Superoxide, H$_2$O$_2$, and iron are required for TNF-α-induced MCP-1 gene expression in endothelial cells: role of Rac1 and NADPH oxidase

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monocyte chemoattractant protein-1; tumor necrosis factor-α; reactive oxygen species; endothelial cells

METHODS

Cell culture, DNA plasmids, and chemical reagents. Human aortic endothelial cells (HAECs) were obtained from Clonetics (San Diego, CA). HAECs were cultured in EGM-2 growth medium. Cells were used between passages 5 and 9. Human dermal microvascular endothelial cells (HMECs) were obtained as described previously (46) and were cultured in modified MCDB 131 (GIBCO-BRL) supplemented with 10% fetal bovine serum and EGM single quote (Clonetics). All cells were maintained at 37°C in a 5% CO$_2$ incubator. Enh-MCP1/Luc, which contains five tandem copies of β-globulin NF-κB sequences linked to a luciferase (Luc) reporter gene, was the generous gift of Dr. Atsuhisa Ueda (Yokohama University, Yokohama, Japan) (42). 5xNF-κB/Luc, which contains five tandem copies of β-globulin NF-κB sequences linked to a luciferase (Luc) reporter gene, was purchased from Promega. Diphenylcyclophosphamide (DPI) was obtained from Toronto Research Chemicals; TNF-α was obtained from Boehringer-Mannheim; 5,10,15,20-tetrakis-(4-sulfonatophenyl)-H$_2$O$_2$, and iron are required for TNF-α-induced MCP-1 gene expression in endothelial cells: role of Rac1 and NADPH oxidase

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prophyrinato iron (III) chloride (FeTPPS) and 1,2-dimethyl-3-hydroxyypyridin-4-one (DMHP) were obtained from Calbiochem; and N,N-dimethylthiourea (DMTU) and dimethyl sulfoxide (Me2SO) were purchased from Sigma.

Preparation of RNA and Northern blot analysis. Total cellular RNA was isolated by a single extraction with TriPure reagent (Roche Diagnostic) and size fractionated using 1% agarose formaldehyde gels. RNA was transferred to nitrocellulose membranes, and hybridizations were performed as described previously (7). The cDNAs used were human MCP-1 and GAPDH CDNA, as described previously (25). Autoradiography was performed with a PhosphorImager 445sI (Molecular Dynamics; Sunnyvale, CA). MCP-1 and GAPDH mRNA levels were also determined with a Quantikine mRNA colorimetric quantification kit (R&D Systems) according to the manufacturer’s instructions. Total RNA samples (2 μg) were hybridized with MCP-1 or GAPDH gene-specific biotin-labeled capture oligonucleotide probes and digoxigenin-labeled detection probes in a 96-well microplate. The hybridization solution was then transferred to a streptavidin-coated microplate, and the RNA-probe hybrids were captured. After a wash to remove unbound material, substrate solution was added. An amplification solution was then added and color developed in proportion to the amount of gene-specific MCP-1 or GAPDH mRNA in the original samples.

Determination of MCP-1 protein levels. HAECs cultured in 24-well plates were infected with Ad.LacZ or Ad.N17Rac1 for 24 h. Cells were then washed, and fresh medium was added. HAECs were left untreated or treated with TNF-α (100 U/ml), and the medium was collected at 16 h. MCP-1 protein levels were determined by quantitative colorimetric sandwich ELISA using a human MCP-1 assay kit according to the manufacturer’s instructions (R&D Systems).

Determination of cell surface expression of ICAM-1 by ELISA. HAECs were plated in 24-well plates and incubated with TNF-α (100 U/ml) for 16 h. Primary mouse antibody for ICAM-1 was obtained from Southern Biotechnology Associates. Cell surface expression of ICAM-1 was determined by primary binding with specific mouse antibodies, followed by secondary binding with a horseradish peroxidase-conjugated goat anti-mouse IgG antibody. Quantification was performed by the determination of colorimetric absorbance at an optical density of 450 nm in triplicate and renilla luciferase activity.

