Overexpression of redox factor-1 negatively regulates NO synthesis and apoptosis in LPS-stimulated RAW 264.7 macrophages

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Abstract Redox factor-1 (Ref-1) is a ubiquitously expressed protein with proven roles as a modulator of redox-sensitive transcription, and as an endonuclease in the base excision repair pathway of oxidatively damaged DNA. Although Ref-1 is induced by a variety of oxidative stress and protects cells against oxidative stress, the function of Ref-1 in regulating nitric oxide (NO) synthesis has not been elucidated to date. We investigated the role of Ref-1 in regulating NO synthesis and NO-mediated apoptosis employing adenoviral-mediated overexpression of Ref-1 in lipopolysaccharide (LPS)-stimulated macrophage RAW 264.7 cells. LPS treatment produced NO synthesis and NO-mediated apoptosis. Forced overexpression of Ref-1 suppressed LPS-stimulated NO synthesis. In parallel with this, Ref-1 also mitigated alteration of inducible NO synthase expression and NO-mediated apoptosis. Our findings suggest that Ref-1 is implicated in protection against cell death resulting from oxidative stimuli containing NO.

Key words: Redox factor-1; Nitric oxide; Macrophage; Lipopolysaccharide; Apoptosis

1. Introduction

Nitric oxide (NO) is a multifunctional biomolecule involved in a variety of physiological and pathological processes [1]. In macrophages, NO contributes to the antimicrobial and tumoricidal activities in pathological conditions. In contrast to the cell protective and anti-inflammatory effects, overproduction of NO has been associated with oxidative stress [2,3] and with the pathophysiology of various diseases such as arthritis, diabetes, stroke, septic shock, autoimmune disease, and chronic inflammation [4,5]. Moreover, NO mediates reactions with proteins and nucleic acids, and causes apoptosis of macrophages. NO-mediated apoptosis is generally considered to be mediated by DNA or mitochondrial damage [6]. Because of the pivotal double-edged role of NO, it is necessary to elucidate intracellular molecules involving in regulatory mechanisms for NO synthesis.

Redox factor-1 (Ref-1) is a ubiquitous 37 kDa bifunctional protein that is transcriptionally upregulated in response to oxidative stresses [7]. It has the two following important functions: (1) a nuclear reducing factor that promotes the DNA binding properties of many redox-sensitive transcription factors that regulate cell growth, differentiation, survival, and death including AP-1, NF-κB, p53, Egr-1, and c-Myb [8–11]; and (2) an endonuclease indispensable in the base excision repair pathway of damaged DNA, which is generated by oxidative damage [12]. However, the role of Ref-1 in the regulation of inducible NO synthase (iNOS) and its importance in macrophage/excess NO-mediated cytotoxicity are not known. Here we report that Ref-1 regulates bacterial lipopolysaccharide (LPS)-induced NO synthesis in RAW 264.7 macrophages.

2. Materials and methods

2.1. Materials

LPS (phenol-extracted *Salmonella enteritidis*), sulfanilamide, *N*-(1-naphthyl)-ethylenediamine dihydrochloride, phosphoric acid (H_3PO_4), and sodium nitrite ($NaNO_2$) were from Sigma Chemical Co. (St. Louis, MO, USA). RPMI containing L-arginine (200 mg/l; 1148 μM), Hanks' balanced salt solution, and other tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD, USA). DAF-2 (4,5-diaminofluorescein) was purchased from Alexis Biochemicals (Carlsbad, CA, USA). L-NAME was purchased from Calbiochem (San Diego, CA, USA). Anti-Ref-1 and anti-iNOS antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Macrophage culture

The murine monocyte/macrophage cell line RAW 264.7 was cultured in 75 cm^2 plastic flasks (Falcon-Becton Dickinson Labware, Franklin Lakes, NJ, USA) and maintained at 37°C with 5% CO_2 in air atmosphere in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum and antibiotics (100 U/ml of penicillin, 100 μg/ml of streptomycin). For experiments, macrophages were detached by vigorous pipetting and, after centrifugation, incubated with fresh medium in either 24 well tissue culture plates (5×10^5 cells/well) or 35 mm tissue culture dishes (1×10^6 cells/dish).

