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Fc-Receptor-Mediated Intracellular Delivery of Cu/Zn-superoxide Dismutase (SOD1) Protects Against Redox-Induced Apoptosis Through a Nitric Oxide Dependent Mechanism

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Abstract

Background: Using specific antibodies against bovine Cu/Zn-superoxide dismutase (EC 1.15.1.1, SOD1) we demonstrated that anti-SOD antibodies (IgG_1) are able to promote the intracellular translocation of the antioxidant enzyme. The transduction signalling mediated by IgG_1 immune complexes are known to promote a concomitant production of superoxide and nitric oxide leading to the production of peroxynitrites and cell death by apoptosis. The Fc-mediated intracellular delivery of SOD1 thus limited the endogenous production of superoxide. It was thus of interest to confirm that in the absence of superoxide anion, the production of nitric oxide protected cells against apoptosis. Study in greater detail clearly stated that under superoxide anion-free conditions, nitric oxide promoted the cell antioxidant armature and thus protected cells against redox-induced apoptosis.

Materials and Methods: The murine macrophage cell-lines J774 A1 were preactivated or not with interferon-γ and were then stimulated by IgG_1 immune complexes (IC), free SOD1 or SOD1 IC and superoxide anion, nitric oxide, peroxynitrite, and tumor necrosis factor-α (TNF-α) production was evaluated. The redox consequences of these activation processes were also evaluated on mitochondrial respiration and apoptosis as well as on the controlled expression of the cellular antioxidant armature.

Results: We demonstrated that SOD1 IC induced a Fcγ receptor (FcγR)-dependent intracellular delivery of the antioxidant enzyme in IFN-γ activated murine macrophages (the J774 A1 cell line). The concomitant stimulation of the FcγR and the translocation of the SOD1 in the cytoplasm of IFN-γ-activated macrophages not only reduced the production of superoxide anion but also induced the expression of the inducible form of nitric oxide synthase (iNOS) and the related NO production. This inducing effect in the absence of superoxide anion production reduced mitochondrial damages and cell death by apoptosis and promoted the intracellular antioxidant armature.

Conclusions: To define the pharmacologic mechanism of action of bovine SOD1, we attempted to identify the second messengers that are induced by SOD1 IC. In this work, we propose that Fc-mediated intracellular delivery of the SOD1 that reduced the production of superoxide anion and of peroxynitrite, promoted a NO-induced protective effect in inducing the antioxidant armature of the cells. Taken together, these data suggested that specific immune responses against antigenic SOD1 could promote the pharmacological properties of the antioxidant enzyme likely via a NO-dependent mechanism.

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Introduction

Since their characterization in 1968 by McCord and Fridovich (1), superoxide dismutases (SOD) have been studied in the treatment of numerous

diseases (2). The initial biological properties of SOD is to promote removal of the superoxide radical by dismutation and therefore constitutes a system for providing protection from the deleterious effects of this free radical, which can be produced in vivo from atmospheric oxygen. Consequently, this enzyme plays a fundamental role in preventing the toxic effects, which could result in an oxygenated microenvironment in which oxygen (a biradical) loses an unpaired electron (reduction).

As free radicals are involved in numerous diseases, the use of SOD in therapeutics has been recommended in different inflammatory processes (rheumatism and fibrosis in particular) (3), viral processes (4,5), and degenerative processes associated with the presence of substantial amounts of oxygen (central nervous system, ischemia, nonvascular gastrointestinal disorders, eye disorders, or control of the undesirable effects of anti-cancer treatments) (6). Bovine Cu/Zn-SOD (Bo-SOD1) has been examined therapeutically in the treatment of patients with various arthritic diseases (3), under radiotherapy or chemotherapy, and of bronchopulmonary dysplasia in premature infants with respiratory distress syndrome. After injection (i.m., s.c. or i.v.) in human, bovine Cu/Zn-SOD seems to be very well tolerated despite its heterologous/antigenic protein character (7). Free bovine Cu/Zn-SOD has no acute toxicity and did not present any delayed toxicity (8). Historically, results obtained with four different types of SOD1 clearly stated that the heterologous/antigenic nature of SOD1 was required to produce optimal antiinflammatory effects (7). Importantly, its mode of action is not linked to the pharmacokinetic parameters of these molecules (3) and the rate of the antiinflammatory activity of a given SOD1 is unrelated to the plasma t1/2 (8). In humans, the total quantity of SOD averages 3.9 g (60 μg/g of tissue for an individual weighing 65 kg) (9). If 8 mg of bovine Cu/Zn-SOD are administered per day, there is an approximate increase of tissue weight of SOD of 0.12 μg/g, which is equally distributed around the body. Based on this evidence, an important question remains: how can such low doses be effective? Apart from the fact that it has been suggested that heterologous, but not homologous, SOD could bind to semispecific sites at the cell surface (10), it has been demonstrated that a negative correlation was drawn up between the antiinflammatory effect of the product and the plasma concentration of anti-SOD antibodies, suggesting that immune stimulation by the heterologous/antigenic protein sequence of the enzyme could promote and strengthen the initial antiinflammatory properties of the enzyme.

