Excess nitric oxide (NO) induces apoptosis in some cell types including macrophages; however, the cascade of NO-mediated apoptosis is not fully understood. We investigated the initial steps of NO-mediated apoptosis in mouse macrophage-like RAW 264.7 cells. When cells were treated with bacterial lipopolysaccharide (LPS) plus interferon-γ (IFN-γ), NO-mediated apoptosis occurred. Under these conditions, p53 accumulation was not observed, indicating that DNA damage is not the main trigger of NO-mediated apoptosis. On the other hand, mRNA and protein for CHOP, a transcription factor known to be induced by endoplasmic reticulum (ER) stress, were induced. The CHOP induction by LPS/IFN-γ treatment preceded cytochrome c release from mitochondria. In addition, p90ATF6, an ER membrane-bound transcription factor involved in ER stress response, was cleaved to its active soluble form p50ATF6, which was transported to nucleus and bound to the ER stress response element of the CHOP gene. In the luciferase reporter assay, both the CHOP-binding element of the Rous sarcoma virus long terminal repeat and ER stress response element of the CHOP gene were activated by LPS/IFN-γ treatment. When RAW 264.7 cells or COS-7 cells were transfected with expression plasmids for CHOP, p90ATF6, or p50ATF6, cell death was observed. In addition, apoptosis induced by p50ATF6 was prevented by a CHOP dominant negative form as well as by an ATF6 dominant negative form, and LPS/IFN-γ-induced apoptosis was prevented by the CHOP dominant negative form. Perinuclear macrophages from CHOP knockout mice showed resistance to NO-mediated apoptosis. These results indicate that the ER stress pathway involving ATF6 and CHOP plays a key role in NO-mediated apoptosis in macrophages.

Nitric oxide (NO) is a multifunctional biomolecule involved in a variety of physiological and pathological processes (1). In pathological conditions, NO functions as a bactericidal or tumoricidal agent. However, excess NO production has been implicated in diseases such as septic shock, autoimmune disease, cerebral infarction, and diabetes mellitus, in which NO-mediated apoptosis is often observed (2, 3). NO has several cytotoxic effects, including reactions with proteins and nucleic acids, and causes apoptosis. NO-induced apoptosis is generally considered to be mediated by DNA damage or mitochondrial damage (4); however, the cascade of the cell death has not been fully clarified.

CHOP, also known as GADD153, is a member of the C/EBP family that heterodimerizes with other members of the C/EBP transcription factor family. This factor is induced in response to cellular stresses, especially endoplasmic reticulum (ER) stress (5–9). CHOP is involved in the process of apoptosis associated with ER stress, although the mechanism is still unclear (6, 10, 11). Recently, ATF6 (12) was shown to be involved in the induction of CHOP in ER stress (13). ATF6 exists constitutively as a transmembrane protein p90ATF6 in the ER under non-stressed conditions (14, 15). ER stress induces proteolysis of p90ATF6 and releases a soluble transcription factor p50ATF6, which is transported into the nucleus, binds to the ER stress responsive element (ERSE) of the CHOP gene, and activates its transcription (13, 16–19). Here we report that the ER stress pathway involving CHOP is also important in bacterial lipopolysaccharide (LPS) plus interferon-γ (IFN-γ)-induced NO-mediated apoptosis in RAW 264.7 macrophages and that this requires ATF6 activation.

 experiment PROCEDURES

Plasmids—A luciferase reporter assay plasmid pGL3-promoter vector that harbors the SV40 promoter was obtained from Promega Corp. (Madison, WI). The PGL3/CHOP-BS plasmid was constructed by inserting the CHOP binding site of Rous sarcoma virus long terminal repeat 5′-TTATGCAATCCT-3′ (20) just upstream of the SV40 promoter after linker attachment. The pGL3/SV40 plasmid was constructed by inserting ERSE of the human CHOP gene promoter 5′-CCAATCAGAGAT-GGCACG-3′ (13) just upstream of the SV40 promoter after linker attachment. A full-length mouse CHOP cDNA clone was isolated by reverse transcription-PCR using total RNA from immunostimulated RAW 264.7 cells. PCR was carried out using mouse CHOP primers corresponding to nucleotides 68–585 (GenBankTM accession number X67083). The product was inserted into the EcoRV site of pT7Blue (Novagen, Madison, WI), yielding pT7Blue-mCHOP. A full-length mouse CHOP cDNA clone was isolated by reverse transcription-PCR, using total RNA from immunostimulated RAW 264.7 cells. PCR was carried out using mouse CHOP primers corresponding to nucleotides 30–734 (GenBankTM accession number X67083). The product was inserted into the EcoRV site of pcDNA3.1 (+) (Invitrogen), yielding pcDNA3.1-mCHOP. The mammalian expression plasmid for CHOP

