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Cu/Zn Superoxide Dismutase Plays Important Role in Immune Response[1]

Moshe Marikovsky,[2]* Vered Ziv,* Nava Nevo,† Catherine Harris-Cerruti,‡ and Ori Mahler§

Activation of macrophages leads to the secretion of cytokines and enzymes that shape the inflammatory response and increase metabolic processes. This, in turn, results in increased production of reactive oxygen species. The role of Cu/Zn superoxide dismutase (SOD-1), an important enzyme in cellular oxygen metabolism, was examined in activated peritoneal elicited macrophages (PEM) and in several inflammatory processes in vivo. LPS and TNF-α induced SOD-1 in PEM. SOD-1 induction by LPS was mainly via extracellular signal-regulated kinase-1 activation. Transgenic mice overexpressing SOD-1 demonstrated a significant increase in the release of TNF-α and of the metalloproteinases MMP-2 and MMP-9 from PEM. Disulfiram (DSF), an inhibitor of SOD-1, strongly inhibited the release of TNF-α, vascular endothelial growth factor, and MMP-2 and MMP-9 from cultured activated PEM. These effects were prevented by addition of antioxidants, further indicating involvement of reactive oxygen species. In vivo, transgenic mice overexpressing SOD-1 demonstrated a 4-fold increase in serum TNF-α levels and 2-fold stronger delayed-type hypersensitivity reaction as compared with control nontransgenic mice. Conversely, oral administration of DSF lowered TNF-α serum level by 4-fold, lowered the delayed-type hypersensitivity response in a dose-dependent manner, and significantly inhibited adjuvant arthritis in Lewis rats. The data suggest an important role for SOD-1 in inflammation, establish DSF as a potential inhibitor of inflammation, and raise the possibility that regulation of SOD-1 activity may be important in the treatment of immune-dependent pathologies. ***The Journal of Immunology*****, 2003, 170: 2993–3001.**

In vivo, oxygen radicals are produced as byproducts of normal oxidative metabolism (1). Hence, activated cells with increased metabolism produce more oxygen radicals. In addition, macrophages, which are phagocytic cells, produce and release reactive oxygen species (ROS)[3] in response to phagocytosis or stimulation with various agents. It has long been known that control of the intracellular redox environment is vital for proper cellular function. To protect themselves from the constant oxidative challenge, cells have developed defense mechanisms that ensure a proper balance between pro- and antioxidant molecules (2). Cu/Zn superoxide dismutase (SOD-1) is a key enzyme in the dismutation of superoxide radicals resulting from cellular oxidative metabolism into hydrogen peroxide (3). Because inflammation is characterized by macrophage activation, we examined the possibility that altered SOD-1 activity would affect the inflammatory process. We speculated that up-regulation of SOD-1 would increase the ability of macrophages to confront an increased level of ROS during inflammation, resulting in increased immune response. In contrast, inhibition of SOD-1 would diminish this ability, resulting in inhibition of the immune response. Disulfiram (DSF), a chelator of heavy metals and the active ingredient of the drug Antabuse used in aversion therapy for chronic alcoholism (4), inhibits SOD-1 in vivo (5). Recently, we demonstrated that transgenic mice overexpressing SOD-1 (Tg-SOD) have a higher angiogenic potential and that DSF inhibits SOD-1, induces oxidative stress and apoptosis in endothelial cells in vitro, and inhibits angiogenesis and tumor growth in vivo (6, 7).

LPS arrests macrophage proliferation and activates them to produce proinflammatory factors such as arachidonic acid metabolites, nitrogen intermediates, and cytokines, such as IL-1, IL-6, and TNF-α, which play important roles in the immune response (8, 9). TNF-α possesses bioactivities that are important in regulating the inflammatory response, including: inducing expression of adhesion molecules (10), and stimulating production of other inflammatory molecules, including IL-1, IL-6, platelet-derived growth factor, TGF, and arachidonic acid metabolites such as PGE_2 and prostacyclin. TNF-α also stimulates the production of reactive oxygen and nitrogen species by leukocytes (11, 12). Early induction of TNF-α therefore initiates a cascade of responses that contribute to the recruitment and activation of inflammatory cells and immune reactions.

In this study, we show that activation of macrophages by LPS or TNF-α increases SOD-1 level in cultured peritoneal elicited macrophages (PEM). We demonstrate that overexpression of SOD-1 increases the release of TNF-α, vascular endothelial growth factor (VEGF), and the metalloproteinases MMP-2 and MMP-9 from cultured PEM, and increases TNF-α levels in the serum, as well as the delayed-type hypersensitivity (DTH) reaction. We also show that inhibition of SOD-1 by DSF strongly inhibits the release of TNF-α, VEGF, and MMP-2, MMP-9 proteinases from cultured, activated PEM. To determine whether systemic administration of DSF would inhibit the immune response, three models were used. DSF lowered TNF-α serum levels in LPS-treated mice, lowered the DTH response in BALB/c mice, and significantly inhibited

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[3] Abbreviations used in this paper: ROS, reactive oxygen species; AA, adjuvant arthritis; DSF, disulfiram; DTH, delayed-type hypersensitivity; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; mTNF, murine TNF; NAC, *N*-acetyl-L-cysteine; ntg, nontransgenic; PEM, peritoneal elicited macrophage; p.o., per os; RA, rheumatoid arthritis; SOD-1, Cu/Zn superoxide dismutase; Tg-SOD, transgenic mice overexpressing SOD-1; VEGF, vascular endothelial growth factor.

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adjuvant arthritis (AA) in Lewis rats. The data suggest an important role for SOD-1 in inflammation, establish DSF as a potential inhibitor of inflammation, and suggest that alterations in the activity of SOD-1 may affect the immune response and pathologies in which inflammation is involved.

Materials and Methods

Materials

DSF was from Aldrich (Milwaukee, WI). *N*-acetyl-L-cysteine (NAC), LPS for in vivo studies from *Escherichia coli* 055:B5, and LPS for in vitro studies from *E. coli* 0111:B4 were all purchased from Sigma-Aldrich (St. Louis, MO). PD98059 (Biomol, Plymouth Meeting, PA) and SB202190 (Calbiochem, San Diego, CA) were used. Human rSOD-1 was kindly donated by Biotechnology General (Rehovot, Israel). Anti-mouse TNF-α-neutralizing Ab was from Endogen (Woburn, MA).