The aim of this study is to demonstrate that SOD1 immune complexes are not only able to promote the cell penetration of the antioxidant enzyme but could also strengthen the antiinflammatory properties of heterologous/antigenic SOD1 in inducing a coordinate and appropriate immune response likely through the activation of the nitric oxide pathway in both normal and activated cells and in the absence of superoxide anion production.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine and 4.5 g/L glucose was obtained from Sigma Chemical Co. (St. Louis, MO, USA), as were all reagents not otherwise noted. Recombinant murine interferon-γ (IFN-γ) was from Immungenex (Los Angeles, CA, USA), the IgG_1 anti-bovine Cu/Zn-SOD was purchased from Valbiofrance (Meudon, France). The cell-permeable superoxide dismutase mimetic, MnT-BAP, was purchased from Alexis Biochemicals (Paris, France).

Cell Culture and Activation

Murine macrophage-like J774.A1 cells (11) were maintained in DMEM containing 3.7 g/l of sodium bicarbonate supplemented with 10% of fetal calf serum (Bioproducts, France) and 1% of nonessential amino acids, in a humidified 37°C atmosphere with 10% CO_2. For experiments, macrophages were seeded at 5×10^5 cells/ml, 100 μl/well in flat bottomed 96-well tissue culture plates (Becton Dickinson, Grenoble, France). Plates were incubated for approximately 1–2 days prior to activation to allow adherence of macrophages. Cells were activated with IFN-γ (1,000 U/ml) and cells were then incubated for an additional 2 days. At the end of the culture period, cell-free supernatants were collected for TNF-α and nitrite measurements. These experiments were always done in endotoxin-free conditions, and all reagents and media were tested for their endotoxin contents by the limulus amebocyte assay.

Flow Cytometry Analysis of Cell Surface Markers

Fcγ receptor (FcγR) expression (CD16/CD32) was done using the 2.4G2 mAb (rat IgG_2b) that was purchased from Pharmingen (Les Ulis, France). mAb were diluted at 1/50 v/v in PBS conatining 1% SVF and 0.2% of azide. 2.4G2 labeling was revealed with anti-IgG_2b coupled to phycoerythrin (Caltag) and cell-related fluorescence was detected by flow cytometry using a FACScan system (Becton Dickinson, Grenoble, France).

Preparation of Immune Complexes and Bo-SOD1 Loading

To prepare the IgG_1/anti-IgG_1 and the Bo-SOD1/ anti-Bo-SOD1 immune complexes, the anti-IgG_1 (20 μg) or the anti-SOD (20 μg) were incubated either with IgG_1 (100 μg) or with bovine Cu/Zn-SOD (different doses) as already described in other systems (11,12). For Bo-SOD1, loading cells were plated in 24-well culture plates at a concentration of 2×10^6 cells/ml with a final volume of 1 ml, and incubated overnight in the presence or absence of the immune complexes. After washing with serum-free medium supplemented with 2 mg/ml bovine serum albumin (Sigma, France), the cells were incubated in this medium in the presence or absence of the indicated concentrations of Bo-SOD1 and incubated for 18 hr, except otherwise indicated in the time-course of the study. As already described (13) for analysis of Bo-SOD1, loading cells were then treated as already described with 0.5% trypsin for 5 min at 37°C to digest residual SOD, which may be bound to the external surface of the cell membrane. After neutralization of the trypsin, the cells were washed and protein extracted with a buffer containing 0.1 M Na_2CO_3 (pH 10.2), 0.2 mM EDTA, 0.2% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The soluble extracts were then assayed for SOD and antioxidant enzymes activities as well as for glutathion (GSH) contents.

TNF-α Measurement

Cell-free supernatants were kept at −80°C to avoid TNF-α degradation. Tumor necrosis factor-α (TNF-α) levels were measured using ELISA kits from British Biotechnology (England) and the threshold of detection was 10 pg/ml.

Assay for $O_2^{\circ-}$, NO, and $ONOO^-$ Production by Resting and Activated Cells

To assess the amount of NO produced, the stable end product of NO, NO_2^- was measured using the Griess reaction as previously described (14).

The generation of $O_2^{\circ-}$ was assayed by measuring the reduction of ferricytochrome c at 37°C by adherent cells (15). Cells were preincubated in the presence of 150 μM ferricytochrome c and, after different times of incubation, the absorbance change at 550 nm was assayed in a spectrophotometer.

To measure $ONOO^-$, production was evaluated after oxidation of dihydrorhodamine 123 to fluorescent rhodamine as previously described (16). Briefly, 5×10^5 cells in 500 μl RPMI medium were pre-incubated for 6 hr in the presence of 100 nM of dihydrorhodamine 123. The suspension was pre-warmed at 37°C, before a 50 μl addition of IgG_1 immune complexes, and fluorescence was then measured on a spectrophotometer (LS-50, Perkin Elmer Corporation, Norwalk, CT, USA) with excitation and emission wavelengths of 500 nm and 536 nm, respectively, and excitation and emission slit widths of 2.5 nm and 3.0 nm, respectively, and 30 min after addition of IC.

Assay for Total and Mn-SOD Activities and Detection of Intracellular Delivery of Bo-SOD1

Total and Mn-SOD activities were assayed as already described (17). In brief, a competitive inhibition assay was performed that used xanthine–xanthine oxidase generated $O_2^{\circ-}$ to reduce nitroblue tetrazolium (NBT) at a constant rate (0.01–0.02 absorbance units per min). The rate of NBT reduction was monitored spectrophotometrically at 560 nm. The inhibition of Cu/Zn-SOD by 5 mM of sodium cyanide was done to differentiate between Cu/Zn-SOD and Mn-SOD. Protein concentrations were determined by the Lowry method (18) and enzymatic activity was expressed in U per mg of protein.

