Induction of CHOP and apoptosis by nitric oxide in p53-deficient microglial cells

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Abstract Excessive nitric oxide (NO) has been implicated in neurotoxicity after stresses such as ischemia. NO toxicity is generally thought to be mediated by the DNA damage–p53 pathway or mitochondrial dysfunction. We investigated the mechanism of NO toxicity by using murine microglial MG5 cells established from p53-deficient mice. When MG5 cells were exposed to bacterial lipopolysaccharide plus interferon-γ, mRNA and protein for inducible NO synthase (iNOS) were markedly induced, and apoptosis occurred. Under these conditions, we found that mRNA and protein for CHOP/GADD153, a C/EBP family transcription factor which is involved in endoplasmic reticulum (ER) stress-induced apoptosis, are induced. iNOS mRNA was induced 2 h after treatment, whereas CHOP mRNA began to increase at 6 h with a time lag. CHOP mRNA was also induced by NO donors *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) or NOC18, or a peroxynitrite generator 3-(4-morpholinyl)-sydnonimine hydrochloride (SIN-1). Bip/GRP78, an ER chaperone which is known to be induced by ER stress, was also induced by SNAP or SIN-1, indicating that NO causes ER stress. These results suggest that NO-induced apoptosis in MG5 cells occurs through the ER stress pathway involving CHOP, but is independent of p53.

Key words: Nitric oxide; Apoptosis; CHOP; Endoplasmic reticulum stress; p53; Microglia; Lipopolysaccharide

1. Introduction

Nitric oxide (NO) is an important physiological signaling molecule in the central nervous system [1–3]. However, under certain pathological circumstances NO is overproduced and excess NO exerts neurotoxicity [4]. Several lines of evidence suggest that excessive glutamate receptor stimulation leads to neuronal death through a mechanism involving neuronal NO synthase (nNOS) activation and overproduction of NO [4]. A role of glial-derived NO in neurotoxicity has also been suggested. NO, which is produced in microglial cells expressing inducible NO synthase (iNOS), has been considered to be cytotoxic to neighboring neurons and oligodendrocytes [5,6]. Pharmacological studies using selective inhibitors of nNOS and experiments using iNOS- and nNOS-deficient mice identified iNOS and nNOS as the primary source of NO in ischemic cell injury [7].

The molecular mechanism underlying NO toxicity has not been fully elucidated, and the primary target of this NO action is largely unknown. It is widely accepted that the NO toxicity is mediated by mitochondrial dysfunction [8–10] or the DNA damage–p53 pathway [11]. By contrast, results from several in vitro experiments suggested contribution of endoplasmic (ER)/sarcoplasmic reticulum dysfunction in functional disturbances induced by ischemia-like insult [12–16].

CHOP/GADD153, a member of the C/EBP family, is induced in response to cellular stresses, especially by ER stress [17–19]. CHOP is involved in the process of apoptosis or programmed cell death associated with ER stress, although the mechanism is still unclear [20–22]. Quite recently, we found that NO depletes ER Ca^{2+}, induces CHOP, and leads to apoptosis of pancreatic β-cells through the unfolded protein response pathway [23]. Here, we report that apoptosis in p53-deficient MG5 microglial cells [24] induced by endogenous or exogenous NO occurs through the ER stress pathway involving CHOP induction.

2. Materials and methods

2.1. Chemicals

S-Nitroso-*N*-acetyl-DL-penicillamine (SNAP), 3-(4-morpholinyl)-sydnonimine hydrochloride (SIN-1), and 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene (NOC18) were purchased from Dojindo Laboratories (Kumamoto, Japan). *Escherichia coli* lipopolysaccharide (LPS) (serotype 0127:B8), Hoechst 33258, and thapsigargin were from Sigma (St. Louis, MO, USA). Mouse interferon-γ (IFN-γ) was from Life Technologies Inc. (Rockville, MD, USA).

2.2. Cell culture and treatment

MG5 microglial and A1 astrocyte-like cell lines were established from p53-deficient mice [24]. MG5 cells were cultured in 100-mm dishes in A1 conditioned medium. A1 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, 20 U/ml penicillin, and 20 μg/ml streptomycin. MG5 cells were treated with a combination of LPS (1 μg/ml) and IFN-γ (100 U/ml), SNAP, NOC18 or SIN-1 for indicated periods.