Animals

Inbred female mice of the BALB/c strain, male mice of the C57BL/6 strain, and female Lewis strain rats were supplied by Harlan Laboratories (Jerusalem, Israel). Animals were 10–14 wk old.

Transgenic mice harboring the human Cu/Zn SOD gene (Tg-SOD) were originally produced by microinjection of fertilized eggs with a linear 14.5-kb fragment of human genomic DNA containing the entire Cu/Zn SOD gene, including its regulatory sequences (13). SOD-1 expression level was 3- to 4-fold higher than in control normal mice. All experiments were performed in male Tg-SOD and age-matched control mice (8–12 wk old). Experiments were repeated twice with groups of five animals.

The animal ethics in all animal experiments meet the standards required by the Hebrew University of Jerusalem Guidelines for the welfare of animals in experimental immunology, and was approved by the Faculty of Agriculture (of the Hebrew University of Jerusalem) Institutional Review Board.

Preparation and stimulation of mouse PEM

PEM were collected by peritoneal washing with DMEM 5 days after i.p. inoculation with 1.5 ml of 3% (w/v) thioglycolate broth (Difco Laboratories, Detroit, MI) from 9- to 10-wk-old C57BL or Tg-SOD mice. Cells were washed twice with DMEM, and 1×10^6 cells were plated in 1 ml in 24-well or 3 ml in 6-well tissue culture plates of DMEM containing 5% (v/v) FCS at 37°C in a humidified incubator (5% CO_2). Nonadherent cells were removed by repeated washing 1 h later.

In vitro activation of macrophages and production of TNF-α or VEGF

PEM were incubated for 36 h in DMEM containing 5% (v/v) FCS at 37°C in a humidified incubator (5% CO_2). Purified cultures of PEM were washed with DMEM and either nontreated or treated with LPS (10–200 ng/ml) for 18–24 h. Supernatants were then collected, centrifuged, and assayed for TNF-α or VEGF. TNF-α was assayed both for biological activity and by ELISA. Briefly, test supernatants were added to cultures of TNF-α-sensitive WEHI 164 cells (14). Cell viability was determined by MTT uptake, as previously described (15). TNF-α protein was determined by using a sandwich OptEIA ELISA kit (BD PharMingen, San Diego, CA). When the effect of various mitogen-activated protein kinase (MAPK) inhibitors was examined on TNF-α secretion, PD98059 or SB202190 was added 60 min before LPS. VEGF was determined using a sandwich ELISA kit (R&D Systems, Minneapolis, MN). DSF or copper was added to PEM for 3 h, then washed three times with DMEM. A total of 50 ng/ml LPS was then added for 18–24 h. Vital staining was performed with trypan blue (Sigma-Aldrich).

Analysis of TNF-α levels in serum

LPS was injected i.p. into male C57BL/6 mice (10 μg), and into male ntg and Tg-SOD mice (5 μg). After 2 h, mice were anesthetized with Ketamin/Rompun (85/15 v/v%) solution. Blood was collected from the heart, and serum was prepared for TNF-α biological activity analysis, as described above. DSF (25 μg) or water was fed per os (p.o.) twice: 1 day and 1 h before LPS injection. Experiments were repeated twice in groups of six animals.

DTH assay

The shaved abdominal skin of inbred BALB/c mice (The Jackson Laboratory, Bar Harbor, ME; groups of 10 females) was sensitized with 100 μl of 2% (v/v) oxazalone dissolved in acetone/olive oil (4:1 (v/v)), applied topically (16). DTH sensitivity was elicited 5 days later by challenging the mice with 10 μl of 0.5% oxazalone in acetone/olive oil, applied topically to each side of the ear. One selected area of the ear was double blindly measured immediately before challenge and 24 h after challenge using a Mitutoyo engineer's micrometer. The DTH reaction is presented as the increment of ear swelling after challenge expressed in units of 10^{-2} mm. DSF (7–14 μg/mouse per application) or water as a control was administered p.o. the day before primary sensitization and every other day thereafter.

AA assay

To induce AA, 9- to 10-wk-old Lewis rats (seven per group) were inoculated intradermally at the base of the tail with 0.1 ml of CFA containing 10 mg/ml *Mycobacterium tuberculosis* (H37RA; Difco), as previously described (17). DSF (300 μg/rat per application) or water as control was administered p.o. 3 days after AA induction, three times per week. Beginning on day 13, rats were assessed for the severity of arthritis. The total arthritis score (0–16) was obtained by adding the score of each of the four joints, double blindly scored, for erythema, swelling, and deformity (17).

Enzymatic assays of SOD-1 activity

SOD-1 activity was determined, as previously described (18). Briefly, inhibition of nitrite formation from hydroxyl-ammonium chloride was determined by a spectrophotometric method (adapted from Elstner and Heupel (19)). Experiments were performed in triplicate. To examine the effect of various MAPK inhibitors on SOD-1, PD98059 or SB202190 was added 60 min before LPS.

Western blotting

Cells were washed twice with ice-cold PBS. Protein extraction for extracellular signal-regulated kinase-1,2 (ERK1,2) determination was performed with lysis buffer: Tris-HCl (20 mM, pH 7.4), NaCl (140 mM), glycerol (10%, v/v), Triton X-100 (1%, v/v), SDS (0.5%, w/v), EDTA (2 mM), PMSF (1 mM), leupeptin (20 μM). Extracts were centrifuged (15,000 $\times$ *g*, 15 min, 4°C), supernatants were collected, and aliquots from each sample (20 μg) were separated by 10% SDS-PAGE, followed by Western blotting. Bovine SOD-1 was detected by probing blots with rabbit anti-bovine SOD-1 primary Ab (Chemicon, Temecula, CA). ERK1,2 were detected by probing blots with anti-activated ERK mAb (Sigma-Aldrich). Total ERK protein was detected using either anti-ERK2 C-terminal Abs (C-14; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-MAPK (Sigma-Aldrich). Goat anti-rabbit or goat anti-mouse secondary Abs conjugated to alkaline phosphatase were purchased from Promega (Madison, WI). Bands were visualized by use of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Promega), and quantitatively analyzed by use of NIH Image 1.61 software.

Gelatin zymography

Aliquots of conditioned medium collected from control and test PEM were concentrated 2- to 3-fold and subjected to substrate gel electrophoresis (20) with modifications. The samples were applied without reduction to a 7.5% polyacrylamide gel impregnated with 1 mg/ml gelatin (Sigma-Aldrich). In this assay, clear zones against a blue background indicate the presence of gelatinolytic activity.