The couple of anti-Cu/Zn-SOD antibodies used to detect the intracellular delivery of Bo-SOD1 were a rabbit polyclonal anti-bovine Cu/Zn-SOD serum to coat the plate (10 μg/ml) and a rabbit IgG-peroxydase anti-bovine Cu/Zn-SOD (5 μg/ml) was purchased from Biodesign International (Kennebunk, ME, USA), the threshold of detection being of 10 ng/ml of Bo-SOD1.

Preparation of Total RNA and Northern Blot

Total cellular RNA was prepared from cell cultures (5×10^6 cells) following the guanidium isothiocyanate method (19,20). Aliquots of total RNA (10 μg) were denatured at 65°C for 15 min in 5% formaldehyde, 50% formamide, and 8% glycerol and then were size separated by electrophoresis onto 0.9% agarose gel containing 2% formaldehyde and 3-(N-morpholino) propane sulfonic acid. After transfer of RNA to nitrocellulose membrane, they were prehybridized for 1 hr at 65°C and then hybridized overnight with the radiolabeled probe piNOS (kindly provided by Dr. Q.W. Xie, Cornell University College, New York, USA) (21).

Detection of iNOS Proteins by FACS Analysis

The presence of iNOS was analyzed by flow cytometry on permeabilized cells, using the Cytoperm permeabilization and fixation kit (Serotec, Oxford, UK) or the IS ULTRA Cell Fix-Perm kit (Bioadvance), according to the manufacturers' specifications. Briefly, 10^6 treated cells were labeled with 10 μl specific anti-iNOS mAb (macNOS, IgG1, clone 54, Transduction Laboratories), directed against the carboxy-terminal portion of the murine macrophage iNOS) followed by incubation with a goat $F(ab')_2$ fragment anti-mouse IgG (H+L)-FITC (Immunotech) the IgG_1-FITC as control isotype (Becton Dickinson). Cells were then analyzed by flow cytometry on a FACScan using Lysys 2 and Procyt software and data were presented as Mean Fluorescence Intensity (MFI).

Quantification of Mitochondrial Respiration

Cell respiration was assessed by the mitochondrial-dependent reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan (22). Cells in 96-well plates were incubated at 37°C with MTT (0.2 mg/ml) for 1 hr. Culture medium was removed by aspiration and the cells were solubilized in DMSO (100 μl). The extent of reduction of MTT to formazan within cells was quantified by the measurement of OD_{550}. All measurements were corrected for the interference with IgG_1 and Bo-SOD1 IC at this wavelength.

Quantification of Apoptosis

Apoptosis-induced DNA fragmentation was estimated as already described (23) with the ApoAlert DNA fragmentation detection kit (Clontech, Palo Alto, CA, USA), based on the terminal deoxynucleotidyl transferase (TdT)-mediated fluorescein-dUTP nick-end (TUNEL) labeling, according to the specifications of the manufacturer.

Antioxidant Enzymatic Activities and GSH Detection

Cells (2×10^6/ml) were measured under the different culture conditions, and the cell pellets were frozen at −80°C prior antioxidant measurement using different colorimetric kits as already described (24,25). Catalase activity was determined in terms of the decrease in the amount of hydrogen peroxide; glutathion peroxidase (Gpx) activity was obtained by colorimetry with 2-nitro-5-thiobenzoic acid, a chemical compound produced through the reaction of glutathion and 5′,5′-dithio(2-nitrobenzoic acid). The levels of GSH were determined using a GSH-400 colorimetric assay kit obtained from R&D diagnostic (Paris, France)

Statistical Analysis

Data shown represent the means and standard deviations for triplicate or quadruplicate cultures in a representative experiment. All experiments were done at least four times with equivalent results. Mann–Whitney U test was used for comparison of populations.

Results

Fc-mediated Intracellular Delivery of Bovine SOD1 in Murine Macrophages Reduced Superoxide Anion Production and Promoted NO-dependent Mechanisms

The murine macrophage cell line J774.A1 spontaneously expressed FcγR (Table 1), expression that was dramatically enhanced after IFN-γ treatment. The ligation of these receptors at the cell surface of IFN-γ preactivated J774.A1 cells by IgG_1 IC induced the concomitant production of superoxide anion and nitric oxide (Fig. 1A and B), the level of response correlating with the expression of FcγR expression ($R = 0.988$ and $R = 0.949$, respectively, according to the Spearman rank test). As already suggested in different other systems (14–16, 26,27), the concomitant induction of $O_2^{\circ -}$ and NO° production led to the final production of peroxynitrites ($ONOO^-$) by IFN-γ preactivated