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Abbreviations: ER, endoplasmic reticulum; LPS, lipopolysaccharide; IFN-γ, interferon-γ; NO, nitric oxide; iNOS, inducible NO synthase; SIN-1, 3-(4-morpholinyl)-sydnonimine hydrochloride; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine

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2.3. Detection of apoptosis

To analyze morphological changes of nuclei, the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), stained with Hoechst 33258 (8 μg/ml) for 10 min, and washed with PBS. The stained cells were observed under a fluorescence microscope.

DNA ladder formation was analyzed as described previously [25]. DNA (10 μg/lane) was separated on agarose gels, stained with ethidium bromide, and photographed with UV illumination.

Caspase-3-like protease activity was determined by using CaspACE Assay System (Promega, Madison, WI, USA) according to the manufacturer's recommendations.

2.4. RNA blot analysis

Total RNA from MG5 cells (2×10^6 cells/100-mm dish) was isolated with the Isogen reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's recommendations. After electrophoresis through formaldehyde-containing agarose gels, RNAs were transferred to nylon membranes. Digoxigenin-labeled antisense RNA probes were synthesized using a transcription kit (Roche Molecular Biochemicals, Mannheim, Germany), from cDNAs for the following proteins: rat iNOS [26], mouse CHOP at nucleotide positions 68–585 (GenBank, accession no. X67083), and mouse Bip at nucleotide positions 1629–1859 (GenBank, accession no. AJ002387). Hybridization, washing and chemiluminescent detection on X-ray films were done as recommended by Roche Diagnostics. Densitometric quantification was done using MacBas software (Fuji Photo Film Co., Tokyo, Japan).

2.5. Immunoblot analysis

MG5 cells (2×10^6 cells/100-mm dish) were homogenized in 25 mM Tris–HCl (pH 7.4) containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor. After centrifugation, the supernatants were subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis, and proteins were electrotransferred to nitrocellulose membranes. Immunodetection was performed using an ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) and antibodies against iNOS (N32020; Transduction Laboratories, Lexington, KY, USA) and CHOP (F-168; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

3. Results

3.1. Induction of iNOS and CHOP in immunostimulated MG5 cells

When p53-deficient MG5 microglial cells were treated with LPS or IFN-γ, iNOS mRNA was induced (Fig. 1A,B). It was induced more strongly by their combination. We found that mRNA for CHOP, a C/EBP family transcription factor involved in ER stress-induced apoptosis, was induced in a similar fashion.

