Role of Neutrophil NADPH Oxidase in the Mechanism of Tumor Necrosis Factor-α-induced NF-κB Activation and Intercellular Adhesion Molecule-1 Expression in Endothelial Cells*

Jie Fan‡, Randall S. Frey, Arshad Rahman, and Asrar B. Malik

From the Department of Pharmacology, University of Illinois College of Medicine, Chicago, Illinois 60612

In this study, we explored a novel function of polymorphonuclear neutrophils (PMN) NAD(P)H oxidase in the mechanism of tumor necrosis factor-α (TNFα)-induced NF-κB activation and intercellular adhesion molecule-1 (ICAM-1) expression in endothelial cells. Studies were made in mice lacking the p47phox subunit of NAD(P)H oxidase as well as in cultured mouse lung vascular endothelial cells (MLVEC) from these mice. In response to TNFα challenge, NF-κB activation and ICAM-1 expression were significantly attenuated in lungs of p47phox−/− mice as compared with wild-type (WT) mice. The attenuated NF-κB activation in p47phox−/− mice was secondary to inhibition of NIK activity and subsequent IκBα degradation. Induction of neutropenia using anti-PMN serum prevented the initial TNFα-induced NF-κB activation and ICAM-1 expression in WT mice, indicating the involvement of PMN NAD(P)H oxidase in signaling these responses. Moreover, the responses were restored upon repletion with PMN obtained from WT mice but not with PMN from p47phox−/− mice. These findings were recapitulated in MLVEC co-cultured with PMN, suggesting that NF-κB activation and resultant ICAM-1 expression in endothelial cells occurred secondary to oxidants generated by the PMN NAD(P)H oxidase complex. The functional relevance of the PMN NAD(P)H oxidase in mediating TNFα-induced ICAM-1-dependent endothelial adhesivity was evident by markedly reduced adhesion of p47phox−/− PMN in co-culture experiments. Thus, oxidant signaling by the PMN NAD(P)H oxidase complex is an important determinant of TNFα-induced NF-κB activation and ICAM-1 expression in endothelial cells.

* The abbreviations used are: TNFα, tumor necrosis factor α; PMN, polymorphonuclear neutrophil; ICAM-1, intercellular adhesion molecule-1; TNFR, tumor necrosis factor receptor; TRADD, TNFα-associated death domain-containing protein; TRAF2, TNFα-associated factor 2; NIK, NF-κB-inducing kinase; MLVEC, mouse lung vascular endothelial cells; WT, wild-type; HBSS, Hanks' balanced salt solution; GSH, reduced glutathione; i.p., intraperitoneal; ANS, anti-mouse neutrophil serum; EMSA, electrophoretic mobility shift assay; Ab, antibody; TRX, thioredoxin; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.

The pro-inflammatory cytokine TNFα, released during sepsis, promotes adhesion of neutrophil (PMN) to the endothelium by inducing the expression of intercellular adhesion molecule-1 (ICAM-1), a counter receptor for the leukocyte β2-integrins LFA-1 and Mac-1 (CD11a/CD18 and CD 11b/CD18) (1–4). The interaction of ICAM-1 with CD11/CD18 integrins enables PMN to adhere firmly to the vascular endothelium and thereby migrate across the microvascular barrier. Studies have shown that the transcription factor NF-κB is the key regulator of ICAM-1 gene expression following TNFα challenge of endothelial cells (4). Signals mediating TNFα-induced NF-κB activation are initiated by the engagement of TNF receptor type I at the plasma membrane and then relayed through specific TNFR-associated proteins. TNFα-associated death domain-containing protein (TRADD) is an adaptor protein that interacts with TNFR type I and is required for TNFα-mediated induction of NF-κB (5). TRADD interacts in turn with two other adaptor proteins, TNFR-associated factor 2 (TRAF2) and receptor-interacting protein (RIP), and forms a complex required for NF-κB activation (6, 7). NF-κB-inducing kinase (NIK) physically interacts with TRAF2 (8), and further, it activates IκBα kinases (IKKs), which in turn phosphorylate IκB proteins, a family of inhibitory proteins that sequester NF-κB as an inactive complex in the cytoplasm. Phosphorylation of IκB triggers the rapid ubiquitination and subsequent degradation of this inhibitor in proteasome complex (9). The liberated NF-κB migrates to the nucleus where it binds to cognate κB enhancer elements and activates target genes such as ICAM-1 (10).