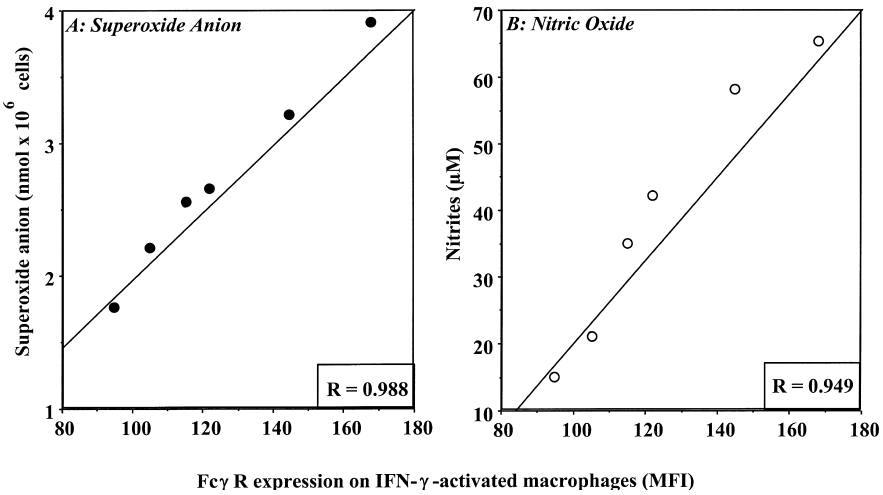

Fig. 1. Correlation between superoxide anion (A) or nitric oxide (B) production and FcγR expression after IC-stimulation. As already described in the Materials and Methods section, J774.A1 cells (2×10^6 cells/ml) were stimulated for 2 days in the presence of IFN-γ (1,000 U/ml) and then cells were incubated in the presence of IgG_1/anti-IgG_1 (100/20 µg/ml), the generation of superoxide anion being assayed by reduction of ferricytochrome c (A), whereas nitric oxide production (B) was evaluated after measurement of NO_2^- production using the Griess reaction, the expression of Fcγ receptor (CD16/CD32) expression was done using the 2.4G2 mAb (rat IgG2b) and cell-related fluorescence intensity (MFI) was detected by flow cytometry using a FACScan system (Becton Dickinson, Grenoble, France). Data are from six independent experiments, and correlation with FcγR expression was evaluated using the Spearman rank test.

cells (Fig. 2) as revealed by oxidation of the dihydro-rhodamine-123 fluorescent probe.

When macrophagic cells were pre-loaded with IgG_1 anti-Bo-SOD1, the subsequent antigenic stimulation with free Bo-SOD1 led to the internalization of the antioxidant enzyme in the cytosol of targeted cells (Fig. 3) as revealed by specific ELISA.

This Fc-mediated cellular penetration of the free Bo-SOD1 considerably limited the capacity of these cells to produce superoxide anion

Table 1. FcγR expression on resting and IFN-γ-activated macrophages

Cells	Isotype Control (Mean Fluorescence Intensity)	2.4G2 anti-FcγR
Resting	65 ± 5	101 ± 4
IFN-γ-activated	75 ± 3	145 ± 15

Cells (2×10^6 cells/ml) were incubated for 48 hr in the presence or absence of 100 U/ml of interferon-γ. Cells were then harvested and stained, as described in the Materials and Methods section, with the 2.4G2 anti-Fcγ receptor monoclonal antibody. The data are expressed as the mean ± SD of one experiment (quadruplicate samples) representative out of six.

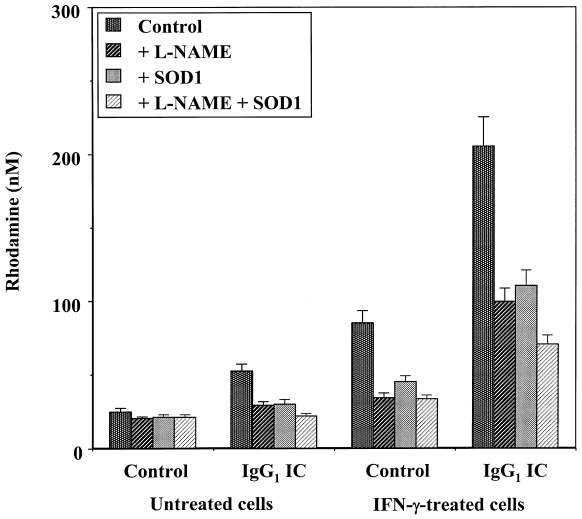

Fig. 2. Immune complexes induced $ONOO^-$ production by IFN-γ-activated macrophages. To measure $ONOO^-$ production of the cellular respiratory burst activity, Rhodamine measurements were monitored as described in Materials and Methods. Interferon-γ-activated J774.A1 cells, (5×10^5 cells in 500 µl) were loaded with dihydroxy-Rhodamine 123, and the suspension was pre-warmed at 37°C, before a 50 µl addition of IgG_1 immune complexes and/or of L-NAME (1 mM) or Free Bo-SOD1 (300 U/ml), rhodamine formation being measured as described in Materials and Methods. The data presented the mean ± SEM of four different experiments.

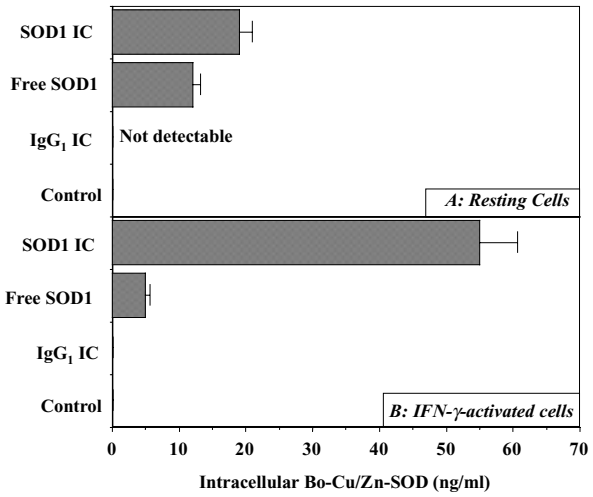

Fig. 3. Fc-mediated intracellular delivery of copper/zinc-superoxide dismutase (SOD1) in macrophages. Cells loading (2×10^6 cells/ml) were plated in 24-well culture plates, and incubated overnight in the presence or absence of the immune complexes (IgG1 IC or SOD1 IC) and/or free SOD1. As already described in Materials and Methods for analysis of Bo-SOD1 loading the cells were then treated as already described with 0.5% trypsin for 5 min at 37°C to digest residual SOD, which may be bound to the external surface of the cell membrane. After neutralization of the trypsin, the cells were washed and protein was extracted with a buffer containing 0.1 M Na_2CO_3 (pH 10.2), 0.2 mM EDTA, 0.2% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The soluble extracts were then assayed by specific ELISA for the presence of bovine SOD1 and data represent the mean ± SEM of four different experiments.

