-ray films with GS-700 Imaging Densitometer (Bio-Rad, Milan, Italy) and a computer program (Molecular Analyst, IBM). β-Actin (Sigma) western blot analysis was performed to ensure equal sample loading.

Electrophoretic mobility-shift assay (EMSA)

Double-stranded oligonucleotides containing the NF-κB recognition sequence (5′-GAT CGA GGG GAC TTT CCC TAG-3′) were end-labeled with [γ-^{32}P]ATP (ICN Biomedicals). Aliquots of nuclear extracts (20 μg of protein for each sample) were incubated for 30 min with radiolabeled oligonucleotides (2.5–5.0 × 10^4 cpm) in 20 μl of reaction buffer containing 2 μg of poly(dI-dC), 10 m*M* Tris-HCl (pH 7.5), 100 m*M* NaCl, 1 m*M* EDTA, 1 m*M* DTT, 1 mg/ml bovine serum albumin, and 10% (vol/vol) glycerol. The specificity of the DNA/protein binding was determined for NF-κB by competition reaction in which a 50-fold molar excess of unlabeled wild-type, mutant, or Sp-1 oligonucleotide was added to the binding reaction 10 min before addition of radiolabeled probe. Protein–nucleic acid complexes were resolved by electrophoresis on 4% nondenaturing polyacrylamide gel in 0.5 × Tris–borate–EDTA buffer at 150 V for 2 h at 4°C. The gel was dried and autoradiographed with an intensifying screen at −80°C for 20 h. Subsequently, the relative bands were quantified by densitometry scanning of the x-ray films with GS-700 Imaging Densitometer (Bio-Rad) and a computer program (Molecular Analyst, IBM).

Measurement of cytokines

TNF-α and IL-1β levels were evaluated in the exudates at 4 h after the induction of pleurisy by carrageenan injection. The assay was carried out by using a colorimetric, commercial ELISA kit (Calbiochem–Novabiochem Corp., San Diego, CA, U.S.A.).

Data analysis

All values in the figures and text are expressed as means ± SEM of *n* observations. For the *in vivo* studies, *n* represents the number of animals studied. The results were analyzed by one-way ANOVA followed by a Bonferroni *post-hoc* test for multiple comparisons. A *p* value of <0.05 was considered significant.

RESULTS

The development of carrageenan-induced pleurisy is attenuated in M40403-treated mice

All mice that had received carrageenan developed an acute pleurisy, producing 0.75 ± 0.02 ml of turbid exudates (Fig. 1A). When compared with the number of cells collected from the pleural space of sham-operated mice, injection of carrageenan induced a significant increase in the number of polymorphonuclear neutrophils (PMNs) (Fig. 1B). Pretreatment of mice with M40403 showed a significant attenuation of the pleural exudates, as well as the number of PMNs within the exudates (Fig. 1). No significant exudates and no significant increase in the number of PMNs were observed in the pleural cavity of sham-operated mice.

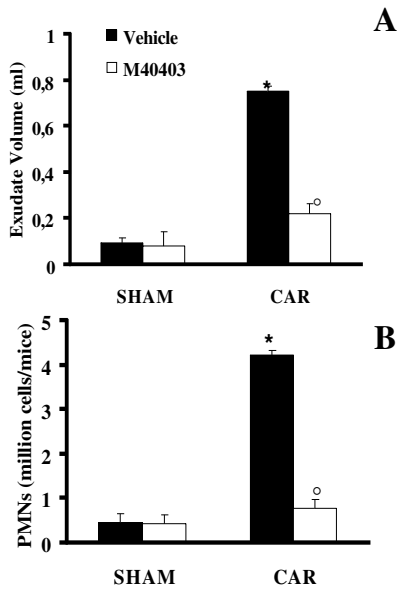

FIG. 1. Effect of M40403 on carrageenan (CAR)-induced inflammation. The increase in volume exudate (**A**) and accumulation of PMNs (**B**) in pleural cavity at 4 h after carrageenan injection were inhibited by M40403 (10 mg/kg i.p.). Each value is the mean ± SEM for $n = 10$ experiments. *$p < 0.01$ versus sham; °$p < 0.01$ versus carrageenan-treated in the absence of M40403.

Effect of M40403 on cytokine increase

The levels of TNF-α and IL-1β were significantly elevated in the exudates from vehicle-treated mice at 4 h after carrageenan administration (Fig. 2). In contrast, the levels of these cytokines were significantly lower in carrageenan-treated mice treated with M40403 (Fig. 2). No significant cytokine increase was observed in the exudates of sham-operated mice.

