Participation of superoxide in neutrophil activation and cytokine production

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Abstract

Reactive oxygen species (ROS) can participate in cellular signaling and have been shown to modulate activation of the transcriptional regulatory factor NF-κB. However, the effects of ROS can differ in various cell populations. To examine the role of superoxide in neutrophil activation, we exposed resting neutrophils and neutrophils stimulated with LPS to paraquat, an agent that specifically increases intracellular superoxide concentrations. Culture of resting neutrophils with paraquat resulted in increased production of the proinflammatory cytokines TNF-α and MIP-2, enhanced degradation of IκB-α, and increased nuclear accumulation of NF-κB. Such effects of paraquat were due to intracellular superoxide (O2−) since they were blocked by the non-specific antioxidant N-acetyl cysteine and the cell permeable superoxide scavenger Tiron, but not by catalase, which facilitates the conversion of H2O2 to H2O and O2. Similar potentiating effects of paraquat were found in LPS-stimulated neutrophils. Exposure of neutrophils to paraquat also enhanced phosphorylation of Ser536 in the p65 subunit of NF-κB an event associated with increased transcriptional activity. Examination of kinases critical for LPS-stimulated gene expression showed that addition of paraquat to resting or LPS exposed neutrophils enhanced activation of p38 MAPK, but not that of Akt or ERK1/2. The potentiation of NF-κB translocation and proinflammatory cytokine production, but not of Ser536 p65 phosphorylation, by paraquat was dependent on activation of p38 MAPK. These results demonstrate that increased intracellular superoxide concentrations are proinflammatory in neutrophils, acting through a p38 MAPK dependent mechanism that results in enhanced nuclear accumulation of NF-κB and increased expression of NF-κB dependent proinflammatory cytokines.

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1. Introduction

Reactive oxygen species (ROS) generated during oxidative metabolism occupy both beneficial and harmful roles in biologic systems. For example, ROS produced by neutrophils can act as bactericidal agents [1]. ROS also are generated in various disease states, such as septic shock [2,3], ischemia–reperfusion injury [4,5], and chronic pulmonary obstructive disease [6,7], where they contribute to organ dysfunction. In addition to their roles in host defense and pathophysiology, ROS also participate in the regulation of cellular signaling, including modulation of protein kinase cascades, protein phosphatases, and transcription factors [8–11].

There is evidence that superoxide (O2−) or H2O2 may behave as classical second messengers, being produced in cells by ligand–receptor activation [8–10]. Engagement of receptors for PDGF [12], EGF [13], angiotensin II [14], GM-CSF [15], TNF-α [16,17], or thrombin [18] results in intracellular generation of O2−. In neutrophils, receptor stimulated the gp91 and gp47 NADPH oxidases lead to oxidative burst, and in other tissues activation of other NADPH oxidases (NOX1–5) may lead to generation of ROS, including O2− and H2O2 [19].

Several reports have suggested that ROS are involved in modulating nuclear translocation and transcriptional activity of the regulatory factor NF-κB. Initial studies indicated that ROS activated NF-κB in what was believed to be a general manner [20–22]. These experiments were primarily based on the ability of antioxidants, such as N-acetyl cysteine (NAC), and...
pyrrolidine dithiocarbamate (PDTC), to block nuclear translocation of NF-κB [21,22]. In addition, ROS themselves, including H₂O₂, result in enhanced nuclear accumulation of NF-κB when added to certain cell populations [21]. However, more recently it has been found that NAC and PDTC interfere with processes relating to NF-κB activation independently of their antioxidant effects [23,24]. Furthermore, ROS have not consistently been found to mediate NF-κB activation in all cell populations [25,26]. In fact, in some cases inhibition of nuclear translocation of NF-κB by cellular exposure to ROS has been reported [27,28]. Thus, the role of oxidants in the regulation of NF-κB is currently controversial [23].

Neutrophils play a central role in response to bacterial infection. Release of O₂ into both the intracellular and extracellular milieu is an important component of bacterial killing by neutrophils [1]. In addition to their role in host defense, neutrophils also participate in the induction of organ system dysfunction in acute inflammatory processes, such as acute lung injury, where their ability to release ROS appears to contribute to cellular and tissue damage [29].

In the present studies, we examined the effects of increased intracellular levels of O₂ on neutrophil activation in both unstimulated cells and after culture with LPS. Exposure of neutrophils to paraquat, was used to increase intracellular O₂ levels [30–35]. These experiments demonstrate that increased intracellular O₂ induces proinflammatory cytokine production in neutrophils through activating p38 MAP kinase and enhancing nuclear translocation of NF-κB.