2.3. Adenoviruses

The replication-deficient adenovirus, AdRef-1, encoding full-length Ref-1 was constructed through homologous recombination in HEK 293 cells, as described previously [13]. AdDl312 encodes an E1-deleted adenovirus without a transgene. AdDl-infected cells or uninfected cells were used as controls in all experiments. Adenoviral stocks were prepared in HEK 293 cells, purified on double cesium gradient, and titered using a standard plaque assay [14]. Infections were carried out at a multiplicity of infection (MOI) of 50, 150 or 300 for 16 h. Protein expression and biochemical or functional assays described below were carried out 28 or 40 h after infection.

2.4. Western blot analysis

Equivalent amounts of total protein were loaded onto 10% sodium

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Abbreviations: Ref-1, redox factor-1; NO, nitric oxide; iNOS, inducible NO synthase; LPS, lipopolysaccharide; ROS, reactive oxygen species

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dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The gels were transferred to nitrocellulose membrane using an electroblotting apparatus (Bio-Rad, Richmond, CA, USA) and reacted with the appropriate primary antibody according to standard methods. Bound immunocomplexes were visualized by ECL reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA). β-Actin was used as an internal control to monitor equal protein sample loading.

2.5. Measurement of nitrite concentration

NO secretion in cultured macrophages was measured by a microplate assay method, as described previously [15]. To measure nitrite (NO_2^-), 100 μl of macrophage culture supernatant were collected, mixed with an equal volume of the Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) and incubated for 10 min at room temperature. Nitrite concentration was determined by measuring the absorbance at 540 nm in an Emax 96 well microtest plate spectrophotometer (Molecular Devices, Menlo Park, CA, USA). $NaNO_2$ was used for external calibration. Cell-free medium alone contained 5–8 μM of nitrite; this value was determined in each experiment and subtracted from the value obtained for each cell sample.

2.6. Detection of NO production in cells

To measure fluorescence intensity, LPS-stimulated cells were washed twice with Krebs–Ringer phosphate buffer (KRP; 120 mM NaCl, 4.8 mM KCl, 0.54 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose, and 15.9 mM sodium phosphate, pH 7.2), and then KRP buffer containing 10 μM DAF-2 and 1 mM L-arginine in the presence or absence of the NO synthase inhibitor L-NAME (1 mM) were added [16]. After incubation for 1 h, supernatants were collected and the fluorescence was measured with a fluorescence microplate reader (Titertek Fluoroscan II, Flow Laboratories, North Ryde, Australia) calibrated for excitation at 485 nm and emission at 538 nm.

2.7. Determination of apoptosis and quantification

Cell suspension was centrifuged onto a clean, fat-free glass slide with a cytocentrifuge. Cells were fixed for 10 min in 4% paraformaldehyde and stained in 4 μg/ml Hoechst 33342 for 30 min at 37°C. The samples were observed and photographed under an epifluorescence microscope [17]. The number of cells showing condensed or fragmented nuclei was determined by a blinded observer from a random sampling of 250–300 cells per experiment.

2.8. Statistics or reproducibility

Data in figures are the mean ± S.D. of at least three different experiments performed in triplicate.

3. Results

3.1. Efficient adenoviral gene transfer into macrophage cell line RAW 264.7

We first determined the level of adenoviral-mediated over-

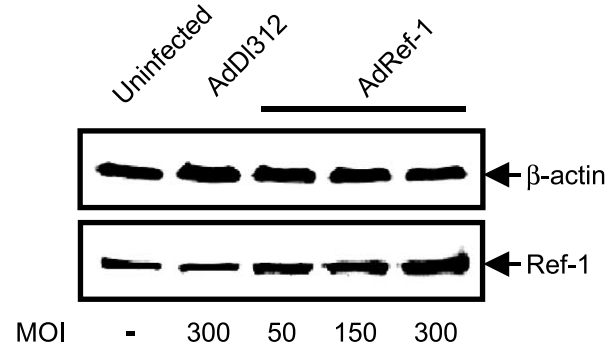

Fig. 1. Demonstration of adenoviral-mediated gene transfer in macrophage cell line RAW 264.7. Cells were infected with AdDl312 or AdRef-1, as indicated, at different MOIs for 16 h, and whole cell extracts were analyzed by Western blotting using a Ref-1 antibody. β-Actin was used as an internal control to monitor equal protein loading.