Generation of oxidants in endothelial cells serves an important signaling function in mediating TNFα-induced NF-κB activation and ICAM-1 expression and, thereby, promoting the stable ICAM-1-dependent endothelial adhesivity and firm PMN adhesion (11–15). Although TNFα has been shown to induce oxidative burst in PMN (16), it remains unclear whether the oxidants thus released can contribute to TNFα-induced NF-κB activation and ICAM-1 expression in the endothelium. The primary source of oxidative burst in PMN is NAD(P)H oxidase, a highly regulated membrane-bound enzyme complex, which catalyzes the production of superoxide by the one-electron reduction of oxygen using NAD(P)H as the electron donor. The core enzyme consists of five subunits: p40phox, p47phox, p67phox, p22phox, and gp91phox. In the basal state, p40phox, p47phox, and p67phox exist in the cytosol as a complex, whereas p22phox and gp91phox are located in membranes of secretory vesicles and specific granules of PMN, where they occur as a heterodimeric flavohemoprotein known as cytochrome b558. Also, two low molecular weight GTP-binding proteins, Rap 1A and rac1/2, are involved in the activation of the NAD(P)H oxidase. Upon stimulation, the cytosolic component p47phox is phosphorylated, and the entire cytosolic complex migrates to the membrane where it associates with cytochrome b558 to assemble the active oxidase (17). In the present study, we addressed the role of PMN NAD(P)H oxidase complex in me-
NAD(P)H Oxidase Contributes to TNFα-induced NF-κB

A. M. White

anti-TNFα-induced NF-κB activation and ICAM-1 expression using mice genetically deficient in p47phox or gp91phox subunit of NADPH oxidase and co-cultures involving mouse PMN and vascular endothelial cells (MLVEC) from these mice. We demonstrate that the functional impairment of neutrophil NADPH oxidase and thereby of oxidant generation significantly delayed the TNFα-induced NF-κB activation and ICAM-1 expression in both lungs and MLVEC of p47phox−/− or gp91phox−/− mice. Our data establish that the effects of NADPH oxidase inhibition were secondary to inhibition of NIK activation and the subsequent IκBα degradation.

EXPERIMENTAL PROCEDURES

Mice—Breeder stocks for p47phox knockout mice were obtained from Dr. Steven Holland (Laboratory of Host Defense, National Institutes of Health) (19). Mice deficient in gp91phox were obtained from Dr. Mary Dinauer (University School of Medicine, Indianapolis, IN) (19). Wild-type (WT) mice of similar genetic background (C57BL/6) were purchased from The Jackson Laboratory. All animals were maintained under specific pathogen-free conditions in a barrier facility. Mice were 9–10 weeks of age at the time of experiments.

Experimental Protocol—Animals were given TNFα (Promega, Madison, WI) 100 units/10 g body weight by intraperitoneal (i.p.) injection at time equals 0, and whole lung tissue was harvested at the ison, WI) 5000 units/10 g body weight or saline by intraperitoneal (i.p.) injection using mice genetically deficient in p47phox or gp91phox. Animals were given TNFα times indicated. In some experiments neutropenia was induced by PMN injection at time equals 0, and whole lung tissue was harvested at the

Western Blot Analysis—Lung tissue homogenate samples or aliquots of MLVEC lysate or non-nuclear protein were separated on a 10% SDS-PAGE under non-reducing condition. Equivalent loading of the gel was determined by quantitation of protein as well as by reprobing membranes for actin detecting. Separated proteins were electroblotted onto a polyvinylidene difluoride membrane and blocked for 1 h at room temperature with Tris-buffered saline containing 1% bovine serum albumin. The membranes were then probed with a 1:1000 dilution of either a purified polyclonal IgG against mouse ICAM-1, or, for non-nuclear protein, monoclonal anti-mouse IκBα antibody (both from Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 1 h. After washing, primary antibodies associated with the membranes were detected on autoradiographic film by horseradish peroxidase-conjugated secondary antibodies and the ECL plus chemiluminescence system (Amersham Biosciences, Inc., Arlington Heights, IL) according to the manufacturer's instructions.

Northern Blot Analysis—Total RNA from lungs was obtained using the guanidine isothiocyanate method (22). Briefly, lungs were homogenized immediately with liquid nitrogen, thawed and homogenized in 4 x guanidine isothiocyanate containing 25 mM sodium citrate, 0.5% Sarkosyl, and 100 mM β-mercaptoethanol. RNA was denatured, electrophoresed through a 1.2% formaldehyde-agarose gel, and transferred to a nylon membrane. Hybridization was carried out using a [32P]dCTP-labeled ICAM-1 cDNA (from ATCC). Blots were then washed under conditions of high stringency, and specific mRNA bands were detected by autoradiography in the presence of intensifying screens. Blots were stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase to control for loading. Expression of mRNA was quantitated using Scion Image software (Scion Corp., Frederick, MD) and was normalized to the glyceraldehyde-3-phosphate dehydrogenase signal.