Effect of M40403 on IκB-α degradation

To elucidate whether induction of pleurisy by carrageenan leads to the degradation of IκB-α in the lung tissue, we have investigated the cytosolic levels of IκB-α by immunoblotting analysis. A basal level of IκB-α was detectable in the cytosolic fraction of lung tissue obtained from normal mice (Fig. 3), whereas in lungs from carrageenan-treated mice IκB-α levels were significantly reduced (Fig. 3). M40403 pretreatment significantly prevented IκB-α degradation following pleurisy induction (Fig. 3). β-actin western blot analysis was performed to ensure equal sample loading (Fig. 3).

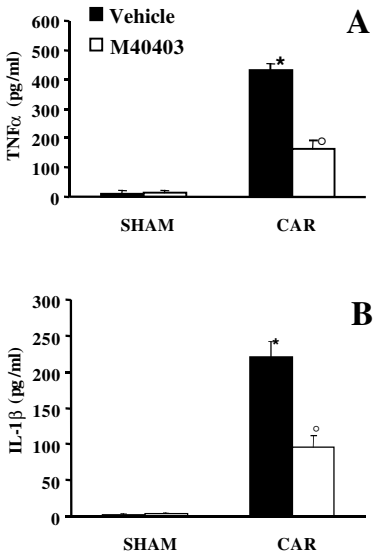

FIG. 2. Pleural injection of carrageenan (CAR) caused at 4 h an increase in the release of the cytokines TNF-α (A) and IL-1β (B). M40403 (10 mg/kg i.p.) inhibited TNF-α and IL-1β production. Each value is the mean ± SEM for $n = 10$ experiments. $*p < 0.01$ versus control; and $°p < 0.01$ versus carrageenan-treated in the absence of M40403.

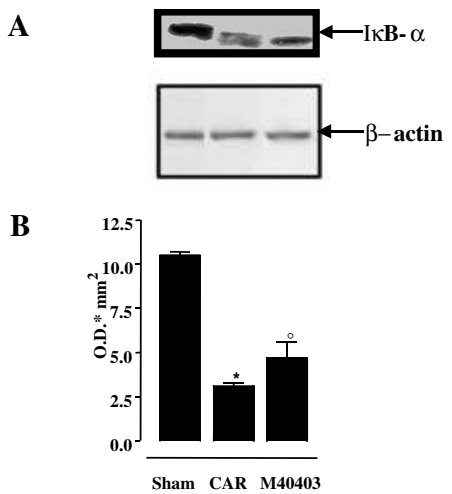

FIG. 3. Effect of M40403 on IκB-α degradation. Western blot analysis (**A**), as well as densitometric analysis (**B**), shows the effect of M40403 on degradation of IκB-α in lung tissue collected at 4 h after carrageenan administration. Sham: basal level of IκB-α band was present in the tissue from sham-treated mice. CAR: IκB-α band has disappeared in the lung tissue from carrageenan-treated mice. M40403: IκB-α band is not completely modified in the lung tissue from carrageenan-treated mice that received M40403. β-Actin western blot analysis was performed to ensure equal sample loading. The data illustrated are representative of a total of three separate experiments carried out on each lung tissue collected from all the mice ($n = 10$).

Effect of M40403 on NF-κB activation in inflamed lungs

To detect NF-κB/DNA binding activity, nuclear extracts from lung tissue of each mouse were analyzed by EMSA. A low basal level of NF-κB/DNA binding activity was detected in nuclear proteins from tissue of untreated mice (naive). The DNA binding activity significantly increased in nuclear extracts obtained from inflamed lungs of control animals 4 h after carrageenan injection. Pretreatment of mice with M40403 (10 mg/kg) caused a significant inhibition of carrageenan-induced NF-κB/DNA binding activity as revealed by specific EMSA (Fig. 4). The specificity of NF-κB/DNA binding complex was demonstrated by the complete displacement of the NF-κB/DNA binding in the presence of a 50-fold molar excess of unlabeled NF-κB probe (W.T. 50x) in the competition reaction. In contrast, a 50-fold molar excess of unlabeled mutated NF-κB probe (Mut 50x) or Sp-1 oligonucleotide (Sp-1 50x) had no effect on this DNA-binding activity (Fig. 4).

DISCUSSION

Several previous studies have suggested that oxidative stress may reflect the inflammatory component of lung inflammation (31).