2. Materials and methods

2.1. Mice

Male BALB/c mice, 8–12 weeks of age, were purchased from Harlan Sprague–Dawley (Indianapolis, IN). The mice were kept on a 12-h light, 12-h dark cycle with free access to food and water. All experiments were conducted in accordance with institutional review board-approved protocols.

2.2. Reagents and antibodies

Isolflurane was obtained from Abbott Laboratories (Chicago, IL). Escherichia coli O111:B4 endotoxin (LPS), N-acetyl cysteine, catalase, Tiron, and paraquat were purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640/25 mM HEPES/L-glutamine was obtained from BioWhittaker (Walkersville, MD). FCS and penicillin/streptomycin were purchased from Mediatech Inc. (Herndon, VA). Bicinchoninic acid protein assay reagent was obtained from BioWhittaker (Walkersville, MD). FCS and penicillin/streptomycin were purchased from Mediatech Inc. (Herndon, VA). Bicinchoninic acid protein assay reagent was purchased from Pierce (Rockford, IL). Activation-specific Abs for phospho-Thr²⁰²/Tyr²⁰⁴ extracellular receptor-activated kinase 1 (ERK1), phospho-Thr³⁸⁰/Tyr³⁸² ERK2, phospho-Thr³⁹²/Tyr³⁸² p38, phospho-Ser⁷²⁷ Akt, phospho-Ser⁷⁸ p65 subunit of NF-B, as well as antibodies for total ERK1/2, p38, Akt, and IB were purchased from Cell Signaling Technologies (Beverly, MA). Protein A/G plus agarose, and IB recombinant protein were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-labeled anti-rabbit antibodies, and ECL reagents were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Western blot analysis

Western blots for phosphorylated and total kinases were performed as described previously [36]. Parallel samples for total kinase were run with samples for activation-specific phosphorylation analysis. Densitometry was performed using chemiluminescence system and analysis software (Bio-Rad) to determine the ratio between phosphorylated and total kinase.

3. Results

3.1. Effects of paraquat on cytokine generation by neutrophils

Addition of paraquat to isolated neutrophils increased cytokine production in a dose dependent manner (Fig. 1A). In particular, at the highest concentrations of paraquat tested, 10⁻² M, levels of MIP-2 and TNF-α in the neutrophil supernatants were greater than 4000 pg/ml and 1000 pg/ml, respectively. Of note, no decrease in cellular viability was found at these concentrations of paraquat, with cell death being less than 2% after 4 h of culture. Dose-dependent potentiation of LPS induced production of MIP-2 and TNF-α was also found in the presence of paraquat (Fig. 1B). These effects of paraquat were due to intracellular superoxide (O₂⁻) generation since they were blocked by the non-specific antioxidant N-acetyl cysteine and the cell permeable O₂ scavenger Tiron, but not by catalase, which facilitates the conversion of hydrogen peroxide (H₂O₂) to H₂O and O₂ (Fig. 2).
3.2. Paraquat increases nuclear translocation of NF-κB in neutrophils

NF-κB plays a central role in the transcriptional regulation of MIP-2 and TNF-α expression [37]. Because paraquat both alone and in the presence of LPS was able to stimulate production of these cytokines by neutrophils, we hypothesized that this effect would be accompanied by enhanced nuclear translocation of NF-κB.

As shown in Fig. 3A, incubation of neutrophils with paraquat resulted in enhanced nuclear translocation of NF-κB, with the greatest increase being found after 40 min of culture. In addition, potentiation of nuclear accumulation of NF-κB was found when paraquat was added to neutrophils cultured with LPS (Fig. 3B). NF-κB that translocated to the nucleus after neutrophil exposure to LPS and paraquat was a heterodimer, composed of p50 and p65 subunits as shown by supershift when incubated with either anti-p50 or anti-p65 antibodies (Fig. 3C).

Nuclear translocation of NF-κB depends on phosphorylation, ubiquitination, and subsequent proteosomal degradation of IκBα [38]. To determine if the effects of paraquat on NF-κB activation were potentially due to effects on IκB-α, we examined degradation of IκB-α in neutrophils cultured with paraquat with or without LPS.