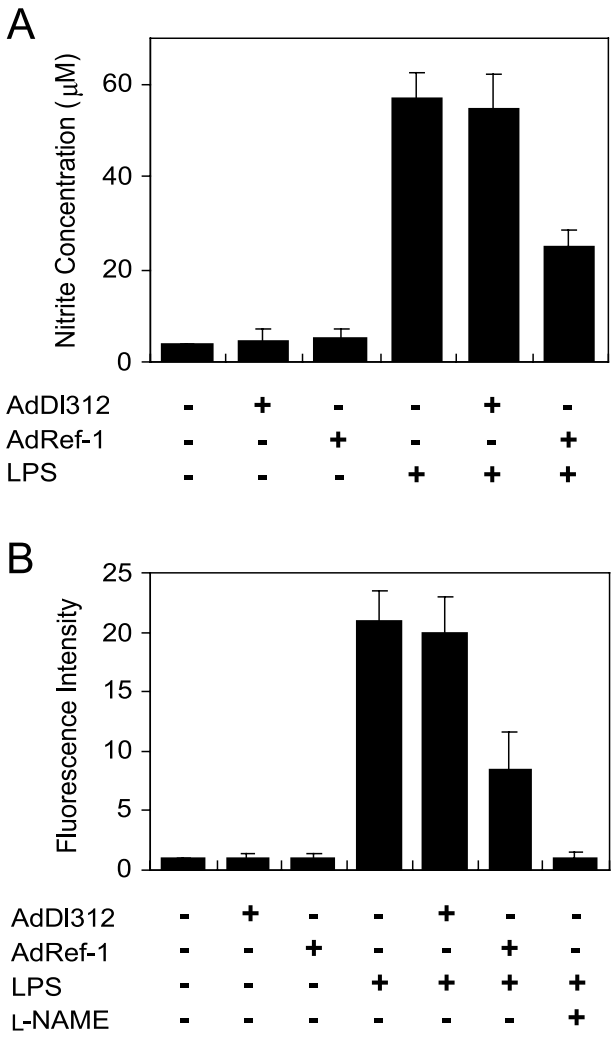

Fig. 2. Effect of Ref-1 on the synthesis of NO by LPS-stimulated macrophages. Cells were infected with AdDl312 (300 MOI) or AdRef-1 (300 MOI) for 16 h. A: Measurement of nitrite. Uninfected, AdDl312- and AdRef-1-infected cells were incubated for another 24 h either in a medium alone or in a medium that contained LPS (200 ng/ml). Then, supernatants were collected, and the amount of nitrite released by macrophages was measured by the method of Griess. Results are presented as means ± S.D. of three independent cell preparations. B: Fluorescence intensity by NO indicator. Cells were infected and stimulated with LPS, as described in A. Then, the cells were washed 12 h after stimulation, incubated for another 1 h in KRP buffer containing DAF-2 in the presence or absence of L-NAME (1 mM), as described in Section 2. Supernatants were measured with a fluorescence microplate reader. Values are means ± S.D. of three experiments.

expression of Ref-1 in RAW 264.7 cells. Infection of cells with AdRef-1 (50, 150, 300 MOI) showed significant expression of Ref-1 compared to cells infected with AdDl312 (300 MOI) or uninfected cells, in a MOI-dependent manner (Fig. 1).

3.2. Ref-1 inhibits NO synthesis in LPS-stimulated macrophages

Next, we assessed the role of Ref-1 in regulating NO synthesis of LPS-stimulated RAW 264.7 cells. Cells were infected with AdDl312 or AdRef-1 as described in Section 2, and then stimulated with 200 ng/ml LPS, the most notable activator for NO synthesis in macrophages, for 24 h. The amount of NO

released was measured by the method of Griess after conversion to nitrite. As shown in Fig. 2A, LPS increased NO synthesis in uninfected and AdDl312-infected macrophages. However, AdRef-1-infected cells resulted in a significant decrease of NO release induced by LPS stimulation.