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1 The abbreviations used are: ER, endoplasmic reticulum; ERSE, ER stress response element; LPS, lipopolysaccharide; IFN-γ, interferon-γ; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SNAP, S-nitroso-N-acetyl-L-cysteine; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; GFP, green fluorescent protein; EGFP, enhanced GFP; TG, thapsigargin.

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dominant negative form (pOPRSVI-L133A/L140ACHOP) was described (10). The mammalian expression plasmids for human full-length ATF6 (pCGN-ATF6 (670)), its active form (pCGN-ATF6 (373)), and its dominant negative form (pCGN-ATF6 (373) AAD) were described (12–14).

The mammalian expression plasmid for CHOP dominant negative form (pOPRSVI-L133A/L140ACHOP) (10) was obtained from Shizuo Akira (Osaka University, Japan). pEGFP-C1 was obtained from CLONTECH Laboratories, Inc. (Palo Alto, CA). A partial rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA clone was isolated by reverse transcription-PCR, using total RNA from rat liver. PCR was carried out using rat GAPDH primers corresponding to nucleotides 238–1042 (GenBank accessions number M17011). The product was inserted into the EcoRV site of pcDNAI (Invitrogen), yielding pcDNAI-GAPDH.

Materials—A polyclonal antibody against mouse CHOP and a monoclonal antibody against p53 were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). A polyclonal antibody against mouse Bip was obtained from StressGen Biotechnologies Corp., Victoria, Canada. Monoclonal antibodies against human/mouse cytochrome c, human hsp60, and rabbit GAPDH were obtained from R&D Systems Inc. (Minneapolis, MN), StressGen Biotechnologies Corp., and CHEMICON International Inc. (Temecula, CA), respectively. A polyclonal antibody against human ATF6 was reported (14). CHOP knockout mice were obtained from Shizuo Akira (Osaka University).

Cell Culture and Transfection—Mouse macrophage-like RAW 264.7 cells were grown in Eagle’s minimum essential medium supplemented with 10% fetal calf serum. COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Mouse peritoneal macrophages were prepared and grown in RPMI 1640 medium supplemented with 10% fetal calf serum as described (21). CHOP knockout mice were described (22). Transfection of RAW 264.7 cells and COS-7 cells with plasmids was carried out using TransIT-LTI polyamine (PanVera Corp., Madison, WI) according to the protocol provided by the manufacturer. In each experiment, the same total amounts of plasmids were transfected by adding insert-less expression plasmids.

Treatment of RAW 264.7 Cells and Mouse Peritoneal Macrophages—Cells were treated with Escherichia coli LPS (serotype 0127:B8, Sigma) plus mouse IFN-γ, a NO donor N-nitroso-N-acetyl-l-arginine (SNAP), a saroplasmine/endoplasmic reticulum Ca2+ ATPase (SERCA) inhibitor thapsigargin, or a topoisomerase inhibitor camptothecin for the indicated periods. A NO scavenger carboxy-PTIO (2-(4-carboxyphenyl)-N(4-iminoethyl)maleimide) and a NO donor SNAP, an ER stress-inducing reagent thapsigargin, or a DNA-damaging reagent camptothecin (CPT, 30 nm) for 12 h (A). The cells were fixed and stained with a DNA-specific fluorochrome Hoechst dye 33258. Phase-contrast images and fluorescence images of the same fields are shown. Original magnifications: ×400. Bars, 10 μm. Cells were treated with LPS (150 μg/ml) plus IFN-γ (100 units/ml), SNAP (1.5 μM), thapsigargin (TG, 2 μM), or camptothecin (CPT, 30 nm) for 6 h, and nuclear extracts (30 μg of protein) were subjected to immunoblot analysis for p53 protein (B). Immunoblots from two dishes for each condition are shown.