Addition of paraquat to otherwise unstimulated neutrophils resulted in decreased amounts of total IκB-α within 7.5 min and continuing for more than 30 min (Fig. 4). As expected, LPS alone also resulted in degradation of IκB-α. Including paraquat in cultures of LPS stimulated neutrophils resulted in even greater degradation of IκB-α than that found with either paraquat or LPS alone during the first 15 min after exposure, with levels of IκB-α being increased above baseline values after 30 min of exposure. Since transcription of IκB-α is dependent on NF-κB activation, the increased generation of IκB-α at the later culture time points may reflect the enhanced nuclear translocation of NF-κB found in neutrophils stimulated with both LPS and paraquat as compared to either stimulus alone.

Phosphorylation of serine 536 in the p65 subunit is associated with increased transcriptional activity of the p50:p65 NF-κB heterodimers [39]. As shown in Fig. 5, exposure of neutrophils to paraquat induced serine 536 phosphorylation with kinetics similar to those found after culture with LPS alone. Co-culture of
neutrophils with paraquat and LPS produced more rapid phosphorylation of p65 and also resulted in a greater degree of phosphorylation than that found with LPS alone.

### 3.3. Effects of paraquat on kinase activation

The kinases Akt and p38 MAPK have been shown to participate in enhancing nuclear translocation of NF-κB in LPS stimulated neutrophils [36,40]. We therefore examined the effects of paraquat on the activation of these kinases both by itself and in the presence of LPS.

As shown in Fig. 6A, the addition of paraquat alone to neutrophils resulted in increased activation of p38. As found in previous studies [40], culture of neutrophils with LPS induced p38 phosphorylation, and this effect was enhanced when paraquat was included in the neutrophil cultures (Fig. 6A). In contrast to the ability of paraquat to activate p38, paraquat did not have any effect on Akt in otherwise unstimulated neutrophils, and did not further potentiate Akt activation in cells stimulated with LPS (Fig. 6B). Similarly, paraquat had no effects on activation of the LPS inducible kinase ERK 1/2 (Fig. 6B).

Because of the participation of p38 in the translocation of NF-κB in neutrophils [41], we investigated whether the ability of paraquat to affect p38 activation was responsible for the effects of this O$_2^-$ generating compound on NF-κB translocation. Addition of SB203580, a specific inhibitor of p38, to neutrophils...
Fig. 5. Paraquat induces phosphorylation of serine 536 in the p65 subunit of NF-κB. Neutrophils were cultured with paraquat (5 mM) (Pq), LPS (100 ng/ml in A or the designated concentrations of LPS in B), or LPS and paraquat together (LPS/Pq). Cell lysates were collected at prespecified times after the initiation of cultures for Western blotting. Serine 536 phosphorylated p65 was detected in the total cell lysates using specific antibodies. Representative gels as well as densitometry results from a single experiment are shown. Two additional experiments yielded similar results.

Fig. 6. Paraquat activates p38, but not Akt or ERK 1/2, in neutrophils. Neutrophils were cultured with paraquat (5 mM) (Pq), LPS (100 ng/ml), or the combination of both paraquat and LPS (LPS/Pq). Cell lysates were obtained after the designated periods of culture and subjected to SDS-PAGE Western blot analysis using antibodies for phosphorylated and total p38, ERK1/2 and Akt. Representative gels are shown as well as densitometry determinations. Two additional experiments provided similar results.
cultured with paraquat diminished nuclear concentrations of NF-κB to levels near those found in unstimulated neutrophils (Fig. 7A). Similarly, inhibition of p38 prevented paraquat induced increases in nuclear translocation of NF-κB in LPS stimulated neutrophils (Fig. 7B). In contrast to the effects of p38 inhibition on paraquat induced nuclear translocation of NF-κB, addition of SB203580 did not produce any changes in the levels of phosphorylation of serine 536 on the NF-κB p65 subunit in neutrophils cultured either with paraquat alone or with LPS plus paraquat, indicating that the paraquat induced phosphorylation of p65 is due to mechanisms independent of p38 activation (data not shown).

Since paraquat by itself and in the presence of LPS resulted in increased production of the NF-κB dependent cytokines MIP-2 and TNF-α by neutrophils (Fig. 1A and B), we wanted to examine if this was due to its effects on p38 activation. Inhibition of p38 in paraquat treated neutrophils decreased concentrations of MIP-2 and TNF-α in culture supernatants to or below baseline levels (Fig. 8A).