The ability of overexpressed Ref-1 to reduce intracellular NO secreted to the medium was also evaluated in macrophages. The *N*-nitrosation of DAFs, yielding the highly green fluorescent triazolofluoresceins, offers the advantages of specificity, sensitivity, and a simple protocol for the direct detection of NO (detection limit 5 nM). As shown in Fig. 2B, there was no significant difference in fluorescent levels between uninfected, AdDl312-infected, and AdRef-1-infected cells under unstimulated conditions (< 5 μM). The supernatant of uninfected and AdDl312-infected macrophages stimulated with LPS produced high fluorescent intensity upon incubation with DAF-2. In comparison, cells infected with Ref-1 showed significant reductions in LPS-induced fluorescence intensity, indicating that overexpressed Ref-1 inhibits LPS-induced NO synthesis in macrophages. And L-NAME, a known inhibitor of NOS, totally blocked the increase of fluorescence intensity, as expected.

3.3. *Ref-1 inhibits the synthesis of iNOS in LPS-stimulated macrophages*

To know the role of Ref-1 on the regulation of iNOS gene expression, we analyzed the amount of expressed iNOS by Western blot analysis (Fig. 3). In unstimulated AdDl312-infected cells, iNOS was not detectable. And LPS induced expressed iNOS level in AdDl312-infected cells. However, when AdRef-1-infected cells were stimulated with LPS, iNOS content was decreased MOI-dependently.

3.4. *Ref-1 inhibits LPS-induced apoptosis in macrophages*

To this end, Hoechst staining was conducted to determine whether the Ref-1-mediated decrease of NO synthesis prevents LPS-induced apoptosis. It was demonstrated that Ref-1 significantly reduced LPS-induced apoptosis, as determined by nuclear morphology. When AdDl312-infected cells were stimulated with LPS, the percentage of dead or dying cells was highly increased (Fig. 4A), compared with the typical round nuclei of unstimulated cells (Fig. 4B). This finding suggests that Ref-1 inhibits apoptosis via the reduction of NO synthesis in LPS-stimulated macrophages.

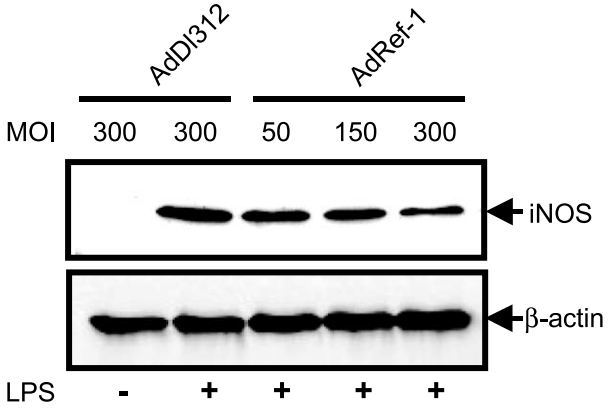

Fig. 3. Effect of Ref-1 on the expression of iNOS in macrophages. Cells were infected with AdDl312 or AdRef-1, as indicated, and stimulated with LPS (200 ng/ml) for another 24 h. Whole cell extracts were subjected to 10% SDS–PAGE and transferred to a nitrocellulose filter.