RESULTS

p53 Is Not Induced in NO-mediated Apoptosis in RAW 264.7 Cells—When mouse macrophage-like RAW 264.7 cells were treated with LPS/IFN-γ, a NO-donor SNAP, an ER stress-inducing reagent thapsigargin, or a DNA-damaging reagent camptothecin for 12 h, morphological changes characteristic of apoptosis were observed (Fig. 1A). 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. We showed previously that apoptosis induced by LPS/IFN-γ treatment is mediated by NO (23). However, the pathway of NO-mediated apoptosis is not fully understood. We asked whether p53 is involved in NO-mediated apoptosis (Fig. 1B). In general, p53 increases in response to DNA damage. In fact, when cells were treated with camptothecin, p53 protein increased. In contrast, treatment with LPS/IFN-γ, SNAP, or thapsigargin did not increase p53.

CHOP Is Induced in NO-mediated Apoptosis in RAW 264.7 Cells—CHOP is a transcription factor that is involved in ER stress-induced apoptosis. We found that CHOP mRNA, which was undetectable in untreated RAW 264.7 cells, was induced when cells were treated with LPS/IFN-γ or SNAP (Fig. 2A). The mRNA was induced by thapsigargin but not by campto-
are shown by means of flow cytometry. The ordinate is set at 100%. Cells were treated with LPS (150 μg/ml) and IFN-γ (100 units/ml), SNAP (1.5 mM), and carboxy-PTIO (2 mM) for 6 h, and total RNAs (2 μg) were subjected to blot analysis for CHOP mRNA (A). RNA blots from two dishes for each condition are shown. The position of 18S rRNA is shown on the right. The lower panel shows ethidium bromide staining of 18S and 28S rRNAs. Cells were treated with a combination of LPS (150 μg/ml) plus IFN-γ (100 units/ml), SNAP (1.5 mM), and carboxy-PTIO (2 mM) for 6 h as indicated on the top, and nuclear extracts (30 μg of protein) were subjected to immunoblot analysis for CHOP protein (B). Cells were treated with LPS (150 μg/ml) plus IFN-γ (100 units/ml) for indicated periods or with TG (2 μM) for 6 h (C). Nuclear extracts (30 μg of protein) were subjected to immunoblot analysis for CHOP protein. The results in panel C and in a parallel experiment were quantified and are shown by means ± ranges (n = 2) (D). The maximal value at 10 h is set at 100%. Cells were treated with LPS (150 μg/ml) plus IFN-γ (100 units/ml), TG (2 μM), or CPT (30 μM) for 10 h, and cell extracts (20 μg of protein) were subjected to immunoblot analysis for Bip (E). Immunoblots from two dishes for each condition are shown. Cells were treated with LPS (150 μg/ml) plus IFN-γ (100 units/ml) for indicated periods and fractionated into the soluble fraction (S) and the particulate fraction (P) as described under “Experimental Procedures” (F). Distribution of total protein in the soluble fraction and the particulate fraction was 65 and 35%, respectively. The fractions (10 μg of protein) were subjected to immunoblot analysis for cytochrome c, hsp60, and GAPDH.

Fig. 2. Effects of various apoptotic stimuli on CHOP induction and cytochrome c release in RAW 264.7 cells. Cells were treated with LPS (150 μg/ml) plus IFN-γ (100 units/ml), SNAP (1.5 mM), camptothecin (CPT, 30 nM), or TG (2 μM) for 6 h, and total RNAs (2 μg) were subjected to blot analysis for CHOP mRNA (A). RNA blots from two dishes for each condition are shown. The position of 18S rRNA is shown on the right. The lower panel shows ethidium bromide staining of 18S and 28S rRNAs. Cells were treated with a combination of LPS (150 μg/ml) plus IFN-γ (100 units/ml), SNAP (1.5 mM), and carboxy-PTIO (2 mM) for 6 h as indicated on the top, and nuclear extracts (30 μg of protein) were subjected to immunoblot analysis for CHOP protein (B). Cells were treated with LPS (150 μg/ml) plus IFN-γ (100 units/ml) for indicated periods or with TG (2 μM) for 6 h (C). Nuclear extracts (30 μg of protein) were subjected to immunoblot analysis for CHOP protein. The results in panel C and in a parallel experiment were quantified and are shown by means ± ranges (n = 2) (D). The maximal value at 10 h is set at 100%. Cells were treated with LPS (150 μg/ml) plus IFN-γ (100 units/ml), TG (2 μM), or CPT (30 μM) for 10 h, and cell extracts (20 μg of protein) were subjected to immunoblot analysis for Bip (E). Immunoblots from two dishes for each condition are shown. Cells were treated with LPS (150 μg/ml) plus IFN-γ (100 units/ml) for indicated periods and fractionated into the soluble fraction (S) and the particulate fraction (P) as described under “Experimental Procedures” (F). Distribution of total protein in the soluble fraction and the particulate fraction was 65 and 35%, respectively. The fractions (10 μg of protein) were subjected to immunoblot analysis for cytochrome c, hsp60, and GAPDH.