in response to PMA (Table 2). In addition, the triggering by IgG1 IC of macrophages preloaded with Bo-SOD1 still induced the production of nitric oxide but in the absence of superoxide anion and peroxynitrite production (Fig. 4A–C). Stimulation of macrophages by IgG_1 IC, under free superoxide anion production, did not affect the expression of iNOS mRNA (Fig. 5A) protein (Fig. 5B) iNOS and up-regulated the NO-dependent production of TNF-α (Fig. 5C). Using a cell-permeable SOD mimetic (MnTBAP) (28), a similar inhibitory effect was also demonstrated on superoxide anion and peroxynitrite production, that of nitric oxide still being unaffected (Table 3). Interestingly, in the presence of an inhibitor of protein synthesis, cycloheximide, the up-regulation of the expression of iNOS as well as the production of TNF-α by SOD1 loaded cells were significantly decreased, whereas those in the presence of MnTBAP were unaffected (Fig. 6). These latter data suggested that in addition to

Table 2. Comparison of the protective effect of Bo-SOD1 before and after intracellular delivery of the antioxidant enzyme to murine macrophages by FcγR-mediated endocytosis

Interferon-γ-Activated Cells	Superoxide Anion Production (nmol × 10^6 cells)
Unloaded cells	0.7 ± 0.2
Unloaded cells + PMA	3.5 ± 0.5
Bo-SOD1-loaded cells	0.4 ± 0.1
Bo-SOD1-loaded cells + PMA	1.3 ± 0.2

IFN-γ-activated cells were treated or not with Bo-SOD1 IC (20 μg/ml of the anti-Bo-SOD1 Ab and 3,000 U/ml of Bo-SOD1) for 6 hr and then were washed and plated in 96-well culture plates (2×10^5 cells per well) and were stimulated or not by 200 nM of PMA to trigger the production of superoxide anion that was measured, as described in Materials and Methods, by reduction of ferricytochrome c. Data represent of the mean ± SEM of three different experiments.

reducing the generation of superoxide anion, the antigenic nature of the SOD1 (Bovine Cu/Zn-SOD) also stimulated innate immunity. Taken together, these data confirmed that NO production, under superoxide anion-free conditions, could be essential in controlling not only the pro-inflammatory state of macrophages but also the pharmacology of SOD1.

Nitric Oxide Generated in Bo-SOD1-loaded Cells Promoted the Expression of the Cellular Antioxidant Armature in Macrophages

The control of the antioxidant capacities of targeted cells generally compensate for the excess of free radical production that is at the origin of the development of inflammatory disorders (29). In this study, we demonstrated that IgG_1 IC induced an up-regulation of the Mn-SOD activity in IFN-γ-activated macrophages and significantly suppressed the glutathion peroxidase (Gpx) activity and the GSH metabolism; the catalase activity remaining unaffected (Table 4). This functional alteration of the antioxidant armature in IFN-γ-activated macrophages is likely the consequence of peroxynitrite production, because it was overcome in the presence of L-NAME and/or of free Bo-SOD1 (Table 5). In addition, Fc-mediated cellular penetration of free Bo-SOD1 in IFN-γ-activated