Nuclear Protein Extraction—Nuclear protein extracts were prepared from lung tissue or MLVEC by the method of Deryckere and Gannon (23). Aliquots of 100 mg of frozen tissue were ground to powder with a mortar in liquid nitrogen. The thawed powder or 1 x 107 cells were homogenized in a Dounce tissue homogenizer with 4 ml of solution A (0.6% Nonidet P-40, 150 mM NaCl, 10 mM HEPES, pH 7.9, 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride). The cells were lysed with 4 ml of solution B supplemented with 10 mM NaF and 150 mM NaCl. The mixture was separated by electrophoresis on a 5% polyacrylamide gel in 1 x SDS-PAGE buffer. Equivalent loading of the gel was determined by Coomassie blue staining.

Electrophoretic Mobility Shift Assay—The probe for EMSA was a 24-bp double-stranded construct of NF-κB consensus binding sequence (5'-AGGAGACTTCTGGGAGGCTTCTT-3'). End labeling was performed by T4 kinase in the presence of [32P]ATP. Labeled oligonucleotides were purified on a Sephadex G-50M column (Amersham Biosciences, Inc., Piscataway, NJ). An aliquot of 5 μg of nuclear protein was incubated with the labeled double-stranded probe (~5,000 cpm) in the presence of 5 μg of non-specific blocker, poly(dI-dC) in binding buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40, and 0.5 mM dithiothreitol) at 25 °C for 20 min. Specific competition was performed by adding 100 ng of unlabeled double-stranded oligonucleotide, whereas for non-specific competition, 100 nM unlabeled double-stranded mutant oligonucleotide (5'-AGCTCAATCTCCCTGGAGGCTTCC-3') that does not bind NF-κB was added. The mixture was separated by electrophoresis on a 5% polyacrylamide gel in 1 x Tris glycine EDTA buffer. Gels were vacuum-dried and subjected to autoradiography and PhosphorImager (Molecular Dynamics) analysis.

PMN Adhesion Assay—The assay was performed as previously de-
sioned (24). PMN isolated from either WT or p47<sup>phox</sup>−/− mice were co-cultured with MLVEC and challenged with 500 units/ml TNFα for 0–6 h. In some groups, anti-ICAM-1 Ab (6 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA) was added to the co-culture system. The percentage of PMN adhering to endothelial cells was determined from the ratio of final reading to initial reading.

Statistics—The data are presented as mean ± S.E. of the indicated number of experiments. Statistical significance among group means was assessed by analysis of variance. The Student Neuman-Keuls post-hoc test was performed. Differences were considered significant when p < 0.05.

RESULTS

Marked Attenuation of TNFα-induced ICAM-1 Expression in Lungs of NAD(P)H Oxidase-deficient Mice—As shown in Fig. 1A, in WT mice TNFα challenge caused a marked increase in ICAM-1 protein expression by 1 h, increasing further by 4 h. However, in p47<sup>phox</sup>−/− mice, the ICAM-1 protein expression at 1 h was abrogated and was significantly attenuated at the later time points. To confirm that this change in p47<sup>phox</sup>−/− was the result of NAD(P)H oxidase deficiency, ICAM-1 protein expression in gp91<sup>phox</sup>−/− mice was also assessed. The observed changes in ICAM-1 protein expression in gp91<sup>phox</sup>−/− mice were consistent with the p47<sup>phox</sup>−/− group. The changes in mRNA expression for ICAM-1 shown in Fig. 1B paralleled the changes in the protein level. The promoter region of the ICAM-1 gene contains an NF-κB consensus binding sequence believed to mediate the regulation of gene transcription (25). In the present studies, gel-shift assay on whole lung tissue was performed to discern whether the attenuation of ICAM-1 mRNA expression in p47<sup>phox</sup>−/− mice was the result of decreased nuclear translocation of NF-κB. As shown in Fig. 1C, the response to TNFα in the WT animals exhibited an increase in NF-κB translocation at 0.5 h, reached a maximum at 1 h, was sustained for 4 h, and decreased over the ensuing 2-h period. In p47<sup>phox</sup>−/− mice, however, TNFα induced a much smaller increase in NF-κB translocation at 1 h, which did not increase further over the next 5 h. The level of NF-κB activity in nuclear fractions in p47<sup>phox</sup>−/− mice during the period of 1–4 h after TNFα was on the average 60% less than in WT mice.