thein. Fig. 2B shows immunoblot analysis of nuclear extracts for CHOP. CHOP, which was barely detectable before LPS/IFN-γ treatment, was induced after treatment. The level of LPS/IFN-γ-induced CHOP was close to that obtained with thapsigargin. This induction was prevented by a NO scavenger carboxy-PTIO, indicating that the induction is mediated by NO. CHOP was induced slightly 2 h after LPS/IFN-γ treatment, increased markedly at 4 h, and increased up to 10 h (Fig. 2, C and D). Bip/GRP78, an ER chaperone that is known to be induced by ER stress (7), was induced by LPS/IFN-γ as well as by thapsigargin (Fig. 2E), indicating that LPS/IFN-γ produces ER stress. Camptothecin did not induce Bip.

We have shown previously that cytochrome c release from mitochondria takes place in LPS/IFN-γ-induced apoptosis of RAW 264.7 cells (27). Fig. 2F shows the time course of cytochrome c release in RAW 264.7 cells treated with LPS/IFN-γ. Cells were fractionated with digitonin into a soluble fraction and a particulate fraction that includes mitochondria, and these fractions were subjected to immunoblot analysis for cytochrome c. Cytochrome c release was not seen up to 6 h after LPS/IFN-γ treatment but was observed at 10 h. At this time, about 70% of cytochrome c was recovered in the soluble fraction. Under these conditions, a mitochondrial matrix protein, hsp60, was recovered exclusively in the particulate fraction, whereas a cytosolic protein, glyceraldehyde 3-phosphate dehydrogenase, was recovered almost exclusively in the soluble fraction. Therefore, we conclude that CHOP is induced prior to cytochrome c release in LPS/IFN-γ-induced apoptosis in RAW 264.7 cells.

ATF6 Is Activated in LPS/IFN-γ-treated RAW 264.7 Cells—Yoshida et al. (13) found that p50ATF6 binds to ERSE of the CHOP gene and transactivates the gene. Therefore, we asked whether ATF6 is activated in LPS/IFN-γ-treated RAW 264.7 cells. Fig. 3A shows the time course of ATF6 activation and CHOP induction. In control cells, p90ATF6, which is the ER transmembrane form of ATF6, was detected, but p50ATF6, which is the processed and active form, was not detected. p90ATF6 was decreased with time after LPS/IFN-γ treatment, whereas p50ATF6 appeared in the nuclei 2 h after LPS/IFN-γ treatment, indicating that p90ATF6 was processed to p50ATF6. CHOP was induced concomitantly with the processing of ATF6. Processed p50ATF6 appeared in the nuclei 2 h after LPS/IFN-γ treatment and increased with time up to 10 h (Fig. 3, B and C). The level at 10 h was about half of that produced in response to thapsigargin. Binding activity to ERSE of the CHOP gene was also monitored (Fig. 3D). When cells were treated with thapsigargin for 6 h, two binding complexes (complexes I and II) were detected. Treatment with LPS/IFN-γ also produced two complexes. An antibody specific to ATF6 diminished complex II, whereas control serum caused no change. Therefore, we conclude that p50ATF6 is involved in complex II. This agrees well with the results obtained with purified p50ATF6 and NF-Y, another transcription factor involved in the complex (13). In that report, it was shown that p50ATF6 and NF-Y were involved in complex II and only NF-Y was involved in complex I. Complex II was not detected in control nuclear extract, began to increase 2 h after LPS/IFN-γ treatment, and increased up to 10 h. These results indicate that LPS/IFN-γ treatment processed to activate ATF6 and that the active form of ATF6 (p50ATF6) binds to ERSE of the CHOP gene.