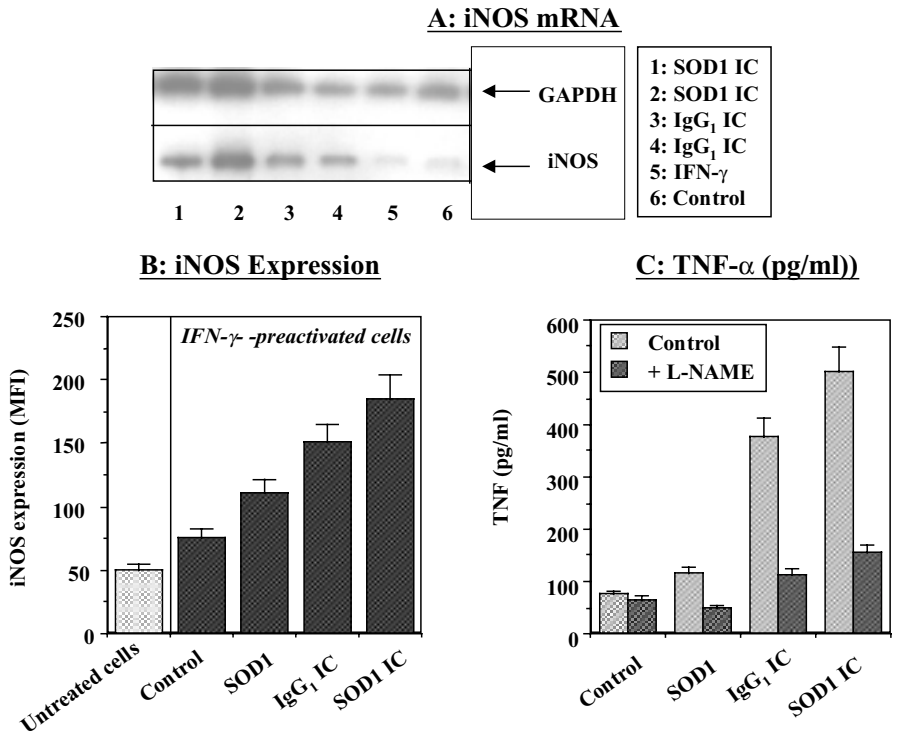

Fig. 4. (A) iNOS mRNA, (B) protein expression, and (C) nitric oxide production after Fcγ receptor ligation by Bo-SOD1 IC. Cells (2×10^6/ml) were incubated overnight with (A) IgG1 IC, or (B) Bo-SOD1 IC prior RNA and iNOS protein expression (B) as described in the Materials and Methods section, the (C) NO-dependent tumor necrosis factor-α production being evaluated, as described in the Materials and Methods section, in ongoing cultures in the cell-free supernatants after 24 hr of culture. Data are from one representative experiment out of four different experiments where data were comparable.

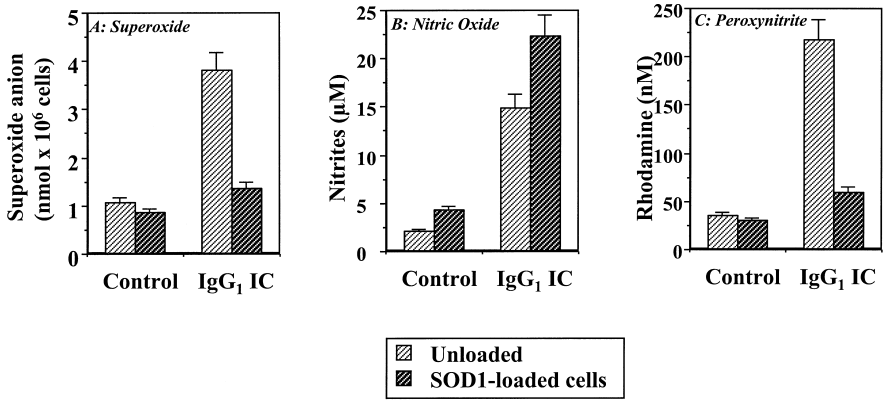

Fig. 5. Effect of IgG1 IC on (A) superoxide anion, (B) nitric oxide, and (C) peroxynitrite production by interferon-γ(IFN-γ)activated macrophages loaded or not with Bo-SOD1. As described in Materials and Methods, IFN-γ-activated J774.A1 cells were loaded or not for 24 hr with specific Bo-SOD1 IC (20 μg of anti-Bo-SOD1 + 100 μg of Bo-SOD1). Then, cells were washed and stimulated with IgG1 IC to produce (A) superoxide anion as revealed by reduction of ferricytochrome c, (B) nitric oxide as measured by NO_2^- production, and (C) peroxynitrites as revealed by rhodamine measurement. Data represent the mean ± SEM of three different experiments.

Table 3. Effect of a cell-permeable SOD mimetic on IgG_1 IC-induced superoxide anion, nitric oxide, and peroxynitrite production by interferon-γ-activated macrophages

Stimulation of IFN-γ-Activated Cells	Superoxide Anion (nmol × 10^6 cells)	Nitrites (μM)	Peroxynitrite Rhodamine (nM)
Control	0.9 ± 0.2	2.4 ± 0.5	98 ± 2.5
MnTBAP	0.3 ± 0.1	0.9 ± 0.2	45 ± 1.2
IgG_1 IC	3.1 ± 0.3	10.5 ± 1.0	208 ± 8.5
IgG_1 IC + MnTBAP	1.2 ± 0.4	18.0 ± 1.2	108 ± 2.4

Resting or interferon-γ-treated cells were challenged overnight by Bo-SOD1 IC (20 μg of anti-Bo-SOD1 and 10 μg of Bo-SOD1) IgG_1IC (20 μg/20 μg) and/or free Bo-SOD1 (10 μg/ml). After extraction of total intracellular proteins, as described in Materials and Methods, the presence of Bo-SOD1 was evaluated by specific ELISA. Data are the mean ± SD (triplicate samples) of one representative experiment out of four.