Role of NAD(P)H Oxidase in TNFα-induced NF-κB Signaling Pathway—To address the role of NAD(P)H oxidase in mediating TNFα-induced IκBα degradation, a requirement for NF-κB activation, the IκBα protein level was measured in the non-nuclear fraction from the same lungs used for NF-κB analysis above. IκBα protein was detected in lungs prior to challenge with TNFα. By 0.5 h after TNFα, there was a significant loss of IκBα to 18 ± 6% of basal content in the WT mice (Fig. 2A). IκBα content remained low with 22 ± 7% of basal value detected at 6 h after TNFα challenge. Thus, TNFα induced a rapid, substantial, and sustained loss of IκBα. However, the loss of IκBα in p47<sup>phox</sup>−/− mice was significantly less by 2 h after TNFα challenge than in WT mice (Fig. 2A). At 30 min after TNFα, IκBα in p47<sup>phox</sup>−/− mice was 74 ± 11% of the basal level, stayed

![Fig. 1. Effect of p47<sup>phox</sup>−/− and gp91<sup>phox</sup>−/− on ICAM-1 expression and NF-κB nuclear translocation.](http://www.jbc.org/content/115/46/3406/F1)

WT, p47<sup>−</sup>- and gp91<sup>−</sup>- mice were harvested at the time indicated after TNFα injection. The data are representative of three independent studies. A, Western blot for ICAM-1 protein in the lungs. Corresponding actin is shown as a control for loading. B, Northern blotting for ICAM-1 mRNA expression in the lungs. Glycer-aldehyde-3-phosphate dehydrogenase mRNA is identified as a control for RNA loading. C, EMSA showing the time course for TNFα-induced NF-κB nuclear translocation in the lung tissue. Cold competition (cold) and mutated probe competition (Mut.) were performed on samples from the time point t = 4 h in the p47<sup>phox</sup>−/− group.
at −55% of the basal level up to 2 h, and then gradually decreased to a level similar to that in WT animals by 6 h after TNFα.

NIK is an upstream signaling molecule integral to the pathway of TNFα induction of NF-κB nuclear translocation through the TNFα receptor (8). We examined whether the impaired IκBα degradation in p47φox−/− is the result of reduced NIK activity. Fig. 2B shows the rapid increase in NIK activity at early time points (as early as 0.5 h) after challenge of WT animals with TNFα. However, lungs of p47φox−/− animals exhibited markedly delayed and attenuated NIK activity. Compared with WT mice, the NIK activity in p47φox−/− at 0.5, 1, and 2 h after TNFα was 7 ± 2%, 31 ± 4%, and 25 ± 5% of the WT values, respectively.

Binding of TNFα with its receptor initiates the recruitment of TNFR-associated proteins to the death domain of TNFR and a complex formation that induces activation of NF-κB (6, 7). In this study, we tested the effects of impairment of NAD(P)H oxidase function in the recruitment of three major TNFR-associated proteins, TRADD, TRAF2, and RIP using co-immunoprecipitation and Western blotting. As shown in Fig. 3, there were no differences between the WT and p47φox−/− mice in TRAF2 and RIP binding to TRADD after the TNFα challenge. Thus, NAPDH oxidase-deficiency-induced decreased signaling and ICAM-1 expression occurred through a mechanism involving inhibition of NIK activity.

Neutrophil NAD(P)H Oxidase Contributes to TNFα-induced ICAM-1 Expression—Because the PMN NAD(P)H oxidase is a major source of superoxide, we hypothesized that NAD(P)H oxidase would contribute to the oxidant-dependent NF-κB activation. To determine the role of PMN, we evaluated the responses to TNFα challenge in lungs of WT and p47φox−/− mice following the depletion of circulating PMN. In some cases, we replenished the PMN in these mice made neutropenic to address the causal role of PMN in the mediating the response. As shown in Fig. 4A, at 2 h after TNFα challenge, neutropenia induced in WT mice reduced the ICAM-1 expression in lungs by −62% (lane 4) as compared with the TNFα alone control group (lane 2). Repletion with WT PMN in these WT neutropenic mice restored the ICAM-1 expression in response to TNFα (lane 6). However, repletion with PMN derived from p47φox−/− mice failed to restore ICAM-1 expression (Fig. 4B). In p47φox−/− mice, a 40% lower expression of ICAM-1 was seen after TNFα challenge (Fig. 4A, lane 8) compared with WT mice (lane 2). Neutropenia in p47φox−/− mice did not further affect the ICAM-1 expression (lane 10), suggesting that the reduced ICAM-1 expression is the result of deficiency of PMN NAD(P)H oxidase. Repletion of p47φox−/− animals with WT PMN caused a complete restoration in ICAM-1 after TNFα (Fig. 4A, lane 12), further indicative of the role of PMN NAD(P)H oxidase in mechanism of TNFα-induced ICAM-1 expression.