Adenoviruses. The adenoviruses encoding myc-tagged cDNA of dominant negative N17Rac1 (Ad.N17Rac1) and CDNA of human Cu/Zn SOD (Ad.SOD) and human catalase (Ad.Catalase) were generous gifts of Toren Finkel (National Institutes of Health) and have been previously described (7, 29, 37, 38). The viruses were amplified in HEK-293 cells and purified on double cesium gradients. Infection was carried out with the indicated multiplicity of infection (MOI) for 18 h, after which the infection medium was aspirated and replaced with fresh medium. Ad.LacZ, an adenovirus encoding the Escherichia coli LacZ gene, was used as a control for adenovirus infection. The ability of infected HAECs to express N17Rac1 was assessed by Western blot analysis via the myc epitope tag using mouse monoclonal anti-myc antibody 9E10, which recognizes the myc peptide only on fusion proteins and not in endogenous myc proteins (Santa Cruz BioTechnology).

Western blot analysis. HAECs were lysed for 30 min on ice in 1 ml of lysis buffer containing 0.5% Nonidet P-40, 50 mM HEPES (pH 7.3), 150 mM NaCl, 2 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium orthovanadate, and 1 mM NaF. Protein samples (15 μg) were subjected to electrophoresis by 10% or 15% SDS-PAGE and transferred to a nitrocellulose membrane. Antibody-bound protein bands were then visualized via horseradish peroxidase-dependent chemiluminescence (Amersham). Anti-rabbit and anti-catalase antibodies were purchased from CalBiochem.

Transfection and assay of reporter gene activity. Because HAECs are relatively resistant to efficient transient transfection, we used HMECs for these experiments. HMECs were grown to 60–70% confluence in six-well plates and transfected with various plasmids (as indicated in the figures) using SuperFect transfection reagent according to the manufacturer’s instructions (Qiagen). After a 24-h recovery, HMECs were exposed to TNF-α (100 U/ml) for 16 h. CAT activity was determined as previously described (7). pRL-TK (renilla luciferase constitutively expressed under the control of thymidine kinase) was used to normalize transfection efficiency. Firefly and renilla luciferase activities were measured using a luciferase reporter system according to the manufacturer’s instructions (Promega). All CAT activities were normalized against the renilla luciferase activity.

Determination of cytotoxicity through lactate dehydrogenase assay. After treatment of HAECS with TNF-α plus various compounds, media were collected. The cytotoxicity was determined by measuring the levels of lactate dehydrogenase (LDH) in the medium using a CytoTox 96 Nonradioactive Cytotoxicity Assay according to the manufacturer’s instructions. LDH is a stable cytosolic enzyme that is released upon cell lysis.

Statistical analysis. Values are expressed as means ± SD of at least three experiments. Statistics were performed using an unpaired Student’s t-test, and values were considered significantly different at the 95% confident levels.

RESULTS

Expression of SOD inhibits TNF-α-induced MCP-1 mRNA accumulation in HAECs. To determine whether O2⁻ is required for TNF-α-activated MCP-1 gene expression, HAECs were infected with the SOD expression vectors Ad.SOD or Ad.LacZ (MOI of 100) for 24 h. HAECs were then treated with TNF-α (100 U/ml) for 4 h. SOD is responsible for the conversion of O2⁻ to H2O2. By Western blot analysis, the Cu/Zn SOD protein level was not detectable in noninfected cells. Infection of HAECs with Ad.SOD resulted in a dramatic increase in the intracellular Cu/Zn SOD protein level (Fig. 1A). By Northern blot analysis, the TNF-α-induced increase in the MCP-1 mRNA level was inhibited by Ad.SOD (Fig. 1B). Expression of SOD also produced a dose-dependent inhibition of MCP-1 protein secretion stimulated by TNF-α (Fig. 1C). These data suggest that O2⁻ is required for TNF-α-induced MCP-1 gene expression in endothelial cells.

Expression of catalase inhibits TNF-α-induced MCP-1 mRNA accumulation in HAECS. O2⁻ generated in cells can be converted spontaneously or enzymatically into H2O2. To determine the role of H2O2 in the regulation of MCP-1 gene expression, we used an adenovirus expressing catalase, which can convert O2⁻ into H2O2. HAECs were infected with Ad.Catalase or Ad.LacZ (MOI of 100) for 24 h, followed by treatment with TNF-α (100 U/ml) for 4 h. Infection of HAECs with Ad.Catalase for 24 h resulted in a fourfold increase in the intracellular catalase protein level by Western blot analysis (Fig. 2A). By Northern blot analysis, infection with Ad.Catalase inhibited the TNF-α-induced MCP-1 mRNA level (Fig. 2B). Similarly, infection with Ad.Catalase produced a dose-dependent inhibition of TNF-α-induced MCP-1 protein secretion (Fig. 2C). These data suggest that H2O2 is involved in TNF-α-induced MCP-1 gene expression.