Induction kinetics of mRNAs and proteins for iNOS and

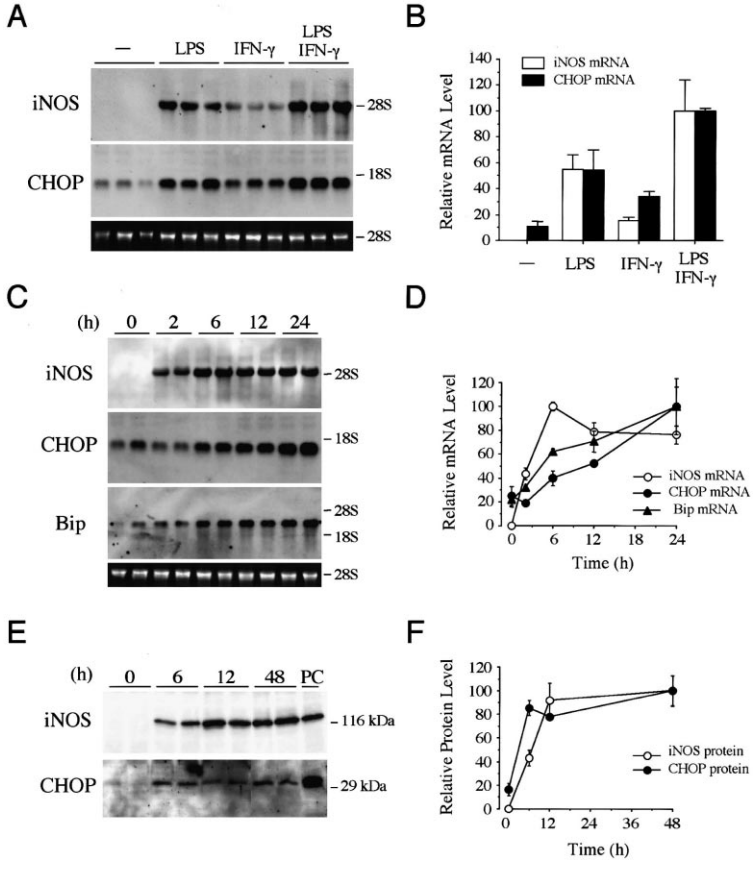

Fig. 1. Induction of iNOS, CHOP and Bip by LPS and IFN-γ in MG5 cells. A: Cells were treated with LPS (1 μg/ml) or IFN-γ (100 U/ml), or their combination for 12 h. Total RNAs (2.0 μg) were subjected to blot analysis. The bottom panel shows ethidium bromide staining of 28S rRNA as a control for RNA loading. B: Results in A were quantified and are shown as means ± S.D. ($n=3$). The maximal values are set at 100%. C: Cells were treated with LPS (1 μg/ml) plus IFN-γ (100 U/ml) for the indicated periods. Total RNAs (2.0 μg) were subjected to blot analysis. The bottom panel shows ethidium bromide staining of 28S rRNA. D: Results in C were quantified and are shown as means ± ranges ($n=2$). The maximal values are set at 100%. E: Cell extracts (20 μg and 30 μg of protein for iNOS and CHOP, respectively) were subjected to immunoblot analysis using the anti-mouse iNOS monoclonal antibody (1:500 dilution) or anti-CHOP antiserum (1:300 dilution). Positive controls (PC) are extracts (20 μg of protein) from LPS/IFN-γ-activated RAW 264.7 macrophages for iNOS and those (30 μg of protein) from 2 μM thapsigargin-treated MG5 cells for CHOP. F: Results in E were quantified and are shown as means ± ranges ($n=2$). The maximal values are set at 100%.

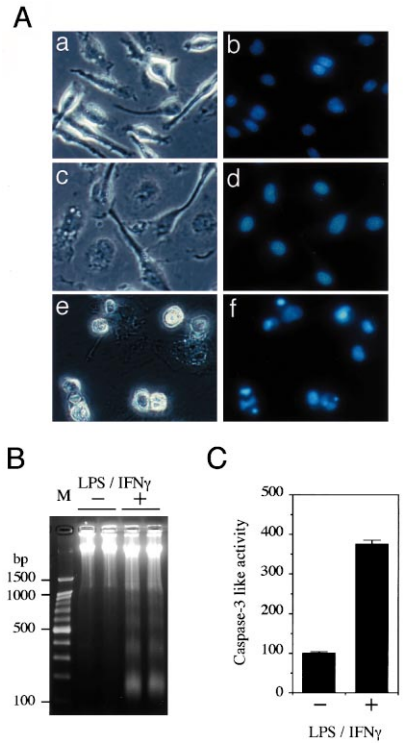

Fig. 2. LPS/IFN-γ-induced apoptosis in MG5 cells. A: Cells were exposed to LPS (1 μg/ml)/IFN-γ (100 U/ml) for 0 h (a and b), 24 h (c and d), or 48 h (e and f). After fixation, the cells were stained with Hoechst dye 33258. Phase-contrast images (a, c, and e) and fluorescence images (b, d, and f) of the same fields are shown. B: Cells were cultured in the presence (+) or absence (−) of LPS (1 μg/ml)/IFN-γ (100 U/ml) for 48 h. DNA was extracted and analyzed in a 2.0% agarose gel as described in the text. 'M' indicates 100-bp DNA ladder marker. C: Cells were cultured in the presence (+) or absence (−) of LPS (1 μg/ml)/IFN-γ (100 U/ml) for 36 h. Caspase-3-like protease activity was measured in lysates with Ac-DEVD-pNA colorimetric assay. Values are means ± S.D. ($n = 3$).

CHOP are shown in Fig. 1C–F. iNOS mRNA was increased 2 h after LPS/IFN-γ treatment and reached a maximum at 6 h. On the other hand, CHOP mRNA was weakly expressed before treatment, began to increase at 6 h with a time lag, and increased up to 24 h. Bip mRNA was also induced by LPS/IFN-γ, indicating that this treatment induces ER stress. iNOS protein was increased at 6 h and reached a near maximum at 12 h. The induction level was close to that in immunostimulated RAW 264.7 macrophages [27]. CHOP protein was also increased at 6 h and remained little changed up to 48 h. The induced level was lower than that in thapsigargin-treated cells. All these results suggest that the CHOP induction is mediated by NO produced by iNOS.