Statistical analyses

Following ANOVA, a two-tailed Student's *t* test was applied.

Results

LPS induces SOD-1 in PEM

LPS activates macrophages and induces the release of various cytokines involved in inflammation. To examine the correlation between SOD-1 level and the immune response, we examined the effect of LPS on SOD-1 induction. In PEM, LPS induced a dose-dependent increase in SOD-1 enzymatic activity (Fig. 1*A*) and protein levels, as demonstrated by Western blot analysis (Fig. 1*B*). At 200 ng/ml LPS, there was a 2.2- and 3-fold increase in protein and enzymatic activity of SOD-1 in PEM, respectively. SOD-1 protein was increased between 4 and 8 h following induction of PEM by LPS (Fig. 1*C*).

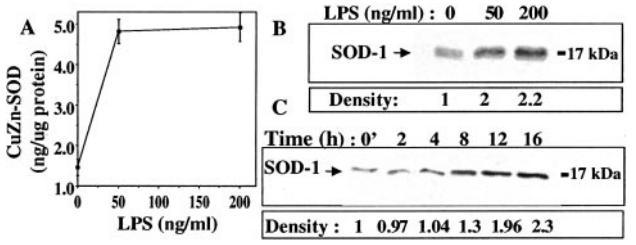

FIGURE 1. LPS induces SOD-1 in PEM. PEM derived from C57BL/6 mice were incubated with various concentrations of LPS for 18 h (*A*, *B*) and for various periods of time with 200 ng/ml of LPS (*C*). SOD-1 level in PEM was determined in the macrophages by enzymatic activity (mean ± SD) (*A*) and by Western blot analysis (*B*), as described in *Materials and Methods*.

LPS induces SOD-1 in PEM via ERK1 activation

Following LPS stimulation, tyrosine phosphorylation of the 44- and 42-kDa isoforms of ERK1,2 is increased in macrophages, and this event appears to mediate some of the responses to LPS (21, 22). We examined whether LPS induces SOD-1 in macrophages via ERK. Western blot analysis demonstrated that LPS induces ERK1 activation in PEM within 30 min (Fig. 2*A1*). Total ERK protein was determined as a control (Fig. 2*A3*). PD98059 (50 μM), an ERK1,2 inhibitor, inhibited, as expected, the activation of ERK1 by LPS, as demonstrated in Fig. 2*A2*. Moreover, PD98059 inhibited the induction of SOD-1 protein by LPS (Fig. 2*B*), as well as, in a dose-dependent manner, SOD-1 enzymatic activity (Fig. 2*C*). SB202190, an inhibitor of P38 MAPK, had a similar, but smaller effect on SOD-1 induction by LPS (Fig. 2*B*). These results indicate that LPS induces SOD-1 in PEM mainly via ERK1 activation.

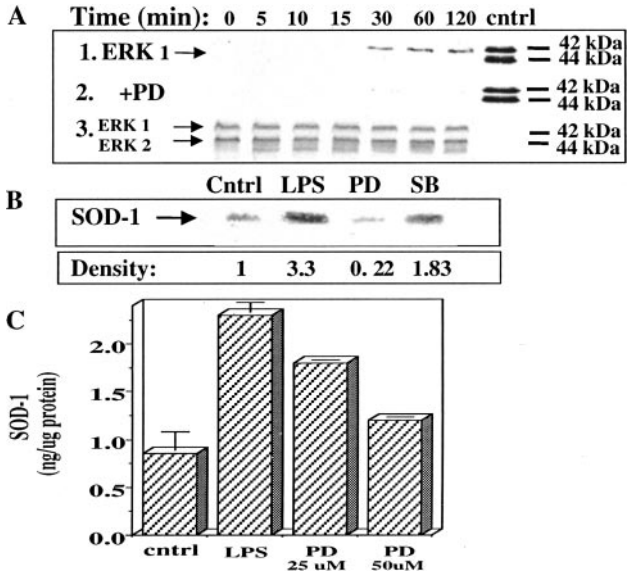

FIGURE 2. LPS induces SOD-1 in PEM via ERK1 activation. PEM derived from C57BL/6 mice were incubated with LPS (200 ng/ml), and the time kinetic of ERK1,2 activation was determined with (*A2*) or without (*A1*) PD98059 (PD; 50 μM). The time kinetic of total ERK protein was determined as a control (*A3*). The effect of MAPK inhibitors PD98059 (25–50 μM in enzymatic activity and 50 μM in Western blot) and SB202190 (SB; 10 nM) compared with control (cntrl) was examined on SOD-1 level in these macrophages after 18 h with LPS, by Western blot analysis (*B*) and by enzymatic activity (mean ± SD) (*C*), as described in *Materials and Methods*.

TNF-α induces SOD-1 in PEM

LPS activates macrophages and induces, among other cytokines, the release of TNF-α. TNF-α was released by activated macrophages within 2–5 h following LPS stimulation (Fig. 3*A*). To examine whether the induction of SOD-1 by LPS is mediated by TNF-α, we examined the effect of TNF-α on SOD-1 induction in PEM. At concentrations similar to those released by LPS-induced PEM, TNF-α induced the level of SOD-1 protein in a dose-dependent manner (Fig. 3*B*), as well as SOD-1 enzymatic activity (Fig. 3*C*). The effect of TNF-α (2 ng/ml) on the induction of SOD-1 protein and enzymatic activity was similar (~2-fold) to that observed with LPS (Fig. 3, *B* and *C*). PD98059 had no effect on the induction of SOD-1 protein by TNF-α (Fig. 3*B*), suggesting that the involvement of ERK1 in inducing SOD-1 by LPS precedes the release of TNF-α.

LPS induces TNF-α secretion from PEM via ERK activation

The release of TNF-α from LPS-stimulated PEM was inhibited in a dose-dependent manner by PD98059; at 25–50 μM, it inhibited the release of TNF-α by 73–93%, as measured by biological activity (Fig. 4*A*). Similar results were demonstrated for TNF-α protein by ELISA (Fig. 4*B*). These results indicate that the release of TNF-α from LPS-stimulated PEM is mainly via the ERK pathway.

LPS induced the release of TNF-α from PEM and increased the level of SOD-1 in PEM mainly via ERK1 activation. Because the induction of SOD-1 in PEM by TNF-α was not via the ERK pathway, it is suggested that the induction of SOD-1 by LPS is through the induction of TNF-α release via the ERK1 pathway. The fact that the increase in TNF-α level preceded the increase in SOD-1 level supports this suggestion. The possibility that LPS induces SOD-1 via ERK in a mechanism additional to the induction of TNF-α release cannot be excluded.