The iron chelator DMHP suppresses TNF-α-induced MCP-1 mRNA accumulation in HAECs. There is a growing body of evidence that intracellular iron may be involved in stress signaling (12, 22). Transition iron is involved in the generation of HO• from H2O2 (12). DMHP is a cell-permeable iron chelator, and it is able to specifically deplete intracellular
Ad.LacZ. inhibits TNF-α/H9251

Me 2 SO. As shown in Fig. 4

HO /HAECs. produced a dose-dependent inhibition of TNF-α/H9251

ments showed similar results.

mRNA levels were determined by Northern blot analysis. Two independent experi-

tions showed similar results. C: HAECs were infected with Ad.LacZ or Ad.SOD (MOI of 100) or Ad.SOD (MOI of 25, 50, and 100) for 24 h and then exposed to TNF-α (100 U/ml) for 16 h. Conditioned media were collected and MCP-1 protein levels were determined as described in METHODS. Values represent means ± SD; n = 4. *P < 0.05 compared with TNF-α-treated cells infected with Ad.LacZ.

A

Fig. 1. Expression of SOD suppresses TNF-α-induced monocyte chemoattractant protein (MCP)-1 mRNA accumulation. A: whole cell lysates from human aortic endothelial cells (HAECs) infected with Ad.SOD (multiplicity of infection (MOI) of 100) for 24 h were analyzed by immunoblotting with antibodies to SOD or β-actin. B: HAECs were infected with Ad.LacZ or Ad.SOD (MOI of 100) for 24 h and then exposed to TNF-α (100 U/ml) for 4 h. MCP-1 mRNA levels were determined by Northern blot analysis. Two independent experiments showed similar results. C: HAECs were infected with Ad.LacZ (MOI of 100) or Ad.SOD (MOI of 25, 50, and 100) for 24 h and then exposed to TNF-α (100 U/ml) for 16 h. Conditioned media were collected and MCP-1 protein levels were determined as described in METHODS. Values represent means ± SD; n = 4. *P < 0.05 compared with TNF-α-treated cells infected with Ad.LacZ.

iron (15). To investigate the role of iron in the regulation of MCP-1 gene expression, we pretreated HAECs with the iron chelator DMHP (1–10 mM). As shown in Fig. 3, DMHP produced a dose-dependent inhibition of TNF-α-induced MCP-1 protein (A) and mRNA levels (B). In contrast, treatment with DMHP had no effect on the TNF-α-induced cell surface expression of ICAM-1 (data not shown). These data suggest that iron or an iron-containing factor may play an important role in TNF-α-induced MCP-1 gene expression. Treatment of HAECs with DMHP at 10 mM for 16 h did not result in any change in LDH levels in the medium (Fig. 3C).

Scavenging of hydroxyl radicals by DMTU and Me 2 SO inhibits TNF-α-induced MCP-1 mRNA accumulation in HAECs. HO· is generated by the reduction of H₂O₂ or through ONOO·. We investigated the role of HO· in TNF-α-induced MCP-1 gene expression using the HO· scavengers DMTU and Me 2 SO. As shown in Fig. 4A, pretreatment with DMTU (5 and 10 mM) produced a dose-dependent inhibition of MCP-1 mRNA accumulation. Similarly, treatment of HAECs with another HO· scavenger, Me 2 SO, suppressed TNF-α-induced MCP-1 protein secretion in a dose-dependent manner (Fig. 4B). In contrast, treatment with DMTU or Me 2 SO had no effect on the TNF-α-induced cell surface expression of ICAM-1 (data not shown). These data suggest that HO· may be involved in TNF-α-induced MCP-1, but not ICAM-1, gene expression. Treatment of HAECs with 10 mM DMTU or 1% Me 2 SO for 16 h did not result in any change in LDH levels in the medium (data not shown).