3.2. Apoptotic cell death in immunostimulated MG5 cells

Because CHOP is known to mediate ER stress-induced apoptosis in some cell types [20,21], we analyzed apoptosis of immunostimulated MG5 cells (Fig. 2). When cells were treated with LPS/IFN-γ, morphological changes characteristic of apoptosis were observed at 48 h (Fig. 2A). Round-shaped cells and apoptotic bodies were observed in phase-contrast images, and chromatin condensation and nuclear fragmentation were seen in Hoechst dye 33258 staining. Treatment with LPS/IFN-γ for 48 h led to formation of DNA ladders which reflects internucleosomal cleavage (185-bp ladder) (Fig. 2B). Caspase-3-like activity, another marker of apoptotic cell death [28], was increased at 36 h after LPS/IFN-γ treatment (Fig. 2C). The increase was not apparent at 24 h (data not shown). Namely, after LPS/IFN-γ treatment, iNOS and CHOP proteins were induced first at 6 h, caspase-3-like activity was then increased at 36 h, and morphological changes characteristic of apoptosis were finally observed at 48 h.

3.3. NO donor-induced apoptotic cell death in MG5 cells

CHOP mRNA was increased by an NO donor SNAP in a dose-dependent manner (Fig. 3A,B). It was also induced by another NO donor NOC18 or a peroxynitrite generator SIN-1 (data not shown). Bip mRNA was also increased by SNAP in a similar dose response, indicating that NO induces ER stress. CHOP and Bip mRNAs were induced by SIN-1 in similar kinetics (Fig. 3C,D).

We next examined whether treatment with NO donors leads to apoptosis in MG5 cells. SNAP induced apoptosis in MG5 cells as revealed by changes in cell and nuclear structures and increased caspase-3-like activity (Fig. 3E,F). SIN-1 induced similar apoptotic changes. However, SIN-1 induced morphological changes more strongly than SNAP and increased caspase-3 activity more strongly. This suggests that peroxynitrite is involved, at least partly, in SIN-1-dependent apoptosis, because SIN-1 generates both NO and superoxide anion, and thus peroxynitrite. Peroxynitrite may also be involved in LPS/IFN-γ-induced apoptosis in MG5 cells in which peroxynitrite is expected to be generated from NO and superoxide anion.

4. Discussion

It is generally believed that NO-induced apoptosis is mediated by the DNA damage pathway involving accumulation of p53 [11]. In the present study, we demonstrated that treatment with LPS/IFN-γ or NO donors leads to apoptosis in p53-deficient microglial MG5 cells. By contrast, we found that CHOP is induced in NO-mediated apoptosis in MG5 cells.

CHOP is induced under various conditions that cause ER stress [29]. The ER is a subcellular compartment exhibiting high calcium activity. Recent reports implicate inositol 1,4,5-triphosphate receptor- and ryanodine receptor-mediated Ca^{2+} release from ER in apoptotic signaling and induction in lymphocytes [30–32]. Quite recently, we found that NO depletes ER Ca^{2+} and causes ER stress and following apoptosis in mouse β-cell-derived MIN6 cells [23]. NO was reported to inhibit Ca^{2+}-ATPase activity of ER by tyrosine nitration within the channel-like domain [33] and to activate ryanodine receptor Ca^{2+} channel by *S*-nitrosylation [34,35]. Therefore, one or both of them may be responsible for the NO-induced depletion of ER Ca^{2+}.

Expression of CHOP is known to be linked to apoptosis. Overexpression of CHOP leads to growth arrest [36] and promotes apoptosis [37]. Mouse embryonic fibroblasts lacking CHOP exhibit significant resistance to ER stress-induced cell death [21,23]. CHOP was found to induce apoptosis in M1 myeloblastic leukemia cells in a p53-independent manner and Bcl-2 delayed this process [20]. Target genes of CHOP were recently identified and designated as DOCs (downstream of CHOP) [22]. However, none of these molecules has been shown to be directly involved in the process of programmed cell death. A novel form of carbonic anhydrase VI, identified as one of the DOCs, may promote apoptosis by increasing