To confirm that LPS/IFN-γ treatment induces CHOP through stimulation of ERSE, we made reporter constructs where ERSE of the CHOP gene or the CHOP binding site of Rous sarcoma virus long terminal repeat was inserted just upstream of the SV40 promoter that drives the luciferase gene. RAW 264.7 cells were transfected with the reporter constructs and treated with LPS/IFN-γ or thapsigargin for 10 h, and then cell extracts were assayed for luciferase activity (Fig. 4). Luciferase activity derived from the SV40 promoter was little af-
ATF6 and CHOP, therefore conclude that activated ATF6 enhances transcription of the CHOP gene through ERSE in vivo in LPS/IFN-γ-treated RAW 264.7 cells.

Overexpression of CHOP, p50ATF6, or p90ATF6 Induces Apoptosis in RAW 264.7 and COS-7 Cells—CHOP was reported to induce apoptosis in M1 myeloblastic leukemia cells (10) and mouse embryonic fibroblasts (6). We asked whether CHOP and ATF6 can induce apoptosis in RAW 264.7 cells. Cells were cotransfected with an EGFP expression plasmid and an expression plasmid for CHOP, p50ATF6, or p90ATF6. When cells were transfected with only the EGFP plasmid, many cells became fluorescent (Fig. 5A). Cotransfection with the CHOP plasmid markedly reduced EGFP-positive cells. When the p50ATF6 plasmid was cotransfected, the number of EGFP-positive cells was even more markedly reduced. In the case of the p90ATF6 plasmid, the number of EGFP-positive cells was moderately reduced. It was shown that overexpression of p90ATF6 leads to partial processing of p90ATF6, resulting in the production of small amounts of p50ATF6 (14) (see also Fig. 6E). These results indicate that ATF6 as well as CHOP induces the death of RAW 264.7 cells.

We then asked whether NO-mediated apoptosis in RAW 264.7 cells can be prevented by expression of the CHOP dominant negative form (Fig. 5C). The number of EGFP-positive cells was decreased markedly by LPS/IFN-γ, and this decrease was effectively prevented by cotransfection with the CHOP dominant negative plasmid. The CHOP dominant negative plasmid alone had little effect on the number of EGFP-positive cells. These results indicate that LPS/IFN-γ-induced apoptosis in RAW 264.7 cells is mediated by CHOP. CHOP-induced apoptosis was prevented by cotransfection with the CHOP dominant negative plasmid.

Overexpression of CHOP, p50ATF6, or p90ATF6 induced apoptosis in RAW 264.7 cells. Treatment with LPS (150 μg/ml) plus IFN-γ (100 units/ml) or thapsigargin (2 μM) was added, and cells were cultured further for 10 h. Luciferase activity was measured, normalized for β-galactosidase activity, and is expressed relative to the activity obtained when cells were cultured in the absence of the reagents. Relative luciferase activities are shown by means + S.E. (n = 4).

Fig. 4. Activation of the CHOP gene through ERSE in vivo in LPS/IFN-γ-treated RAW 264.7 cells. The ERSE site or CHOP-binding consensus sequence (CHOP-BS) was inserted just upstream of the SV40 promoter of the pGL3-promoter luciferase vector. Resulting reporter plasmids (10 μg/10-cm dish) and a control β-galactosidase plasmid pAc-LacZ (5 μg/10-cm dish) were cotransfected into RAW 264.7 cells. After 24 h, LPS (150 μg/ml) plus IFN-γ (100 units/ml) or TG (2 μM) was added, and cells were cultured further for 10 h. Luciferase activity is shown by means + S.E. (n = 4).