Scavenging of ONOO· by FeTPPS did not suppress TNF-α-induced MCP-1 mRNA accumulation in HAECs. O₂·⁻ can rapidly react with nitric oxide (NO), yielding another reactive species, ONOO·. To investigate the role of ONOO· in TNF-α-induced MCP-1 gene expression, we used the ONOO· scavenger FeTPPS, which catalyzes the isomerization of ONOO· to nitrate anion and thereby decreases its decomposition to highly reactive intermediates such as nitrogen dioxide and hydroxyl radicals (27). Pretreatment of HAECs with FeTPPS (50 and 100 μM) did not suppress TNF-α-induced MCP-1 gene expression (Fig. 4A), suggesting that ONOO· is not involved in TNF-α’s signaling in MCP-1 gene expression.

The NADPH oxidase inhibitor DPI inhibits TNF-α-induced MCP-1 mRNA accumulation in HAECs. The NADPH oxidase system is responsible for the transfer of electrons from NADPH to molecular oxygen with the subsequent production of superoxide anion. Pretreatment of HAECs with DPI (50 and 100 μM) did not suppress TNF-α-induced MCP-1 mRNA accumulation in HAECs.

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DPI is an inhibitor of flavine-binding proteins such as NADPH oxidase. We (41) have previously reported that treatment of HAECs with DPI suppressed TNF-α-induced O2- production and VCAM-1 mRNA accumulation. HAECs were pretreated with or without DPI (20 and 40 μM) for 1 h and then treated with TNF-α (100 U/ml) for 4 h. Pretreatment with DPI produced a dose-dependent inhibition of TNF-α-induced MCP-1 mRNA accumulation (Fig. 5). These data suggest that flavin-binding proteins such as NADPH oxidase are involved in the TNF-α-induced MCP-1 gene expression.

Dominant negative N17Rac1 inhibits TNF-α-induced MCP-1 gene expression in HAECs. Rac1 regulates NADPH oxidase activity (2). Activation of Rac1 by cytokines is associated with increased production of O2- in a variety of cell types, including endothelial cells (29, 36, 37). To investigate the role of Rac1 in TNF-α-activated MCP-1 gene expression, we used a recombinant adenovirus expressing a dominant negative mutant, N17Rac1. HAECs were infected with Ad.LacZ or Ad.N17Rac1 (MOI of 100) for 24 h. By Western blot analysis, the myc-tagged N17Rac1 protein level was marked increased after infection with Ad.N17Rac1, whereas HAECs mock infected or infected with Ad.LacZ had no myc-N17Rac1 protein expression (Fig. 6A). To determine the role of Rac1 in the regulation of MCP-1 expression, HAECs infected with Ad.N17Rac1 (MOI of 25, 50, and 100) or Ad.LacZ (MOI of 100) for 24 h were treated for 4 h with TNF-α (100 U/ml). As shown in Fig. 6B, the TNF-α-induced increase in MCP-1 mRNA accumulation is suppressed by the hydroxyl radical scavenger N,N-dimethylthiourea (DMTU) and Me2SO but not by the peroxynitrite scavenger 5,10,15,20-tetakis-(4-sulfonatophenyl)porphyrinato iron (III) chloride (FeTPPS). A: HAECs were pretreated with DMTU (10 or 5 mM) or FeTPPS (100 or 50 μM) for 1 h and then exposed to TNF-α (100 U/ml) for 3 h. MCP-1 mRNA levels were determined by Quantikine mRNA kits and normalized to GAPDH levels. B: HAECs were pretreated with Me2SO (0.5 or 1%) for 1 h and then exposed to TNF-α (100 U/ml) for 4 h. Conditioned media were collected and MCP-1 protein levels were determined as described in METHODS. Values represent means ± SD; n = 4. *P < 0.05 compared with TNF-α alone-treated cells.