Overexpression of SOD-1 in macrophages induces increased release of TNF-α

Because LPS enhanced both TNF-α release and SOD-1 induction in PEM, we examined the possibility that increased expression of SOD-1 enables elevated release of TNF-α from PEM. The level of SOD-1 enzymatic activity in PEM derived from Tg-SOD mice was 2.1-fold higher than in PEM derived from control ntg mice (Table I). At low concentrations, LPS (10–20 ng/ml) induced a 30- to 40-fold increase in TNF-α release from PEM derived from Tg-SOD mice as compared with PEM derived from control ntg mice,

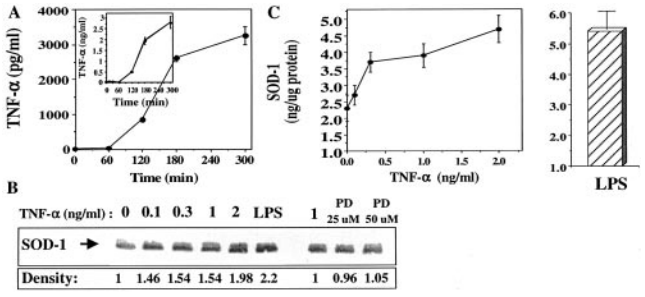

FIGURE 3. TNF-α induces SOD-1 in PEM. PEM derived from C57BL/6 mice were incubated with LPS (200 ng/ml) for various periods of time, and TNF-α release (mean ± SD) was determined by ELISA (*A*) or by biological activity (*A*, *inset*), as described in *Materials and Methods*. PEM were incubated with various concentrations of rmTNF-α for 18 h, and SOD-1 level was determined by Western blot analysis (*B*) and by enzymatic activity (mean ± SD) (*C*), as described in *Materials and Methods*. LPS (200 ng/ml) served as a positive control. MAPK inhibitor PD98059 (PD; 25–50 μM) was added in presence of TNF-α (1 ng/ml).

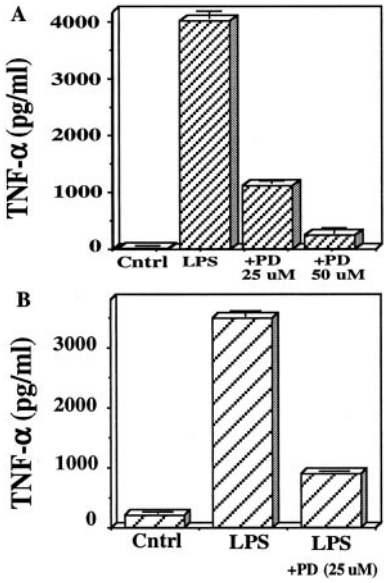

FIGURE 4. LPS induces TNF-α secretion via ERK activation. PEM derived from C57BL/6 mice were incubated with LPS (200 ng/ml) for 18 h with or without PD98059 (PD). TNF-α release (mean $\pm$ SD) was determined by biological activity (*A*) or by ELISA (*B*), as described in *Materials and Methods*.

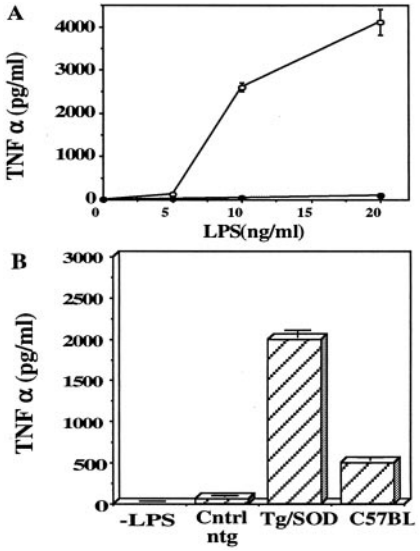

FIGURE 5. Overexpression of SOD-1 in macrophages induces increased release of TNF-α. PEM derived from control ntg (●) or from Tg-SOD mice (○) were incubated with various concentrations of LPS for 18 h, and TNF-α release (mean $\pm$ SD) was determined by biological activity (*A*) or by ELISA (*B*), as described in *Materials and Methods*. LPS concentration in *B* was 20 ng/ml. Macrophages derived from C57BL/6 mice were incubated with 20 ng/ml of LPS and served as controls. The basal level of TNF-α was determined in PEM derived from control ntg, Tg-SOD, and C57BL/6 mice, and incubated without LPS (−LPS).

as shown by both biological activity (Fig. 5*A*) and ELISA (Fig. 5*B*). LPS-stimulated macrophages derived from Tg-SOD released 4-fold more TNF-α than macrophages derived from C57BL/6 mice (Fig. 5*B*).

Inhibition of TNF-α release from PEM by DSF is reversed by antioxidants and copper

Increased expression of SOD-1 resulted in increased TNF-α release from LPS-activated PEM. We examined the possibility that inhibition of SOD-1 reduces TNF-α release from activated PEM. DSF, an inhibitor of SOD-1 (6), was added for 3 h to PEM derived from C57BL/6 mice and washed away, and then LPS (50 ng/ml) was added for activation. DSF (0.3 and 1 μM) inhibited TNF-α release from activated macrophages in a dose-dependent manner by 86 and 95%, respectively (Fig. 6*A*). Cu/ZnSOD depends on copper for its enzymatic activity. The inhibitory effect of DSF, which is a heavy metal chelator, was abolished in a dose-dependent manner by preincubation with 0.3–1 μM Cu^{2+} (Fig. 6*A*), but not with other metals such as Ni^{2+}, Zn^{2+}, or Mn^{2+} (data not shown). Because DSF is capable of inhibiting SOD-1 activity both in vivo (5) and in vitro (6), it is likely that DSF inhibits TNF-α release from activated macrophages by inhibiting SOD-1 via the chelation of its copper moiety.

Inhibition of SOD-1 activity may alter the delicate balance of ROS within the cell. We therefore examined the effect of the antioxidant NAC on DSF inhibition. NAC by itself had no effect on TNF-α release from PEM (Fig. 6*A*). Addition of NAC significantly reversed, in a dose-dependent manner, the inhibitory effect of DSF on TNF-α release, as determined by ELISA (Fig. 6*A*) and by biological activity (Fig. 6*B*). This indicates that the inhibitory effect of DSF on TNF-α release involves increased production of ROS in activated macrophages. Neutralizing anti-murine TNF-α (mTNF-α) Ab inhibited 75% of the biological activity of TNF-α, demonstrating that the cytotoxic activity derived from LPS-activated macrophages belongs to TNF-α (Fig. 6*B*). To verify macrophage viability, we used trypan blue staining, which demonstrated over 99% viability following the various treatments.