Fig. 3. Activation of ATF6 in LPS/IFN-γ-treated RAW 264.7 cells. Cells were treated with LPS (150 μg/ml) plus IFN-γ (100 units/ml) for indicated periods, and whole cell extracts (100 μg of protein for ATF6 and CHOP, 10 μg of protein for GAPDH) were subjected to immunoblot analysis for ATF6, CHOP, and GAPDH (A). Cells were treated with LPS (150 μg/ml) plus IFN-γ (100 units/ml) for indicated periods or TG (2 μM) for 6 h, and nuclear extracts (30 μg of protein) were subjected to immunoblot analysis for the activated form of ATF6, p50ATF6 (B). The results in panel B and in a parallel experiment were quantified and are shown by means ± ranges (n = 2) (C). The relative value at 10 h is set at 100%. Gel shift analysis of a factor(s) binding to ERSE of the CHOP gene is shown (D). Cells were treated with LPS (150 μg/ml) plus IFN-γ (100 units/ml) for indicated periods or TG (2 μM) for 6 h, and nuclear extracts were prepared. A digoxigenin-labeled probe for the ERSE site of the CHOP gene was incubated with nuclear extracts (5 μg of protein), and a gel shift assay was performed as described under “Experimental Procedures.” An antiserum against ATF6 (αATF6, 1 μl) or a nonimmune serum (Control Ab, 1 μl) was added to the binding mixture halfwary through the reaction. As competitors, unlabeled oligonucleotides for the ERSE site (ERSE) or the p53 binding site (Control oligo) were added in the reaction mixture. The positions of complexes I and II are indicated on the right.

fected by treatment with LPS/IFN-γ or thapsigargin. When ERSE of the CHOP gene was inserted, luciferase activity was enhanced 2.2-fold by LPS/IFN-γ treatment and 3.4-fold by thapsigargin treatment. When the CHOP binding site was inserted, luciferase activity was enhanced 3.2-fold by LPS/IFN-γ treatment and 5.9-fold by thapsigargin treatment. We therefore conclude that activated ATF6 enhances transcription
The number of EGFP-positive cells was decreased, and the results were similar to those for RAW 264.7 cells (Fig. 6A). Expression of EGFP was quantified by immunoblot analysis, and the results are shown in Fig. 6B and C. CHOP protein, which was not detectable in nuclear extracts from control cells, was detected in nuclear extracts from cells transfected with the plasmid for p50ATF6 or p90ATF6 as well as for CHOP (Fig. 6D). In cells transfected with the p50ATF6 plasmid, p50ATF6 protein was detected in addition to endogenous p90ATF6 (Fig. 6E). When the p90ATF6 plasmid was transfected, processed p50ATF6 as well as unprocessed p90ATF6 was detected. Therefore, overexpression of p90ATF6 leads to a partial processing to active p50ATF6, which in turn induces CHOP and following apoptosis. Fig. 6F shows the time course of GFP expression cotransfected with the EGFP expression plasmid and an expression plasmid for CHOP or p50ATF6 (F). After incubation for indicated times, cell extracts (20 μg of protein) were subjected to immunoblot analysis for GFP and GAPDH.
cells. The decrease in EGFP-positive cells by p50ATF6 was partially reversed by the dominant negative forms of ATF6 and CHOP, and the decrease by CHOP was almost completely reversed by its dominant negative form. Expression of p50ATF6 and its dominant negative form (Fig. 7D) and that of CHOP and its dominant negative form (Fig. 7E) were confirmed by immunoblot analysis.

We then asked whether expression of CHOP or ATF6 induces apoptosis in COS-7 cells (Fig. 8). When CHOP or p50ATF6 was coexpressed with EGFP, 68 or 79% of EGFP-positive cells showed apoptotic changes, respectively. When p90ATF6 was coexpressed, 48% of EGFP-positive cells were apoptotic, apparently due to partial processing to p50ATF6 (see Fig. 6E).

**Peritoneal Macrophages from CHOP-deficient Mice Are Resistant to NO-mediated Apoptosis**—We finally asked whether CHOP is involved in NO-mediated apoptosis in primary-cultured peritoneal macrophages by using CHOP knockout mice. When peritoneal macrophages from wild-type mice were treated with 1.5 mM SNAP, CHOP mRNA was induced with time (Fig. 9A). When wild-type macrophages were treated with

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**DISCUSSION**

Excess NO production induces apoptosis in various cell types. It is generally believed that NO-induced apoptosis is mediated by the DNA damage pathway involving accumulation of p53 (4). However, in this report we showed that p53 induction is not evident in NO-mediated apoptosis in RAW 264.7 macrophages. It was reported that there is a p53-independent pathway in addition to a p53-dependent one in NO-mediated
apoptosis (28). From the results shown here, we conclude that the p53-independent pathway is the major one in NO-mediated apoptosis in RAW 264.7 cells. Recently, we showed that NO induces apoptosis in p53-deficient microglia (29).