Fig. 4C shows the effects of PMN depletion and repletion on lung tissue IκBα level at 0.5 h after TNFα challenge. In WT animals, TNFα alone caused an 82 ± 9% decrease in IκBα (lane 2), whereas in the neutropenic WT group, TNFα caused a 38 ± 8% reduction in IκBα (lane 4). Repletion with WT PMN restored the decrease in IκBα (lane 6). In contrast, neutropenia in p47φox−/− mice did not significantly affect IκBα level in response to TNFα (lane 10) as compared with TNFα alone group (lane 8); although in the latter group the IκBα level was −52% higher than that in WT animals (lane 2). Repletion with WT PMN markedly reduced IκBα in response to TNFα in the p47φox−/− animals (lane 12), and the value reached the same level seen in WT animals. Thus, the results in Fig. 4 show that the PMN NAD(P)H oxidase complex plays a critical role in the mechanism of TNFα-induced ICAM-1 expression through the NF-κB pathway.

Enhanced TNFα Induction of ICAM-1 in Endothelial Cells Depends on Neutrophil NAD(P)H Oxidase—Because endothelial cells express ICAM-1, we address the possibility in a endo-
thelial cell/PMN co-culture system whether the PMN NAD(P)H oxidase complex could induce ICAM-1 in endothelial cells. We used MLVEC in which we measured ICAM-1 expression after TNFα stimulation. TNFα induced a gradual increase in ICAM-1 expression during a 1- to 4-h period in both WT and p47phox−/− MLVEC (Fig. 5A). The level of ICAM-1 expression in p47phox−/− MLVEC at each time point was lower than at the corresponding time points in WT MLVEC, suggesting that endogenous endothelial NAD(P)H oxidase is also involved in regulating TNFα-induced ICAM-1 expression. However, co-culture of either WT or p47phox−/− MLVEC with WT PMN caused a rapid and augmented ICAM-1 expression in response to TNFα in both groups (Fig. 5A). To exclude the possibility that the increased ICAM-1 expression resulted from the ICAM-1 present in PMN, we also studied PMN obtained from ICAM-1−/− mice and used these in the co-culture experiment. As shown in Fig. 5A, addition of the ICAM-1−/− PMN still induced an augmented ICAM-1 expression in MLVEC, indicating that the increased ICAM-1 expression in MLVEC was the result of signals emanating from the PMN.

To address the role of oxidants derived from PMN in TNFα-induced ICAM-1 expression in endothelial cells, GSH (5 mM) was applied to the co-culture system. In biological systems, O2− is rapidly reduced by superoxide dismutase to H2O2, and GSH serves as an effective H2O2 scavenger. As shown in Fig. 5B, GSH eliminated the effect of PMN in increasing ICAM-1 expression in TNFα-stimulated MLVEC.

**Adhesion Molecules Are Required for Neutrophil NAD(P)H Oxidase-induced NF-κB Activation and ICAM-1 Expression in Endothelial Cells**—The data presented above in mouse

---

**Fig. 4.** A, Western blot showing the effect of PMN depletion and repletion on expression of ICAM-1 protein in the lung tissue of WT and p47phox−/− animals 2 h after TNFα (i.p.). Neutrophil depletion was performed with rabbit anti-mouse neutrophil serum (150 μl, i.p.) 16 h before injection of TNFα. Neutrophil repletion was performed to the neutropenia mice with the neutrophils isolated from WT mice whole blood in the amount of 1 × 106 cells in 150 μl of saline solution via tail vein. Actin was detected for normalizing the densitometry of ICAM-1. The graph depicts the mean and S.E. from three mice. The asterisks show p < 0.01 compared with WT/TNFα (lane 2). B, representative Western blot showing PMN from p47phox−/− mice failed to restore ICAM-1 expression in the lungs of WT neutropenia mice. PMN depletion and repletion as described in A. The lungs are harvested at 2 h after TNFα i.p. C, Western blot showing the effect of PMN depletion and repletion on degradation of IxBα in the non-nuclear fraction of lungs in WT and p47phox−/− animals 0.5 h after TNFα, i.p. The graph depicts the mean and S.E. of densitometry of IxBα, which normalized by corresponding actin density, from three mice. The asterisks show p < 0.01 compared with WT control group (lane 1).
Figs. 5 and 6. The changes in ICAM-1 expression were correlated with adhesion of PMNs to endothelial cells (MLVEC). However, anti-CD18 Ab resulted in a lesser extent by E-selectin. PMN to endothelial cells (MLVEC) was assessed. As shown in Fig. 5, it is evident that endothelial cells induced by PMN requires an adhesive interaction mediated by CD18 and expression of ICAM-1 in endothelial cells induced by PMN contributes to the oxidant signals to the endothelial cell. To determine the role of adhesive interactions in mediating the PMN-dependent NF-κB activation and ICAM-1 expression in endothelial cells, we studied the effects of anti-E-selectin and anti-CD18 Abs (both from Santa Cruz Biotechnology, Santa Cruz, CA). As shown in Fig. 6, at 2 h after TNFα, anti-E-selectin Ab caused a ~26% reduction in ICAM-1 expression in MLVEC. However, anti-CD18 Ab resulted in a ~68% reduction of ICAM-1 expression in MLVEC. The changes in ICAM-1 expression were correlated with alterations in NF-κB nuclear translocation at 1 h after TNFα as shown in Fig. 6. Thus, the augmented activation of NF-κB and expression of ICAM-1 in endothelial cells induced by PMN requires an adhesive interaction mediated by CD18 and to a lesser extent by E-selectin.