Table I. *Macrophages from Tg-SOD express higher levels of SOD-1*[a]

	SOD-1 (ng/μg protein)
Control ntg	2.31 $\pm$ 0.21
Tg-SOD	4.88 $\pm$ 0.33

[a] PEM were collected from 12-wk-old control ntg or Tg-SOD mice, as described in *Materials and Methods*, and cultured for 48 h. SOD-1 enzymatic activity was determined, as described in *Materials and Methods.*

DSF inhibits release of VEGF from PEM

In this study, we observed that DSF inhibits the release of TNF-α from activated macrophages (Fig. 6, *A* and *B*). In addition to its proinflammatory effect, TNF-α is an angiogenic factor in vivo (23). Angiogenesis is often a normal process inherent to inflammation. We therefore examined the effect of DSF on the release of VEGF, another angiogenic factor. DSF, dose dependently and eventually completely (0.1–0.3 μM), inhibited the release of VEGF from LPS-stimulated macrophages (Fig. 6*C*).

Collagenase secretion from macrophages is regulated by SOD-1

Macrophages mediate the destruction of connective tissues during inflammatory responses by secreting matrix-degrading MMP. LPS stimulates the secretion of both the 72-kDa (MMP-2) and 92-kDa (MMP-9) MMP that degrade type IV, V, and XI collagen. These 72- and 92-kDa collagenases constitute the key and rate-limiting proteinases governing the degradation of basement membrane collagens.

We examined the effect of SOD-1 regulation on collagenase secretion by activated macrophages. PEM derived from Tg-SOD mice expressed 2.1-fold higher SOD-1 activity than PEM derived from control ntg mice (Table I). LPS stimulated increased secretion of MMP-2 and MMP-9 (Fig. 7*A*) from PEM derived from both

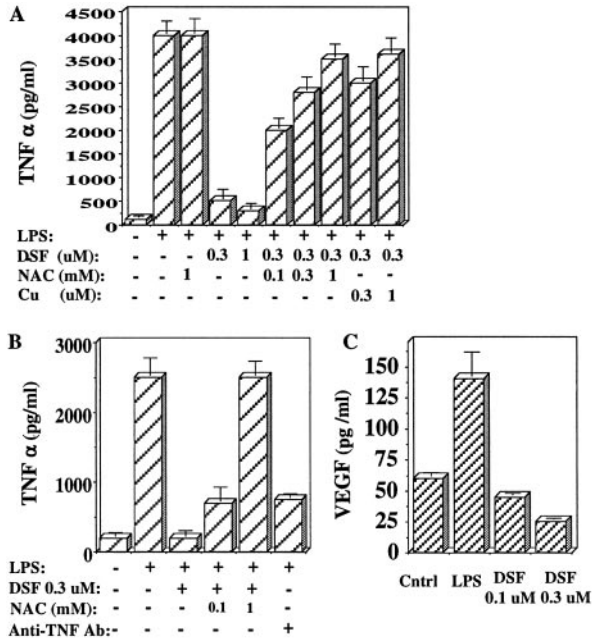

FIGURE 6. DSF inhibits release of TNF-α and VEGF from PEM. PEM derived from C57BL/6 mice were incubated with or without LPS (50 ng/ml) for 18 h with or without various concentrations of DSF, NAC, copper, and neutralizing anti-mTNF-α Ab. TNF-α release (mean $\pm$ SD) was determined by ELISA (*A*) or by biological activity (*B*), as described in *Materials and Methods*. VEGF (*C*) release (mean $\pm$ SD) was determined by ELISA, as described in *Materials and Methods*.

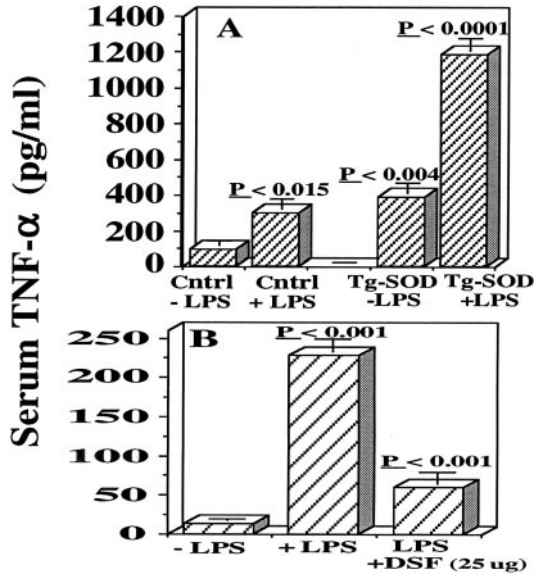

FIGURE 8. TNF-α levels in serum are higher in LPS-treated Tg-SOD mice (*A*) and decreased in DSF-treated mice (*B*). Groups ($n = 6$) of control parental (ntg) and Tg-SOD (*A*) or C57BL/6 (*B*) mice were examined for TNF-α levels (mean $\pm$ SD) in their serum following LPS or PBS treatment. LPS or PBS were injected i.p. into C57BL/6 (10 μg) or Tg-SOD and control ntg mice (5 μg). TNF-α was analyzed 2 h later by biological activity, as described in *Materials and Methods*. DSF (25 μg/mouse) or water as control (*B*) was administered p.o. twice: 1 day and 1 h before LPS injection.

mice strains. The basal level of both MMP-2 and MMP-9 secreted by nonstimulated PEM was higher (2.6- to 1.33-fold, respectively) in macrophages derived from Tg-SOD than from control ntg mice (Fig. 7*A*), indicating that increased SOD-1 level in PEM leads to a higher activation potential. The secretion of MMP-9 from LPS-stimulated macrophages derived from Tg-SOD mice was 2.15-fold higher than that from LPS-stimulated macrophages derived from control ntg mice (Fig. 7*A*). In contrast, DSF dose dependently inhibited the secretion of both MMP-2 and MMP-9 from LPS-stimulated PEM derived from C57BL mice. As shown for TNF-α, addition of NAC significantly reversed the inhibitory effect of DSF on MMP-2 and MMP-9 secretion (Fig. 7*B*), indicating that this inhibition involves an increase in ROS production in activated macrophages.