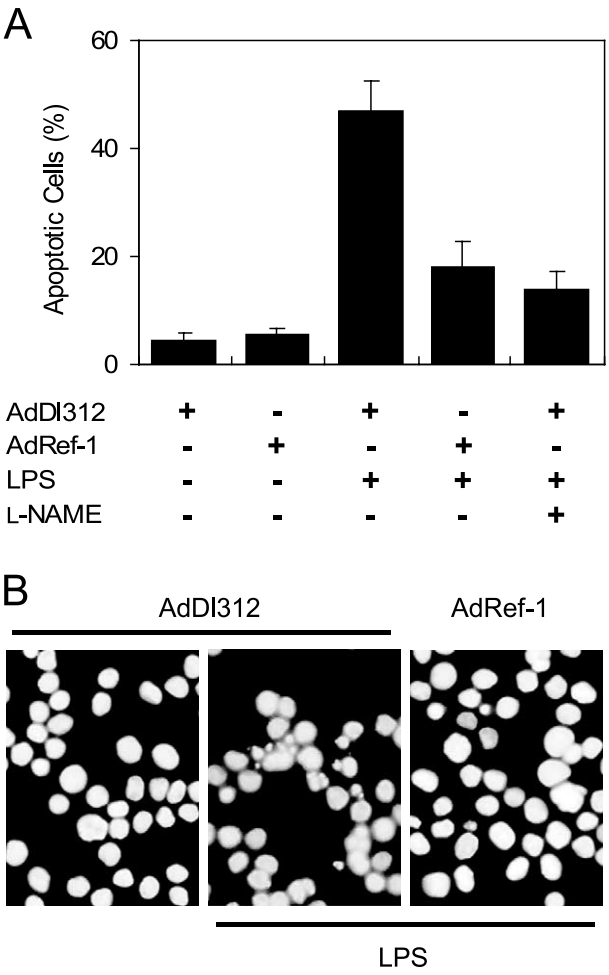

Fig. 4. Effect of Ref-1 on NO-induced apoptosis in stimulated macrophages. A: Measurement of cell apoptosis. Uninfected and infected cells were stimulated with LPS (200 ng/ml) in the presence or absence of L-NAME (1 mM) for 24 h. Cells were fixed and stained with the Hoechst dye 33342. Images were obtained with a fluorescence microscope. Values are means ± S.D. of three independent experiments. B: Hoechst staining pictures demonstrating nuclear condensation or fragmentation.

4. Discussion

Excess NO released by iNOS reveals antimicrobial and tumoricidal effects [1,18], but can also affect the surrounding tissue, suggesting a pathological role of NO for several inflammatory diseases [4,5,18]. And NO can induce apoptosis in various cell types, including macrophages [19,20], β-cells [21], and thymocytes [22]. NO induced accumulation of the tumor suppressor protein p53, which was described as an essential and early indicator of NO-induced apoptosis [23]. Subsequently, cleavage of poly(ADP-ribose) polymerase indicating the activation of caspases or related proteases may occur [24,25]. However, there may also be p53- and caspase-independent pathways indicating that NO-induced apoptosis more likely is the result of direct DNA damage [25–27].

This report provides evidence that overexpression of Ref-1 inhibits NO synthesis and NO-mediated apoptosis in LPS-

stimulated macrophages. Ref-1 is described in the literature as a nuclear protein, and would therefore not be predicted to regulate cytoplasmic factors. However, Ref-1 is also expressed in the cytoplasm of a variety of cell types, including endothelial cells, hepatocytes, and thyroid cells [13,28,29]. Although mitochondria play a key role in the regulation of apoptosis as a predominant source of reactive oxygen species (ROS) generated in most apoptotic cells, the most important system for ROS generation in phagocytes is NADPH oxidase [30,31]. The observation that Ref-1 modulates the activity of a Rac1-regulated oxidase may also have relevance to immune cell function. Rac proteins regulate the activity of the NADPH oxidase that is responsible for the microbial oxidative burst in macrophages and neutrophils. Furthermore, differentiation of circulating monocytes to tissue macrophages is associated with a shift of Ref-1 expression from the nucleus to the cytoplasm [32,33] with a concomitant decrease in phorbol ester-stimulated ROS generation [34], which suggests a role for cytoplasmic Ref-1 in the regulation of the macrophage oxidative burst.

These accumulated lines of evidence provide a functional connection between inhibition of NO synthesis mediated by NF-κB [35], which is the primary transcription factor regulating iNOS gene expression after LPS stimulation, and decrease of intracellular ROS via the inhibition of NADPH oxidase in the activation of macrophages. In fact, in knockout mice with defective NADPH oxidase, LPS-induced NF-κB activation is deficient, indicating the NADPH oxidase pathway is important for maximal activation of NF-κB following treatment with LPS [36]. Indeed, overexpression of Ref-1 attenuated NF-κB activation in LPS-stimulated RAW 264.7 cells (data not shown).

In conclusion, the results presented in this report demonstrate that Ref-1 inhibits NO synthesis in LPS-stimulated macrophage RAW 264.7 cells. The results also suggest that the level of intracellular Ref-1 may negatively regulate NO-mediated apoptosis of activated macrophages. Further studies are needed to determine whether Ref-1 is involved in regulation of iNOS in other cell types with iNOS. And this finding prompts future experiments of the underlying molecular mechanisms in regulation of the induction of NO synthesis and NO-mediated apoptosis in the development of activation and death in macrophages.

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