Fig. 3. The iron chelator 1,2-dimethyl-3-hydroxypyridin-4-one (DMHP) suppresses TNF-α-induced MCP-1 protein secretion and mRNA accumulation. A: HAECs were pretreated the iron chelator DMHP (1–10 mM) for 4 h and then exposed to TNF-α (100 U/ml) for 4 h. Conditioned media were collected and MCP-1 protein levels were determined as described in METHODS. B: HAECs were pretreated with the iron chelator DMHP (5 or 10 mM) for 4 h and then exposed to TNF-α (100 U/ml) for 3 h. MCP-1 mRNA levels were determined by Quantikine mRNA kits and normalized to GAPDH levels. C: HAECs were pretreated with the iron chelator DMHP (10 mM) for 4 h and then exposed to TNF-α (100 U/ml) for 16 h. Lactate dehydrogenase (LDH) levels in the medium were determined by using a CytoTox96 Cytotoxicity Assay. Values represent means ± SD; n = 4. *P < 0.05 compared with TNF-α alone-treated cells.
mRNA levels was inhibited by dominant negative Ad.N17Rac1 in a dose-dependent manner. Similarly, dominant negative N17Rac1 suppressed the TNF-α-induced increase in MCP-1 protein secretion (Fig. 6C). These data suggest that Rac1 is involved in TNF-α-induced MCP-1 gene expression.

Dominant negative N17Rac1 suppresses TNF-α-induced transactivation of NF-κB-driven promoters and MCP-1 promoter. The promoter regions in the MCP-1 gene contains three recognition sites for the transcription factor NF-κB (42). To explore whether N17Rac1 inhibits NF-κB activation in HMECs, we transiently transfected HMECs with a 5×NF-κB/Luc plus N17Rac1 expression vector. HMECs were treated with TNF-α (100 U/ml) for 16 h. As shown in Fig. 7A, TNF-α induced a marked increase in NF-κB promoter activity in the presence of an empty vector. Expression of dominant negative N17Rac1 dramatically inhibited TNF-α-induced transactivation of NF-κB promoter activity (Fig. 7A). These data suggest that the inhibition of Rac1 suppresses TNF-α-induced transactivation of NF-κB.

To investigate whether dominant negative N17Rac1 can inhibit MCP-1 gene transcription, Enh-MCP1/CAT was co-transfected with an expression vector for N17Rac1. As expected, TNF-α induced a marked increase in Enh-MCP1/CAT promoter activity in the presence of an empty vector (Fig. 7B). Expression of dominant negative Rac1 inhibited TNF-α-induced transactivation of Enh-MCP1/CAT promoter activity (Fig. 7B). These data suggest that dominant negative Rac1 suppresses TNF-α-induced MCP-1 gene transcription at least in part through inhibition of NF-κB transactivation in endothelial cells.

DISCUSSION

Using multiple complementary molecular and biochemical interventions, we explored the relative roles of the specific oxidants involved in MCP-1 gene expression in endothelial cells. Our finding that TNF-α-induced MCP-1 gene expression can be suppressed by SOD provides the first direct evidence that O_{2}^{-} is involved in redox-sensitive regulation of MCP-1 gene expression. O_{2}^{-} may participate in the signaling pathway by generating downstream radicals such as HO•. O_{2}^{-}• stimulates HO-production through the Haber-Weiss reaction by acting as the rate-limiting electron donor for a preexisting pool of free iron (12). O_{2}^{-} also promotes HO-formation by elevating free iron levels (17). Similarly, Volk and co-workers (44) reported that
treatment of endothelial cells with iron, a superoxide scavenger and antioxidant (19), inhibited cytokine-induced ROS generation and MCP-1 release. These findings are consistent with our early study (8) showing that expression of SOD suppresses TNF-α-induced VCAM-1, ICAM-1, and E-selectin gene expression, suggesting that O$_2^{-}$ may serve as a common early signal for the activation of these inflammatory gene expression.

Our study also demonstrated that H$_2$O$_2$ is required for activation of MCP-1 gene expression. In contrast, we reported early that expression of catalase only partially suppressed TNF-α-induced E-selectin gene expression but had no effects on VCAM-1 and ICAM-1 gene expression (8). These findings suggest that the signaling pathway is diverged after the initial oxidant signal of O$_2^{-}$, where H$_2$O$_2$ mediates MCP-1, but not VCAM-1 and ICAM-1, gene expression. H$_2$O$_2$, by itself, is a poorly reactive oxidant. However, H$_2$O$_2$ can react with intracellular iron through the Haber-Weiss reaction to form HO-. HO- is cytotoxic by virtue of its ability to initiate lipid peroxidation, damage membranes, oxidize sulfhydryl compounds, and inactivate enzymes and transports (12). The suppression of MCP-1 gene activation by a HO- scavenger suggests that HO-may mediate TNF-α-induced inflammatory gene expression.