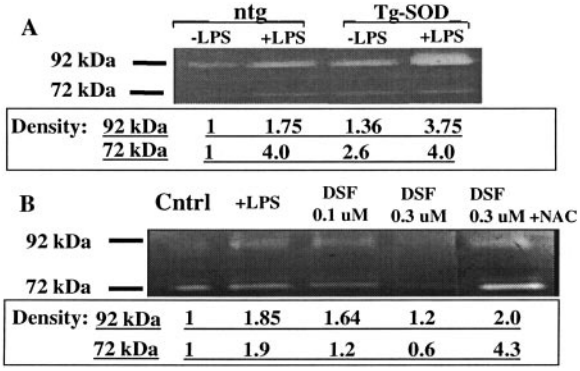

FIGURE 7. Collagenase secretion is increased in macrophages derived from Tg-SOD mice. PEM derived from Tg-SOD or control ntg (*A*) and from C57BL/6 mice (*B*) were incubated with (+LPS) (50 ng/ml) or without LPS (cntrl) for 18 h, and supernatant was collected, concentrated, and analyzed for gelatinolytic activity, as described in *Materials and Methods*. DSF with or without NAC was added to PEM for 3 h before stimulation with LPS (*B*), as described in *Materials and Methods*. This is one representative experiment of two.

TNF-α levels in serum of LPS-treated mice are regulated by SOD-1

Following the observation that overexpression of SOD-1 in macrophages induces increased release of TNF-α and that inhibition of SOD-1 by DSF decreases the release of TNF-α from activated PEM in vitro, we examined the role of SOD-1 in release of TNF-α in vivo. The basal level of TNF-α in the serum of nonstimulated Tg-SOD mice was 4-fold higher than that in control ntg mice (Fig. 8*A*), indicating a higher basal level of macrophage activation in animals overexpressing SOD-1. Similarly, the serum level of TNF-α in LPS-stimulated mice was 4-fold higher in Tg-SOD mice than that in control ntg mice (Fig. 8*A*), indicating an increased immune response in animals overexpressing SOD-1. To examine the effect of SOD-1 inhibition on the immune response, we introduced DSF (25 μg) orally to LPS-stimulated C57BL/6 mice. Two hours after LPS stimulation, TNF-α level in the serum of DSF-treated mice was 4-fold lower than that in control mice (Fig. 8*B*), demonstrating the role of SOD-1 in the immune response.

DTH reactivity is regulated by SOD-1

The role of SOD-1 in cell-mediated inflammation was further studied in the DTH reaction in vivo. The DTH response was measured by challenging the ears with oxazalone 5 days after primary sensitization. Overexpression of SOD-1 in transgenic mice increased the DTH response to oxazalone by 2.15-fold compared with control ntg mice (Fig. 9*A*). To examine the effect of SOD-1 inhibition on the immune response in the DTH reaction, we introduced DSF (7 and 14 μg/mouse per application) orally to oxazalone-sensitized BALB/c mice. DSF reduced in a dose-dependent manner (33% $\pm$

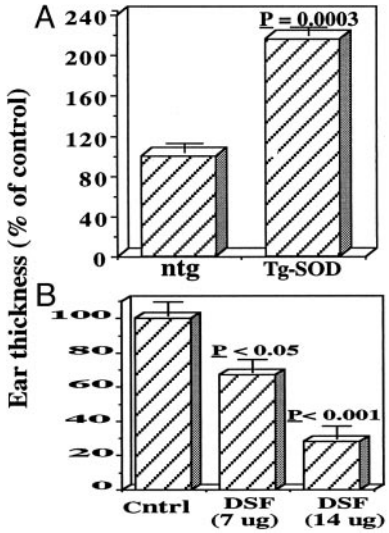

FIGURE 9. DTH reactivity is elevated in Tg-SOD mice (*A*) and inhibited in DSF-treated mice (*B*). Groups ($n = 10$) of control parental (ntg) and Tg-SOD (*A*) or BALB/c (*B*) mice were sensitized to oxazalone by skin painting, as described in *Materials and Methods*, and the degree of DTH reactivity was assessed 6 days later by measuring increase in ear thickness 24 h after the second challenge. DSF (7–14 μg/mouse per application) or water as control was administered p.o. the day before primary sensitization and every other day thereafter (*B*). The DTH reaction is presented as the percentage of ear swelling compared with control-sensitized ntg mice in *A* and with control-sensitized BALB/c mice in *B*. DTH reaction is expressed as the mean ± SEM.

6.8 and 72% ± 7.5, respectively) the DTH response to oxazalone (Fig. 9*B*). Classical dexamethasone (40 μg) treatment reduced the DTH response to oxazalone by 47% ± 5.8 (data not shown).

DSF inhibits AA

To further test whether DSF might be effective in reducing the immune response in vivo, we induced AA in Lewis rats with *M. tuberculosis* Ag and treated the rats 3 days later. Oral administration of DSF (300 μg/rat per application) significantly inhibited AA in Lewis rats (Fig. 10).

Discussion

In vivo, normal oxidative metabolism results in the production of oxygen radicals as byproducts (1). Activated cells with increased metabolism therefore demonstrate enhanced oxygen radical production. When macrophages are activated, oxygen consumption increases markedly, resulting in the production of various ROS such as superoxides (24). Some of the ROS, produced during macrophage activation, serve as pathogen-killing agents, and some may affect the intracellular oxygen-redox balance. A physiological need exists to metabolize the oxygen radicals that may cause damage and apoptosis of macrophages. Alterations in the activity of SOD-1 may therefore affect immune reactions and inflammation-dependent pathologies. The data presented in this work demonstrate that alterations in SOD-1 activity do indeed affect the inflammatory response. Increase in SOD-1 levels does not necessarily mean a change in the oxidative-redox balance of the cell, but may rather affect the potential of the cell to deal with increased superoxide radical production (i.e., to regulate metabolism and as a result synthesis of factors). Increased potential of the cell to deal with elevated superoxide radical production does not directly mean that the oxidative-redox level in the cell has changed. It may rather mean an ability to maintain a normal steady state of oxidative-redox balance, despite an increase in superoxide radical production, and therefore, it does not directly imply an effect on the oxidation or reduction of the inflammatory factors. However, the possibility that alterations in SOD-1 activity may also affect a possible oxidation of inflammatory mediators, therefore their activity or their stability, cannot be excluded.