One consequence of increased oxidative stress is the activation and inactivation of redox-sensitive proteins (37). Re-

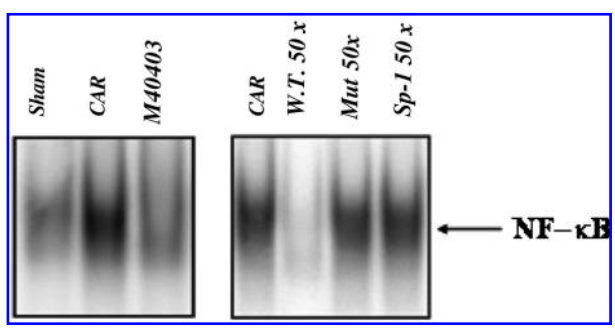

FIG. 4. Effect of M40403 on NF-κB/DNA binding activity in mouse lung. Whole extracts from carrageenan-treated (CAR) or vehicle-treated (sham) mouse lung were prepared as described in Materials and Methods and incubated with ^{32}P-labeled NF-κB probe. Representative EMSA of NF-κB shows the effect of M40403 (M40403) on NF-κB/DNA binding activity evaluated in lung tissue 4 h after carrageenan administration. In competition reaction, whole-cell extracts were incubated with radiolabeled NF-κB probe in the absence or presence of identical but unlabeled oligonucleotides (W.T. 50x), mutated nonfunctional κB probe (Mut 50x), or unlabeled oligonucleotide containing the consensus sequence for Sp-1 (Sp-1 50x). Data illustrated are from a single experiment and are representative of three separate experiments.

cent evidence suggests that the activation of NF-κB may also be under the control of oxidant/antioxidant balance (19, 35). This hypothesis is based primarily on the observation that low doses of peroxides, including H_2O_2 and *tert*-butyl hydroperoxide, can induce NF-κB activation, whereas some antioxidants prevent it (10, 25). NF-κB is normally sequestered in the cytoplasm, bound to the regulatory protein IκB. 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 (9). The net result is the release of the NF-κB dimer, which is then free to translocate into the nucleus. The exact mechanisms by which M40403 suppresses NF-κB activation in inflammation are not known. However, in this study, the transient loss of IκB-α, which occurs in lung tissue from carrageenan-treated mice, was significantly reduced by M40403 treatment, suggesting that M40403 may also inhibit NF-κB activation via a significant prevention of IκB-α degradation. This is in agreement with a recent study that demonstrated that M40403 prevents *N*-methyl-D-aspartate-induced NF-κB activation *in vitro* (27). Most notably, we report here that M40403 prevents the nuclear translocation of NF-κB in the lung of mice subjected to experimental pleurisy. These results support the view that carrageenan-induced pleurisy leads to the activation of NF-κB (at least in macrophages and epithelial cells), and also that the dose of M40403 used in our study is sufficient to inhibit the activation of NF-κB *in vivo*. Furthermore, the ability of M40403 to prevent nuclear translocation of NF-κB strongly suggests that O_2^- is a key ROS in this pathway.

NF-κB has been indicated to play a role in the development of numerous pathological states (2, 11, 17). The inflammatory products of NF-κB activation have been demonstrated in several cellular models (36), and a critical role of NF-κB in the inflammatory cascade *in vivo* is now emerging (1, 7, 23) In fact, several studies suggest that the nuclear translocation of NF-κB is a prerequisite for the full development of lung inflammatory injury. Furthermore, the activation of NF-κB is a common end point of various signal transduction pathways, including the activation of phosphatidylcholine-specific phospholipase C, protein kinase C, protein tyrosine kinases, and mitogen-activated protein kinases and other signaling factors (22, 28). Binding of NF-κB to the respective binding sequence on genomic DNA encoding for IL-1 and TNF-α results in a rapid and effective transcription of these genes (12, 40). There is good evidence that TNF-α and IL-1β help to propagate the extension of a local or systemic inflammatory process (5, 6, 30). Recently, we have demonstrated that M40403 inhibits TNF-α and IL-1β production in an experimental model of pleurisy in rats (33). Therefore, the inhibition of the production of TNF-α and IL-1β by M40403 confirmed in the present study is most likely attributed to the inhibitory effect on the activation of NF-κB.

The results of our studies suggest that one mechanism through which O_2^- influences the inflammatory response is through the activation of the redox-sensitive transcription factor NF-κB. Removal of O_2^- with selective superoxide dismutase mimetic represents a viable strategy to modulate inflammatory responses governed by overproduction of this ROS.

ABBREVIATIONS

DTT, DL-dithiothreitol; EMSA, electrophoretic mobility-shift assay; H_2O_2, hydrogen peroxide; IκB-α, inhibitory protein κB-α; IL-1β, interleukin-1β; NF-κB, nuclear factor-κb; O_2^-, superoxide anion; PBS, phosphate-buffered saline; PMNs, polymorphonuclear neutrophils; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; TNF-α, tumour necrosis factor-α.

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Received for publication February 26, 2004; accepted April 19, 2004.

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