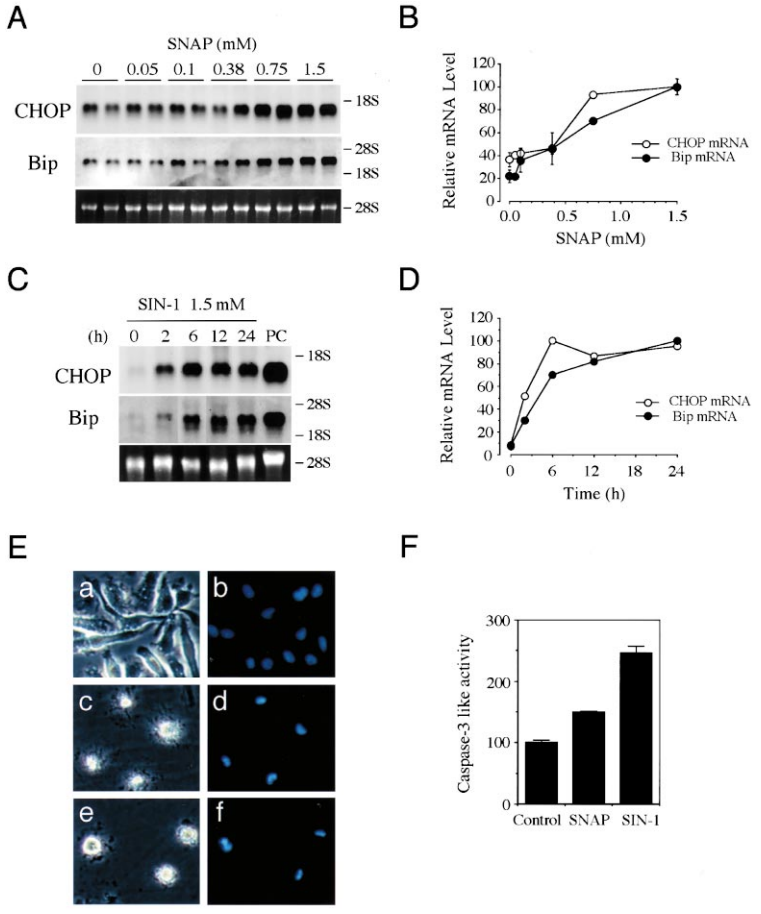

Fig. 3. Effect of NO donors on expression of CHOP and Bip and on apoptosis in MG5 cells. A: Cells were treated with indicated concentrations of SNAP for 6 h, and total RNAs (2.0 μg) were subjected to blot analysis for CHOP and Bip mRNAs. The bottom panel shows ethidium bromide staining of 28S rRNA. B: Results in A were quantified and are shown as means ± ranges ($n = 2$). The maximal values are set at 100%. C: Cells were treated with SIN-1 (1.5 mM) for the indicated periods. Total RNAs (2.0 μg) were subjected to blot analysis. Positive controls (PC) are total RNA (2.0 μg) from 2 μM thapsigargin-treated MG5 cells for CHOP and Bip mRNAs. The bottom panel shows ethidium bromide staining of 28S rRNA. D: Results in C were quantified and are shown as means ± ranges ($n = 2$). The maximal values are set at 100%. E: Cells were treated with vehicle (a and b), SNAP (1.5 mM) (c and d), or SIN-1 (1.5 mM) (e and f) for 24 h. After fixation, the cells were stained with Hoechst dye 33258. Phase-contrast images (a, c, and e) and fluorescence images (b, d, and f) of the same fields are shown. F: Cells were treated with vehicle (control), SNAP (1.5 mM), or SIN-1 (1.5 mM) for 12 h. Caspase-3-like protease activity was measured in lysates with Ac-DEVD-pNA colorimetric assay. Values are means ± S.D. ($n = 3$).

intracellular proton concentrations [38,39]. Recently, McCullough et al. [40] reported that CHOP expression results in down-regulation of Bcl-2 expression, depletion of cellular glutathione, and exaggerated production of reactive oxygen species. However, the precise apoptosis cascade downstream of CHOP remains to be clarified.

In the present study, we showed that overactivation of microglial MG5 cells with immunostimulants induces apoptotic cell death. Activation of microglia results in the production of large amounts of cytotoxic factors which cause secondary nerve cell damages [41–44]. Therefore, overactivation-induced apoptosis may be an essential self-regulatory mechanism for microglia in order to limit bystander killing of vulnerable neurons.

Taken together, results of the present and previous studies suggest that the ER calcium pool is a target of NO toxicity. Depletion of ER calcium stores by NO induces a stress response, characterized by induction of CHOP. The present results on NO toxicity may provide a basis for new therapeutic intervention in various pathological states of the brain in which NO plays a major role in the development of cell injury.

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