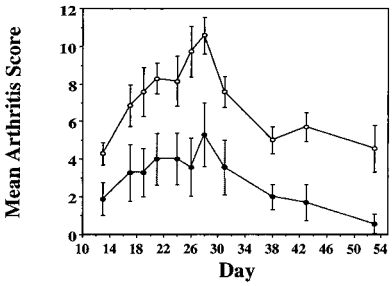

FIGURE 10. Treatment of rats with DSF inhibits AA. AA was induced in Lewis rats ($n = 7$), and the arthritis severity (mean ± SEM) was scored as described in *Materials and Methods*. Beginning on day 3, the rats were treated p.o., three times per week with water (○) or DSF (●) (300 μg/rat per application). Significant differences from the water control were $p < 0.05$ on day 38; $p < 0.03$ on days 17, 21, 24, 26, and 53; $p < 0.01$ on day 13; and $p < 0.005$ on days 26, 28, and 43.

We show that macrophage activators, such as LPS and TNF-α, increase SOD-1 in cultured PEM. All macrophages express the multicomponent enzyme, NADPH oxidase, and generate superoxides that are then dismutated by SOD-1 to various extents into hydrogen peroxide. LPS- and TNF-α-elicited macrophages display higher NADPH oxidase activity (9). Elevation of SOD-1 in macrophages has been previously demonstrated by oxidative stress induced by UVB radiation (25) or by hyperoxia and ischemia-reperfusion (26).

The three MAPK family members, ERK1,2, p38, and Jun N-terminal kinase, and their upstream activators are stimulated by LPS in a variety of macrophage cell types (21, 27, 28). The experimental work in macrophages has revealed considerable differences in MAPK responses to stimuli between primary cells and different cell lines (29).

The ERK pathway is required for both macrophage proliferation and the correct production of cytokines (30). Following LPS stimulation, tyrosine phosphorylation of the 44- and 42-kDa isoforms of ERK1,2 is increased in macrophages, and this event appears to mediate several responses to LPS (21, 22). We demonstrate in this study that LPS induces SOD-1 in macrophages mainly via ERK1 activation. SB202190, an inhibitor of P38 MAPK, had only a partial inhibitory effect on SOD-1 induction by LPS.

LPS activates macrophages and induces, among other cytokines, the release of TNF-α. We show in this study that TNF-α is released via ERK1 activation by LPS-activated PEM within 2–3 h. This is in accordance with previous studies demonstrating that ERK1,2 is partially involved in TNF-α mRNA expression (30), production, and release (31) from LPS-stimulated marrow-derived macrophages.

TNF-α induced SOD-1 to a level similar to that induced by LPS and at concentrations similar to those released by LPS-stimulated PEM. The fact that LPS induces both SOD-1 and TNF-α via ERK1 and that TNF-α also induces SOD-1, but not via ERK1, suggests that LPS elevates SOD-1 in PEM through the induction of TNF-α via the ERK1 pathway. Nevertheless, the possibility that LPS induces SOD-1 via ERK1 in a mechanism additional to the induction of TNF-α release cannot be excluded.

Monocyte apoptosis has emerged as a central regulatory event in hemopoiesis and inflammation. TNF-α has been previously shown to induce apoptosis in macrophage-like U937 cells (32) and to

inhibit growth of bone marrow progenitor cells. Overexpression of SOD-1 in these cells renders them resistant to the toxic effect of TNF-α, indicating that TNF-α-mediated growth inhibition of hemopoietic cells occurs via the production of superoxides (33).

We examined the possibility that increased expression of SOD-1 enables macrophages to confront the increased oxidative stress induced by LPS and, as a result, to demonstrate increased secretion of TNF-α when they are activated. A 2-fold increase in SOD-1 activity in PEM derived from Tg-SOD mice enabled a 30- to 40-fold increase in TNF-α secretion as compared with PEM derived from control ntg mice. As we have seen, LPS induces TNF-α release and elevates SOD-1 levels via ERK1 activation. Elevation of SOD-1 may be induced by LPS and/or the secreted TNF-α. Elevation of SOD-1 results in return in increased release of TNF-α. Both LPS and TNF-α induce also the production of ROS in macrophages. SOD-1 decreases superoxide radicals level. Therefore, induction of SOD-1 enables the macrophages to confront the increase in superoxide production and to allow further increased macrophage activation and increased TNF-α secretion (schematic model presented in Fig. 11). Inhibition of SOD-1 by DSF will result in increased superoxide radicals level. The possibility that the effect of DSF on inflammation may also be by affecting some inflammatory mediators, whose activities may be sensitive to superoxide radicals, cannot be excluded.

Inhibition of SOD-1 by DSF reduced TNF-α release in a dose-dependent manner from LPS-activated PEM. CuZn/SOD depends on copper for its enzymatic activity. The inhibitory effect of DSF, which is a heavy metal chelator, was specifically and dose dependently abolished by preincubation with copper, but not Zn^{2+}, Ni^{2+}, Fe^{2+}, or Mn^{2+}. Because DSF inhibits SOD-1 activity both in vivo (5) and in vitro (6), it is likely that the inhibitory effect of DSF on TNF-α release from activated macrophages is through the chelation of the copper moiety from SOD-1. These data are in good agreement with recent findings demonstrating that dietary copper deficiency impairs both innate and acquired branches of immunity (34) and decreases SOD-1 activity and TNF-α levels in bovine plasma (35). It is thus possible that, in addition to other mechanisms, Cu deprivation affects SOD-1 activity and as a result exerts an anti-inflammatory effect.

LPS stimulates the secretion from PEM of both the 72-kDa (MMP-2) and 92-kDa (MMP-9) MMP, which degrade type IV, V, and XI collagen (36, 37). MMP-2 and MMP-9 constitute the key and rate-limiting proteinases governing the degradation of basement membrane collagens during inflammatory responses. DSF completely inhibited the release of MMP-2 and MMP-9 collagenases from macrophages. The role of SOD-1 in collagenase secretion from activated macrophages is further demonstrated in this study by the fact that PEM overexpressing SOD-1 activity 2-fold, secreted, upon stimulation with LPS, levels of MMP-2 and MMP-9 that were 2-fold higher than those from PEM derived from control ntg mice.