We found that CHOP is induced in NO-mediated apoptosis in RAW 264.7 cells and peritoneal macrophages. NO is known to inhibit many enzymes and ion channels (3). We found that NO depletes ER Ca\(^{2+}\) and causes ER stress in mouse β cell-derived MIN6 cells (22). NO was reported to inhibit Ca\(^{2+}\)-ATPase activity of SERCA2a by tyrosine nitration within the channel-like domain (30). We have therefore proposed that NO induces ER stress by disturbing ER Ca\(^{2+}\) homeostasis in cells (22).

CHOP induces apoptosis in some cell types (6). Embryonic fibroblasts derived from CHOP knockout mice exhibit significantly less apoptosis as compared with wild-type cells when challenged with ER stress-inducing reagents (6). CHOP was found to induce apoptosis in M1 myeloblastic leukemia cells in a p53-independent manner, and Bcl-2 delayed this process (10). In the present work, we showed that CHOP-deficient peritoneal macrophages are more resistant to NO-induced apoptosis than wild-type cells. Because CHOP functions as a transcription factor, there must be a target gene(s) whose transcription is activated by CHOP and whose product(s) works in the apoptosis signal cascade. Wang et al. (11) found candidate target genes of the CHOP protein using representational difference analysis. However, these genes are distinct from known factors involved in the ER stress response and apoptosis. Recently, McCullough et al. (31) reported that CHOP expression results in down-regulation of Bcl-2 expression, depletion of cellular glutathione, and exaggerated production of reactive oxygen species. The precise apoptosis cascade downstream of CHOP remains to be clarified.

ATF6 is a type 2 ER membrane protein with its NH\(_{2}\) terminus in the cytosol (15). When ER stress such as unfolded protein accumulation in ER occurs, ATF6 (p90ATF6) is cleaved to release its cytosolic domain (p50ATF6), which is a transcription factor of the basic leucine zipper family (14). Recently, Ye et al. (16) reported that this proteinolyis is mediated by the Site-1 protease (S1P) and Site-2 protease (S2P). However, it is still unknown how the ER stress signal triggers proteolysis of ATF6. At least four genes, GRP78, GRP94, CHOP, and calreticulin, have been shown to be activated by p50ATF6 (13, 17, 32). The cleavage of the transmembrane protein in response to cell signaling to liberate cytosolic fragments that enter the nucleus to control gene transcription is called regulated intramembrane proteolysis (Rip) (15). In the present work, we found that the Rip type proteolysis of ATF6 takes place in the process of NO-mediated apoptosis in RAW 264.7 cells.

An ERSE was identified in the CHOP gene. The consensus sequence of ERSE is CCAAT-N\(_{9}\)-CCACG, and this cis-acting element is necessary and sufficient for the response to ER stress (32, 33). The sequence CCACG provides specificity for the response to ER stress. The general transcription factor NF-Y binds to CCAAT and p50ATF6 binds to CCACG, thereby accounting for the specificity of the ER stress response (13). p50ATF6 was shown to bind directly to CCACG only when CCAAT exactly 9 bp upstream of CCACG is bound by NF-Y. We suggest that complex II in Fig. 3C consists of ATF6 and NF-Y, whereas complex I consists of NF-Y, as shown previously by Yoshida et al. (13). On the other hand, Fawcett et al. (34) reported that the CHOP gene can be activated by ATF4, another basic leucine zipper-type transcription factor, via its binding to the C/EBP-ATF site present in the CHOP promoter region and distinct from the ERSE. Recently, Harding et al. (35) revealed that translation of ATF4 is selectively increased during ER stress and that this translational induction is mediated by PERK, a type 1 transmembrane protein kinase in the ER, which senses ER stress and transmits signals by phosphorylating the α subunit of eukaryotic initiation factor 2. Therefore, it is very likely that not only ATF6 but also ATF4 is activated in RAW 264.7 cells by LPS/IFN-γ and that both factors are involved in NO-induced apoptosis. Islet cells and macrophages are highly active in protein secretion, and it is tempting to speculate that “secretory” cells active in protein secretion are more sensitive to NO-mediated apoptosis than “non-secretory” cells, which are less active in protein secretion. This remains to be tested.
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