As shown in Fig. 1, it is evident that NAD(P)H oxidase deficiency did not block NF-κB translocation and ICAM-1 mRNA and protein expression in response to TNFα at the later time points (4 and 6 h after TNFα), suggesting that there is also an NAD(P)H oxidase-independent mechanism responsible for ICAM-1 expression. To address the time-dependent role of NAD(P)H oxidase after TNFα challenge, the adhesion of mouse PMNs to endothelial cells (MLVEC) was assessed. As shown in Fig. 7, at time = 0, there was similar basal adhesion of PMN from WT and p47phox −/− to MLVEC. At 1 and 2 h after TNFα stimulation, there were 2.4- and 3.2-fold increases in adhesion of WT PMN to MLVEC. This adhesion response was dependent on the expressed ICAM-1, because it was blocked by the anti-ICAM-1 Ab, whereas control IgG had no effect. In contrast, adhesion of PMN from p47phox −/− mice to MLVEC failed to increase at 1 h after TNFα stimulation, and was increased 1.6-fold at 2 h after TNFα. However, at 4 and 6 h, when WT PMN exhibited 4.3- and 4.6-fold increases in adhesion, respectively, the p47phox −/− PMN showed similar increases in adhesion of 4.2- and 4.6-fold increase in adhesion, respectively. These data indicate an important role of the PMN NAD(P)H oxidase system in the early phase (up to 2 h) of the TNFα-induced PMN adhesion response, which paralleled the early ICAM-1 expression.

**DISCUSSION**

TNFα has been shown to play an important role in the adhesion of neutrophils to endothelial cells (1, 13). Firm neutrophil adhesion requires the activation of NF-κB and the increased expression of ICAM-1 in endothelial cells (24, 26, 27). Oxidant production is considered as a key event in mediating the TNFα-induced NF-κB activation and initiation of NF-κB-dependent transcription of ICAM-1 (13, 28, 29); however, the fundamental question of whether oxidants derived from adherent neutrophils can contribute to and thus amplify TNFα-induced ICAM-1 expression in endothelial cells remains to be addressed. In the present study, we show a novel function of phagocytic NAD(P)H oxidase in TNFα-induced NF-κB signal-

---

**References:**

1. Fig. 5 A. Effect of PMN co-culture on MLVEC ICAM-1 protein expression in response to TNFα. MLVECs were isolated and cultured as described under "Experimental Procedures" and treated with TNFα (500 units/ml) for the times indicated. Co-cultured PMNs were isolated from WT and ICAM-1 −/− mice, respectively, and were in the concentration of 1 × 10⁶ cells/ml. At the end of incubation with TNFα, MLVECs were washed with HBSS for three times and followed by cell lysis using lysis buffer. The cell lysates were then subjected to Western analysis with anti-ICAM-1 antibody. B. Representative Western blot showing the effect of GSH on PMN-mediated enhanced ICAM-1 expression in MLVEC. Confluent MLVECs were treated with TNFα (500 units/ml) in the presence or absence of WT PMN (1 × 10⁶ cells/ml) and/or GSH (5 mM) for 2 h, followed by washing with HBSS for three times, and subsequent Western analysis.