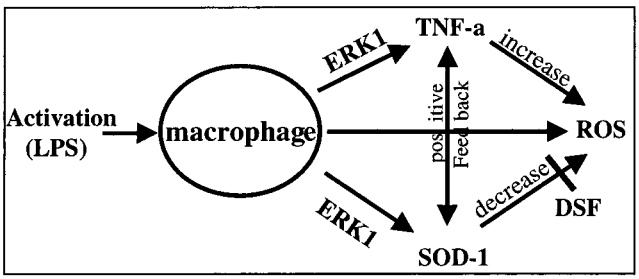

FIGURE 11. Positive feedback control of TNF-α and SOD-1 level in activated macrophages. Macrophages are activated by various signals, one of which is LPS. LPS induces TNF-α release via ERK1 activation, as well as elevates SOD-1 level via ERK1 activation. Elevation of SOD-1 may be either by LPS and/or the secreted TNF-α. Elevation of SOD-1 results in turn in increased release of TNF-α. Both LPS and TNF-α induce also production of ROS in macrophages. SOD-1 decreases superoxide radicals level. Therefore, induction of SOD-1 enables the macrophages to confront the increase in ROS production and to allow further increased macrophage activation and increased TNF-α secretion.

Our results show that the inhibitory effect of DSF on the release of TNF-α, MMP-2, and MMP-9 involves increased production of ROS in PEM. These results confirm a previous study, demonstrating that inhibition of SOD-1 by DSF induces oxidative stress, which results in endothelial cell growth arrest (6).

Macrophages are known to induce angiogenesis (38, 39) that is often inherent to inflammation. VEGF is a well-known angiogenic factor (40), as are TNF-α (23) and MMP-2, MMP-9 proteinases (41, 42), which, in addition to their proinflammatory effect, are also proangiogenic in vivo. The inhibition of angiogenesis by DSF previously shown to be via induction of apoptosis in endothelial cells (6) may, therefore, also result from the inhibitory effect of DSF on the release of the proangiogenic factors TNF-α, VEGF, and MMP-2, MMP-9 proteinases from macrophages.

Involvement of macrophages in various acute and chronic inflammatory pathologies has been demonstrated in AA (43), rheumatoid arthritis (RA) (44), DTH (45–47), and sepsis (48). More precisely, TNF-α, VEGF, and MMP-2, MMP-9 proteinases derived from macrophages have been shown to be involved in many acute and chronic inflammatory pathologies. TNF-α is capable of a broad range of immunoresponsive actions that are central to the pathophysiology of acute and chronic inflammation and cancer (49). Overproduction and secretion of TNF-α contribute to the acute symptoms of septic shock (50, 51). TNF-α is involved in the chronic effects of diseases such as RA (52) and collagen-induced arthritis (53), as well as DTH (46, 47).

Monocyte/Macrophage production of MMP-2 and MMP-9 is considered to be of major importance in the pathology of diseases such as RA (54), AA (55), and DTH (47). Monocyte-derived MMP-9 is also involved in sepsis (56). VEGF is involved in RA (57) and in collagen-induced arthritis (58). In addition, neovascularization, to which TNF-α, MMP-2, MMP-9, and VEGF are important contributors, plays an important role in RA (58), AA (59), and DTH (60).

Macrophages and many of their secretion products, such as TNF-α, MMP-2, MMP-9, and VEGF, are involved in sepsis, DTH, and AA. Therefore, we examined the role of SOD-1 in these pathologies in vivo. In accordance with the data demonstrated in vitro, Tg-SOD mice reacted 4-fold more strongly to sepsis elicited by LPS and 2-fold stronger in DTH reaction compared with control ntg mice. This indicates a higher level of immune response potential in animals overexpressing SOD-1. Similarly, orally introduced DSF decreased TNF-α level in sepsis 4-fold, strongly reduced the DTH response, and significantly inhibited AA in Lewis rats, demonstrating the role of SOD-1 in the immune response of various acute and chronic inflammatory pathologies.

LPS-treated PEM from transgenic mice overexpressing SOD-1, under the control of the mouse hydroxyl-methyl coenzyme A reductase promoter, demonstrated a significant reduction in microbicidal and fungicidal activity (61). Another study demonstrated that increases in SOD-1 reduce the production of superoxides and induce expression of the inducible form of NO synthase and the related NO production in macrophages (62).

Superoxide and NO produced by endothelium, macrophages, and neutrophils can react rapidly to form peroxynitrite, a potent ROS (63). Because macrophages produce both superoxide and

NO, they may be particularly vulnerable to oxidant injury by this mechanism. The important role of SOD-1 was demonstrated when overexpression of SOD-1 in RAW 264.7 macrophages protected them against NO cytotoxicity (64). As expected, SOD-1-overexpressing cells coped with the higher levels of oxygen radicals generated by oxidative stress (65), paraquat (18), DSF (6), thiram (7), and NO cytotoxicity (64), thereby preventing the initiation of apoptotic or other arrest-signaling pathways.

Hydrogen peroxide and ROS are agents commonly produced during inflammatory processes. ROS and specifically hydrogen peroxide at relatively low concentrations serve as messengers mediating directly or indirectly the activation of transcription factors such as NF-κB, and as a result the induction of various proinflammatory genes as well as of SOD (66, 67), which in turn enables the cell to handle toxic concentrations of ROS. At low concentrations, ROS may serve as second messengers. However, at higher concentration, ROS are toxic and need to be scavenged by the antioxidant defense mechanism of the cell. The pathways of SOD-1 and NF-κB may be separated: the SOD-1 pathway scavenges high concentrations of toxic ROS and enables an increased metabolism and synthesis of proinflammatory cytokines, and the NF-κB pathway, via low concentrations of ROS, especially hydrogen peroxide, induces proinflammatory cytokines. The two pathways may also be coupled. Induction of SOD-1, which transmutates superoxide radicals into hydrogen peroxide, may result in NF-κB activation, and as a result induction of various proinflammatory genes. Thus, scavenging of superoxide radicals may also involve activation of NF-κB and induction of inflammation.

mRNA and protein levels of manganese superoxide dismutase, another key enzyme in the metabolism of oxygen radicals, were also previously shown to be induced by LPS (68–70). Some of the ROS produced during macrophage activation act as pathogen-killing agents, while others may affect the delicate intracellular oxygen-redox balance. Therefore, SOD-1, a key enzyme in ROS metabolism, plays an important role in immune processes, because modulation of its activity may differentially affect the NO-dependent microbicidal activity and release of cytokines by activated macrophages. Specific regulation of antioxidant enzymes may thus provide a new way to prevent and treat diseases of the immune system.

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