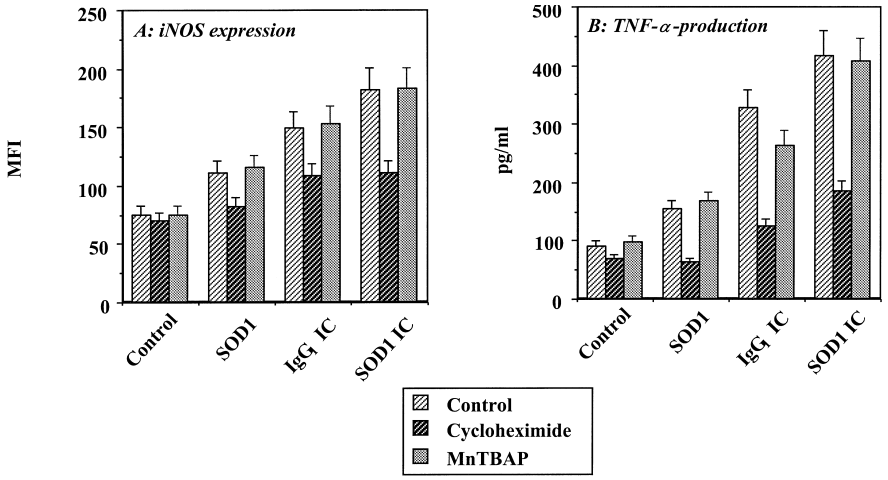

Fig. 6. Effect of a SOD mimetic and of an inhibitor of protein synthesis on the (A) Bo-SOD1-induced iNOS expression, and (B) tumor necrosis factor-α (TFN-α) production. As described in Materials and Methods, IFN-γ-activated J774.A1 cells were stimulated or not for 24 hr with IgG1 IC or specific Bo-SOD1 IC (20 μg of anti-Bo-SOD1 + 100 μg of Bo-SOD1) and in the presence or absence of MnTBAP (50 μM) or cycloheximide (50 μM). Then (A) iNOS expression or, (B) TNF-α production were evaluated and data represent the mean ± SEM of three different experiments.

Table 4. Intracellular delivery of Bo-SOD1 by immune complexes promote the antioxidant armature in interferon-γ-treated macrophages

Cell Treatment	Mn-SOD	Glutathion Peroxidase (U/mg/protein)	Catalase	Glutathion nmoles/10^6 cells
Control	95 ± 3	9.3 ± 1	870 ± 31	22 ± 3
IgG_1 IC	125 ± 1	2.1 ± 2	850 ± 15	8 ± 2

Interferon-γ-treated cells (2 × 10^6 cells/ml) were challenged overnight by Bo-SOD1 IC (20 μg of anti-Bo-SOD1 and 10 μg of Bo-SOD1) and/or of 1 mM L-NAME. After extraction of total intracellular proteins, antioxidant enzymes activities and GSH measurements were done as described in Materials and Methods. Data are the mean ± SD (triplicate samples) of one representative experiment out of four.

Table 5. Role of nitric oxide in the control of the anti-oxidant armature in interferon-γ-treated macrophages stimulated by SOD1 IC

Interferon-γ-Treated Cells+	Mn-SOD	Glutathion Peroxidase (U/mg/protein)	Catalase	Glutathion nmoles/10^6 Cells
None	75 ± 2	9.3 ± 1	870 ± 31	22 ± 3
L-NAME	7 ± 1	9.1 ± 2	520 ± 15	15 ± 2
SOD1	12 ± 2	8.4 ± 1	695 ± 25	19 ± 1
SOD1 IC	212 ± 1	15.5 ± 2	920 ± 15	30 ± 3
SOD1 IC + L-NAME	85 ± 2	29.0 ± 2	1025 ± 30	55 ± 4

Interferon-γ-treated cells (2×10^6 cells/ml) were challenged overnight by Bo-SOD1 IC (20 μg of anti-Bo-SOD1 and 10 μg of Bo-SOD1) and/or of 1 mM L-NAME. After extraction of total intracellular proteins, antioxidant enzymes activities and glutathion measurements were done as described in Materials and Methods. Data are the mean ± SD (triplicate samples) of one representative experiment out of four.

macrophages also inhibited IgG_1 IC-induced antioxidant imbalance (Table 5). Taken together, the data clearly indicates that peroxynitrites produced by macrophages after IgG_1 IC-stimulation contributes to the development of a redox imbalance that could contribute to the development of pro-inflammatory disorders.

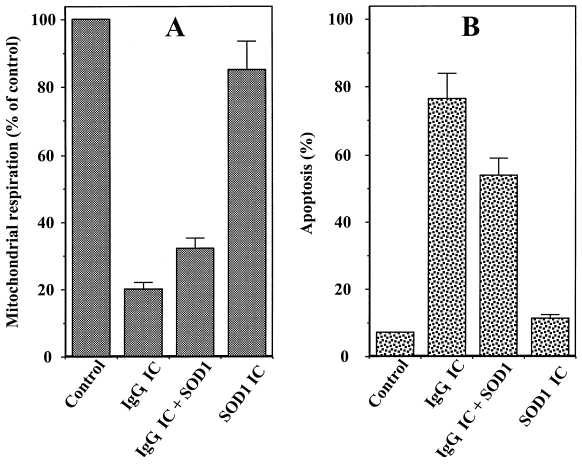

Fig. 7. Mitochondrial (A) respiration, and (B) apoptosis after ligation of Fcγ receptor by IgG1 or Bo-SOD1 IC. Cell respiration was assessed by the mitochondrial-dependent reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan as described in the Materials and Methods section. Cells (10^6 cells/ml) in 96-well plates were incubated at 37°C with MTT (0.2 mg/ml) for 1 hr. Culture medium was removed by aspiration and the cells were solubilized in DMSO (100 μl). The extent of reduction of MTT to formazan within cells was quantified by the measurement of OD_{550}. Data represent the mean ± SEM of four different experiments. Apoptosis-induced DNA fragmentation was estimated as described in the Materials and Methods section using the ApoAlert DNA fragmentation detection kit (Clontech, Palo Alto, CA, USA). Data represent the mean ± SEM of four different experiments.