2. Fig. 6 Effect of anti-E-selectin and anti-CD18 Abs on PMN-mediated enhanced ICAM-1 expression and NF-κB nuclear translocation in MLVEC. Confluent MLVECs were treated with TNFα (500 units/ml) in the presence or absence of WT PMN (1 × 10⁶ cells/ml) and/or either anti-E-selectin Ab or anti-CD18 Ab for 2 h, followed by washing with HBSS for three times, and cell lysis. Western blot was performed with the cell lysates, and EMSA was performed using the nuclear protein extracted from the lysates. E = anti-E-selectin Ab; CD = anti-CD18 Ab. The figure is representative of three studies.
NAD(P)H Oxidase Contributes to TNFα-induced NF-κB

As shown in Fig. 5, WT neutrophils induced a rapid and augmented increase in ICAM-1 expression in both WT and p47phox−/− MLVEC, suggesting a direct interaction between neutrophils and MLVEC. Addition of the antioxidant GSH to the co-culture prevented the effect of WT neutrophil in inducing ICAM-1 expression in endothelial cells, indicating that the interaction between neutrophils responsible for ICAM-1 expression in MLVEC is mediated through oxidants. Thus, the early phase of neutrophil adhesion that was ICAM-1-dependent, because anti-ICAM-1 antibody prevented the response. In contrast, p47phox−/− neutrophils failed to adhere at these early time points consistent with the hypothesis that exogenously released neutrophil oxidants were critical for the early-onset ICAM-1 expression and resultant neutrophil adhesion. However, by 4 and 6 h after TNFα challenge, there was no difference in neutrophil adhesion between the WT and p47phox−/− neutrophils. Thus, it appears that the early phase of neutrophil adhesion is dependent on expression of ICAM-1 secondary to the release of neutrophil NAD(P)H oxidase-derived oxidants, whereas the latent ICAM-1-dependent neutrophil adhesion response is the result of other factors such as activation of the endothelial oxidant signaling machinery. Fig. 8 shows a model for the interaction of neutrophils and endothelial cells and the basis of neutrophil NAD(P)H oxidase-induced transcription of ICAM-1 in endothelial cells. TNFα has been reported to activate neutrophil NAD(P)H oxidase through stimulation of tyrosine kinases (35, 36). On the basis of our data, we postulate that reactive oxygen species generated by neutrophil NAD(P)H oxidase serve a signaling function in activating NF-κB in endothelial cells.

Although oxidants are involved in the NF-κB signal transduction pathways, the molecular targets have not yet been defined. The contribution of redox regulation and location of potential redox-sensitive sites within the NF-κB activation pathway are the subject of controversy (37). It has been reported that NF-κB itself is a molecular target for redox regulation (38, 39). DNA binding activity of NF-κB can be regulated through reduction of a disulfide bond involving the cysteine residue 62 in p50 by thioredoxin (TRX) (38). Takeuchi and associates (40) showed that TRX inhibited TRAF2-, TRAF5-, and TRAF6-induced NF-κB activity but did not affect NIK-,
NAD(P)H Oxidase Contributes to TNFα-induced NF-κB

FIG. 3. Hypothetical model for interaction of the neutrophil and endothelial cell. TNFα stimulation results in NAD(P)H oxidase activation and production of reactive oxygen species in neutrophils, as well as initiation of NF-κB signaling in endothelial cell. Adhesion of neutrophils to endothelial cells is primarily mediated by binding of constitutive ICAM-1 to CD18 and provides the appropriate coupling required for neutrophils to transmit oxidant signals to endothelial cells. The oxidants activate NF-κB and ICAM-1 expression. Neutrophil NAD(P)H oxidase mediates these responses through its ability to activate NIK. The results indicate that the endothelial cell is an important target for the neutrophil NAD(P)H oxidase activity resulting in stable endothelial expression of ICAM-1. The functional relevance of neutrophil NAD(P)H oxidase-induced endothelial ICAM-1 expression may be to induce the early-onset firm neutrophil adhesion and enable the rapid migration of neutrophils across the vessel wall to site of infection.