Activation of p38 has been shown to have a central role in modulating LPS induced generation of TNF-α by neutrophils [42–44]. Consistent with those findings, addition of SB203580 to LPS stimulated neutrophils prevented LPS associated increases in TNF-α and MIP-2 production (Fig. 8B). In addition, inhibition of p38 also reduced cytokine production in neutrophils cultured with both LPS and paraquat to near baseline levels (Fig. 8B), indicating that p38 participates in the potentiating effects of paraquat in LPS stimulated neutrophils.

4. Discussion

In the present studies, exposure of neutrophils to paraquat, a potent inducer of increased intracellular superoxide (O$_2^-$) [31–35,45,46], robustly stimulated the production of TNF-α and MIP-2. Proinflammatory cytokine expression was increased by addition of paraquat to resting neutrophils, and paraquat also potentiated the effects of LPS on neutrophil activation. The transcriptional factor NF-κB occupies a central role in modulating the expression of many proinflammatory mediators, including MIP-2 and TNF-α [37], and nuclear translocation of NF-κB was enhanced in paraquat exposed neutrophils. The principal mechanism controlling nuclear accumulation of NF-κB is degradation of the inhibitory protein IκB-α as a result of phosphorylation-stimulated ubiquitination [37]. IκB-α prevents nuclear entry of NF-κB by masking the nuclear localization sequence [37]. Exposure of neutrophils to paraquat produced enhanced degradation of IκB-α. These results therefore indicate that increased intracellular O$_2^-$ promotes the proinflammatory properties of neutrophils, and particularly their ability to produce proinflammatory cytokines, through enhancing IκB-α degradation, thereby resulting in greater nuclear accumulation and transcriptional activity of NF-κB.

The mitogen activated kinase p38 participates in the nuclear translocation of NF-κB in LPS stimulated neutrophils [40]. In the present experiments, the addition of paraquat to resting neutrophils resulted in p38 activation, and paraquat also enhanced p38 activation in neutrophils cultured with LPS. Previous studies have also shown that reactive oxygen species participate in the phosphorylation and activation of p38, although the mechanism through which such activation occurs has not been well delineated [47,48]. Paraquat had no effects on the phosphorylation of Akt or ERK 1/2, showing that the effects of increased intracellular O$_2^-$ on kinase activation are specific, and similar to those of classically described second messengers. In addition, activation of p38 participates in O$_2^-$ induced enhancement of nuclear translocation of NF-κB and
proinflammatory cytokine production, since inhibition of p38 prevented both of these events in paraquat treated neutrophils. In neutrophils cultured with LPS and paraquat, inhibition of p38 reduced cytokine production to baseline levels, indicating that all of the effects of increased intracellular O$_2^-$ in this setting were due to p38 activation.

A number of phosphorylation sites within the p65 subunit of NF-$\kappa$B are known to be important for regulation of its transcriptional activity [39,49]. In particular, phosphorylation of Ser536 in the NF-$\kappa$B p65 subunit promotes transactivating ability. This phosphorylation event occurs independently of nuclear accumulation of NF-$\kappa$B, and a number of protein kinases, including Akt and IKK$\beta$, have been presumed to be involved [49–52]. Phosphorylation of p65 increases the transcriptional ability of NF-$\kappa$B through mechanisms that appear to involve assembly of transcriptional complexes at the $\kappa$B promoter sites of NF-$\kappa$B target genes [49,53]. We found that even though paraquat increased phosphorylation of p65Ser$^{536}$, this event was unlikely to be responsible for the increased production of the NF-$\kappa$B dependent cytokines. In particular, while inhibition of p38 diminished paraquat induced nuclear translocation of NF-$\kappa$B and cytokine production, it had no effect on p65 phosphorylation. These results are consistent with previous experiments demonstrating that transactivation of NF-$\kappa$B is independent of nuclear accumulation [39].

Recent evidence from our laboratory and others indicates that different ROS can have distinct effects on cellular activation and also that the effects of ROS on NF-$\kappa$B differ among various cell populations [25,26,54,55]. In particular, we previously found that H$_2$O$_2$ decreased nuclear accumulation of NF-$\kappa$B and TNF-$\alpha$ expression in LPS stimulated neutrophils [56]. These effects of H$_2$O$_2$ were not related to activation of the p38 kinase, but rather appeared to be due to inhibition of I$\kappa$B-$\alpha$ degradation. Such results, showing an inhibitory role of H$_2$O$_2$ on neutrophil activation, are consistent with reports that H$_2$O$_2$ diminished IL-8 production in fMLP stimulated neutrophils [57]. We also demonstrated inhibitory effects of H$_2$O$_2$ on nuclear accumulation of NF-$\kappa$B in neutrophils cultured with TNF-$\alpha$, indicating that this was a more general effect, and not limited to TLR4 signaling [56]. Those findings are concordant with previous reports in nonmyeloid cell populations that H$_2$O$_2$ can inhibit LPS-stimulated activation of NF-$\kappa$B [27,58]. Although previous studies [27,58] suggested that the mechanism by which H$_2$O$_2$ diminished nuclear translocation of NF-$\kappa$B was through inactivation of IKK, we did not find any effect of H$_2$O$_2$ on LPS induced activation of the IKK$\alpha/\beta$ complex nor on phosphorylation of I$\kappa$B-$\alpha$ in neutrophils [56].