Nitric Oxide Generated in the Absence of Superoxide Anion Production Protects Macrophages Against Mitochondrial Damages and Apoptosis

In addition to the regulation of the pro-inflammatory status of macrophages, it is also known that coordinate induction of $O_2^{\circ-}$ and NO° production by in IFN-γ-preactivated cells induces a $ONOO^-$-dependent induction of mitochondrial damages and apoptosis (29–31). Indeed, FcγR cross-linking induced similar $ONOO^-$-dependent-induced (i) alteration of mitochondrial respiration (Fig. 7A), and (ii) concomitant apoptosis (Fig. 7B). In these experimental conditions, the specific cellular translocation of Bo-SOD1 was found to protect actively against $ONOO^-$-induced suppression of mitochondrial respiration (Fig. 7A) and apoptosis induction (Fig. 7B). Taken together, the data suggested that induction of NO° production under free superoxide anion production is mostly associated with the protection of mitochondrial damages and apoptosis (23), whereas the concomitant production of $O_2^{\circ-}$, NO°, and more specifically of $ONOO^-$ led to the induction of mitochondrial damages and cell death by apoptosis.

Discussion

The results obtained in this study indicate that antibodies specific to Bo-SOD1 may assume the cellular penetration of the antioxidant enzyme

in the cytoplasm of macrophages through a Fc-dependent mechanism. Here, we confirmed (data not shown) and enlarged the data from different groups suggesting that exogenous SOD may penetrate cells and affect the oxidative metabolism of the targeted cells. In addition, our data suggest that in the context of a specific immune response (e.g., production of specific antibodies) after injection and/or oral ingestion (3,32) the pharmacology of antigenic Cu/Zn-SOD could be multifactorial. It (i) reduces the oxygen toxicity by suppressing the intracellular production of $O_2^{\circ-}$ and $ONOO^-$, (ii) promotes NO° production and NO°-related biological events after iNOS induction (33–35), and (iii) promotes the antioxidant armature in targeted cells. In fact, we suggest that the principal in vivo pharmacological characteristics of antigenic Cu/Zn-SOD are to promote an immune response that will strengthen the anti-inflammatory properties of this antioxidant and likely via the induction of NO° production. Such production of NO°, in the absence of superoxide anion production, protected cells principally against redox-mediated cell death (mitochondrial alterations and apoptosis) and promoted the cell antioxidant enzyme armature as suggested schematically in Figure 8.

In addition to providing new highlights in the pharmacology of Bo-SOD1, these data suggest that the development of a coordinate and appropriate immune response against this antigenic antioxidant enzyme could be essential for induction of its full pharmacologic effect. These data thus provide new insights for the development of heterologous SOD as therapeutic agents, as already suggested for other antioxidant enzymes such as catalase (36), and could explain, at least in part, the poor pharmacologic effect of human SOD in humans, the product being in that case not antigenic but homologous. However, the development of Bo-SOD1 as a therapeutic agent has been stopped because of the prion-mediated disease (37,38), and, until now, no other sources of heterologous Cu/Zn-SOD have been developed as pharmaceutical products. However, the existence of different sources of vegetal SOD led us to develop a new heterologous SOD product deliverable by the oral route (US patent #6,045,809 and B. Dugas et al., manuscript in preparation) and that is under development as a new antiinflammatory drug.

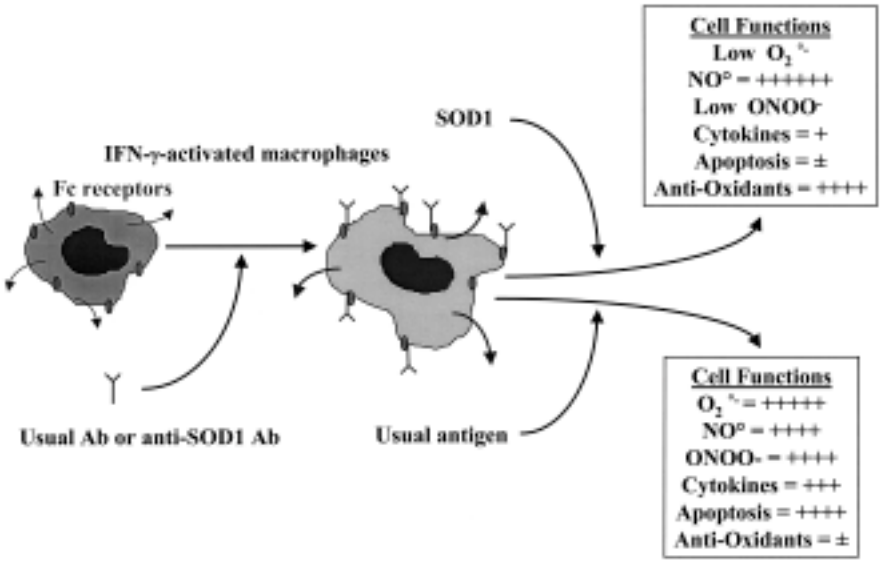

Fig. 8. Schematic representation of the putative mechanism of action of heterologous Cu/Zn-SOD on Fc receptor-bearing cells. Macrophages expression Fcγ receptors can trigger immune complexes to generate the concomitant production of superoxide anion (high oxygen toxicity) and of nitric oxide, which then are chemically combined to generate peroxynitrites that are principally responsible in cell degenerescence notably by affecting cell respiration and inducing cell death by apoptosis. In the present work, we demonstrate that when the triggering antigen is Bo-SOD1, the production of superoxide anion, after intracellular antigen translocation, is inhibited (low oxygen toxicity) whereas NO° production was maintained and in that case is protective notably by inducing the expression of protective antioxidant enzymes.

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