REFERENCES
1. Diamond, M. S., Staunton, D. E., Marlin, S. D., and Springer, T. A. (1991) Cell 65, 961–971
2. Carlos, T. M., and Harlan, J. M. (1994) Blood 84, 2068–2101
3. Hou, J., Baichwal, V., and Cao, Z. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11641–11645
4. Ledebur, H. C., and Parks, T. P. (1995) J. Biol. Chem. 270, 933–943
5. Hou, H., Shu, H. B., Pan, M. G., and Goeddel, D. V. (1996) Cell 84, 299–308
6. Liu, Z. G., Hou, H., Goeddel, D. Y., and Karin, M. (1996) Cell 87, 565–576
7. Hou, H., Huang, J., Shu, H. B., Baichwal, V., and Goeddel, D. V. (1996) Immunity 4, 397–396
8. Malinin, N. L., Boldin, M. P., Kovalenko, A. V., and Wallach, D. (1997) Nature 385, 540–544
9. May, M. J., and Ghosh, S. (1998) Immunol Today 19, 80–88
10. Baeuerle, P. A., and Baichwal, V. R. (1997) Adv. Immunol. 61, 111–137
11. Ashkenazi, A., and Diciz, V. M. (1998) Science 281, 1305–1308
12. Buttke, T. M., and Sandstrom, P. A. (1994) Immunol Today 15, 7–10
13. True, A. L., Rahman, A., and Malik, A. B. (2000) Am. J. Physiol. 278, L302–L311
14. Peng, Q., Wei, Z., and Lau, B. H. (2000) Cell Mol. Life Sci. 57, 834–841
15. Manns, S. K., Mukhopadhyay, A., and Aggarwal, B. B. (2000) J. Immunol. 164, 6569–6519
16. Balazovich, K. J., Suchard, S. J., Remick, D. G., and Boxer, L. A. (1996) Blood 88, 690–696
17. Bevan, B. M. (1999) Blood 93, 1464–1476
18. Jackson, S. H., Gallin, J. I., and Holland, S. M. (1995) J. Exp. Med. 182, 751–758
19. Paluck, J. D., Williams, D. A., Gifford, M. A., Du, X., Fisherman, J., Orkin, S. H., Doerschuk, C. M., and Dinauer, M. C. (1995) Nat. Genet. 9, 202–209
20. Szecs, S., Varga, C., Ember, I., and Kertai, P. (1994) J. Immunol. Methods 167, 245–251
21. Hohler, B., Holzapfel, B., and Kummer, W. (2000) Histochem. Cell Biol. 114, 29–37
22. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
23. Deryckere, F., and Gannon, F. (1994) BioTechniques 16, 405
24. Lo, S. K., Janakidevi, K., Lai, L., and Malik, A. B. (1993) Am. J. Physiol. 264, L406–L412
25. Paxton, L. L., Li, J. L., Serc, V., Duff, J. L., Naik, S. M., Shibagaki, N., and Caughman, S. W. (1997) J. Biol. Chem. 272, 15928–15935
26. Korn, S. H., Wouters, E. F., Vos, N., and Janssen-Heininger, Y. M. (2001) J. Biol. Chem. 276, 35106–35113
27. Manna, S. K., Mukhopadhyay, A., and Aggarwal, B. B. (2000) J. Immunol. 162, 6569–6519
28. Balazovich, K. J., Suchard, S. J., Remick, D. G., and Boxer, L. A. (1996) Blood 88, 690–696
29. Baeuerle, P. A., and Baichwal, V. R. (1997) Adv. Immunol. 61, 111–137
30. Balazovich, K. J., Suchard, S. J., Remick, D. G., and Boxer, L. A. (1996) Blood 88, 690–696
31. Yoshida, M., Korfhagen, T. R., and Whitsett, J. A. (2001) Am. J. Physiol. 281, L406–L412
32. Satinian, J. A., Shuldiner, M., Hora, K., Xing, Y., Shan, Z., and Schindler, D. F. (1993) J. Clin. Invest. 92, 1564–1571
33. Hubbard, A. K., and Rothlein, R. (2000) Free Radic. Biol. Med. 28, 1379–1386
34. Dustin, M. L., and Springer, T. A. (1988) J. Cell Biol. 107, 321–331
35. Dusi, S., Della Bianca, V., Donini, M., Nadalini, A. K., and Rossi, F. (1996) J. Immunol. 157, 4615–4623
36. Schonberg, M. A., Leung, C. C., and Raffin, T. A. (1995) J. Biol. Chem. 270, 13124–13132
37. Bowie, A., and O’Neill, L. A. (2000) Biochem. Pharmacol 59, 13–23
38. Matthews, J. R., Wouters, E. F., Vos, N., and Janssen-Heininger, Y. M. (2001) J. Biol. Chem. 276, 35593–35700
39. Hayashi, T., Ueno, Y., and Okamoto, T. (1993) J. Biol. Chem. 268, 11380–11388
40. Takeuchi, J., Hirota, K., Itoh, T., Shinkura, R., Kitada, K., Yodoi, J., Namba, T., and Fukuda, K. (2000) Antioxid. Redox Signal. 2, 83–92
41. Korn, S. H., Wouters, E. F., Vos, N., and Janssen-Heininger, Y. M. (2001) J. Biol. Chem. 276, 35593–35700
42. Science 244, 357–359
Role of Neutrophil NADPH Oxidase in the Mechanism of Tumor Necrosis Factor-α-induced NF-κB Activation and Intercellular Adhesion Molecule-1 Expression in Endothelial Cells

Jie Fan, Randall S. Frey, Arshad Rahman and Asrar B. Malik

J. Biol. Chem. 2002, 277:3404-3411.
doi: 10.1074/jbc.M110054200 originally published online November 29, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M110054200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 42 references, 17 of which can be accessed free at http://www.jbc.org/content/277/5/3404.full.html#ref-list-1