In contrast to the inhibitory properties of H$_2$O$_2$ on neutrophil activation, exposure of RAW264.7 macrophages to superoxide anion (O$_2^-$) enhanced LPS induced NF-$\kappa$B translocation to the nucleus [59]. In the present studies, exposure of neutrophils to paraquat, which increases intracellular concentrations of O$_2^-$, was also associated with enhanced nuclear accumulation of NF-$\kappa$B in resting neutrophils and in those stimulated with LPS. Such results are consistent with a pro-inflammatory role for O$_2^-$, in contrast to the apparent anti-inflammatory properties of H$_2$O$_2$, on neutrophil activation. Additionally, treatment of neutrophils with the non-specific

![Fig. 8. Inhibition of p38 prevents paraquat induced enhancement of cytokine release. Neutrophils were left unstimulated without (Con) or with SB203580 (12 $\mu$M) (Con+SB), or were cultured with paraquat (5 mM) (Pq), LPS (100 ng/ml) (LPS), or LPS and paraquat (LPS+Pq) with or without SB 203580 (12 $\mu$M) (SB). Production of MIP-2 and TNF-$\alpha$ into the culture supernatants was determined after 4 h of culture. Data (mean±SD) from an experiment performed in triplicate are shown. Two additional experiments produced similar results.](image-url)
antioxidant N-acetyl cysteine and the cell permeable O$_2$ scavenger Tiron reduced paraquat induced cytokine production, but addition of calafate, which facilitates the conversion of hydrogen peroxide (H$_2$O$_2$) to H$_2$O and O$_2$, resulted in a further increase in MIP-2 levels in the culture supernatants. These data are consistent with a proinflammatory role for O$_2$, but an anti-inflammatory role for H$_2$O$_2$.

In vivo studies indicate that enhanced generation of O$_2$ is proinflammatory. In particular, we found that inhibition of xanthine oxidase with allopurinol or tungsten containing diets decreased the severity of hemorrhage induced acute lung injury, including diminishing pulmonary accumulation of neutrophils and proinflammatory cytokine production in the lungs [60–62]. Because the early stages of hemorrhage-induced acute lung injury are largely neutrophil dependent [63] and because xanthine oxidase generates O$_2$ [60] those experiments suggested that O$_2$ had an important proinflammatory role in contributing to neutrophil dependent lung injury. Similarly, overexpression of extracellular superoxide dismutase, a condition associated with diminished effects of O$_2$ as a result of increased dismutation to H$_2$O$_2$, is associated with decreased pulmonary NF-κB activation, neutrophil accumulation, and indices of lung injury after hemorrhage [64].

The present experiments, demonstrating the proinflammatory effects of O$_2$ on neutrophil activation, when combined with previous studies from our laboratory and others showing that H$_2$O$_2$ is anti-inflammatory for neutrophils [28,58], suggest that the balance between these two ROS may be important in modulating neutrophil driven inflammatory responses in vivo. For example, although reduction in cellular exposure to O$_2$ is the presumed mechanism by which administration of superoxide dismutase (SOD), overexpression of extracellular superoxide dismutase (EC-SOD), or administration of extracellular superoxide dismutase mimetics decrease NF-κB activation and the severity of lung injury in situations, such as hemorrhage, associated with increased generation of ROI [62,64], other mechanisms affecting the balance between O$_2$ and H$_2$O$_2$ may also be involved. EC-SOD and SOD facilitate through the dismutation reaction the conversion of O$_2$ to H$_2$O$_2$ (2O$_2$ + 2 H$^+$ → O$_2^+$ + H$_2$O$_2$). It is therefore possible that the anti-inflammatory properties of EC-SOD are due to its ability to generate H$_2$O$_2$, an apparent anti-inflammatory ROS, and not from more general antioxidant effects. Future experiments will be necessary to explore this hypothesis more completely.

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References