Intracellular iron plays a role in regulating gene expression (11, 32). The induction of MCP-1 by TNF-α was completely inhibited by DMHP, suggesting that iron or iron-containing cellular protein is involved in MCP-1 expression stimulated by TNF-α. Iron is required for the generation of the potent oxidant HO- through the iron-catalyzed Haber-Weiss reaction. In this reaction, O$_2^{-}$ reduces iron, which in turn reduces H$_2$O$_2$ to form HO- (12). In addition to reducing iron, O$_2^{-}$ also univalently oxidizes the [4 Fe-4S] clusters of dehydratases, which causes the release of iron. The “free” iron, which is kept reduced by O$_2^{-}$ or other cellular reductants, then reduces H$_2$O$_2$ to form HO- (22). The inhibition of MCP-1 expression by iron depletion and a HO- scavenger suggests that iron-mediated generation of HO- via the Haber-Weiss reaction may mediate TNF-α-induced MCP-1 gene expression.

O$_2^{-}$ also rapidly reacts with NO, yielding another reactive species, ONOO$^-$ (33). ONOO$^-$ is highly reactive toward all classes of biomolecules, including proteins, lipids, and nucleic acids, and is a potential candidate for effecting the production of proinflammatory cytokines. In addition, ONOO$^-$ is capable of generating highly reactive HO$^-$ (33). A recent study (49) has indicated that ONOO$^-$ may function as an intracellular signal for the production of IL-8. Exogenous ONOO$^-$ has been shown to stimulate NF-κB activation in endothelial cells (9) and monocytes (26). ONOO$^-$ also activates expression of inducible NO synthase and IL-6 (9, 26). FeTPPS is a scavenger for ONOO$^-$ and catalyzes the isomerization of ONOO$^-$ to nitrate anion and thereby decreases its decomposition to highly intermediates such as nitrogen dioxide and hydroxyl radicals (27). In the present study, however, the scavenging of ONOO$^-$ by FeTPPS had no effect on TNF-α induced MCP-1 gene expression, suggesting that ONOO$^-$ may not be involved in MCP-1 gene upregulation. In support of these observations, it was reported that inhibition of generation of NO by the NO synthase inhibitor N-monomethyl-L-arginine had no effect on cytokine-induced MCP-1 generation in endothelial cells (44).

The present study demonstrates that Rac1 and downstream NADPH oxidase are involved in the regulation of MCP-1 gene expression in endothelial cells. We provide evidence that dominant negative Rac1 inhibits MCP-1 gene transcription and suppressed NF-κB-mediated transcription, suggesting that Rac1 mediated TNF-α-induced MCP-1 gene expression through an NF-κB-dependent pathway. It has been previously reported that Rac1 is required for nutrient deprivation-induced superoxide generation and MCP-1 production (23). These data also suggest that Rac1-mediated superoxide generation plays an important role in MCP-1 production.

Rac1 is involved in the activation of NADPH oxidase in endothelial cells (1) and is required for superoxide generation in response to inflammatory stimuli in a variety of cell types, including endothelial cells (10, 18, 31, 45, 47). Using the NADPH oxidase inhibitor DPI, we reported that flavin-binding proteins such as NADPH oxidase are required for TNF-α-induced O$_2^{-}$ generation and activation of VCAM-1 gene expression (41). In the present study, we used both an NADPH oxidase inhibitor and a dominant negative N17Rac1 expression vector to inhibit NADPH oxidase and demonstrated that TNF-α-induced MCP-1 gene expression is mediated by an NADPH oxidase-dependent pathway. These data suggest that NADPH oxidase-mediated generation of ROS plays an important role in MCP-1 gene expression in endothelial cells.

Our data suggest that activation of Rac1 and an NADPH oxidase-dependent pathway and O$_2^{-}$ generation are required for MCP-1 gene upregulation in endothelial cells. We further demonstrated that iron, an iron-containing factor, or iron-dependent generation of ROS is involved in TNF-α-induced inflammatory gene expression. Together, these findings provide a molecular link between Rac1, NADPH oxidase, and ROS in the signaling pathways that mediate TNF-α-mediated MCP-1 gene expression.

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REFERENCES