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# Molecular mechanisms of the LPS-induced non-apoptotic ER stress-CHOP pathway

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**The expression of C/EBP homologous protein (CHOP), which is an endoplasmic reticulum (ER) stress-induced transcription factor, induces apoptosis. Our previous study demonstrated that lipopolysaccharide (LPS)-induced CHOP expression does not induce apoptosis, but activates a pro-IL-1β activation process. However, the mechanism by which CHOP activates different pathways, depending on the difference in the inducing stimuli, remains to be clarified. The present study shows that LPS rapidly activates the ER function-protective pathway, but not the PERK pathway in macrophages. PERK plays a major role in CHOP induction, and other ER stress sensors-mediated pathways play minor roles. The induction of CHOP by LPS was delayed and weak, in comparison with CHOP induction by ER stress-inducer thapsigargin. In addition, LPS-pre-treatment or overexpression of ER chaperone, IgH chain binding protein (BiP), prevented ER stress-mediated apoptosis. LPS plus IFN-γ-treated macrophages produce a larger amount of nitric oxide (NO) in comparison with LPS-treated cells. Treatment with the NO donor, SNAP (*S*-nitro-*N*-acetyl-DL-penicillamine), induces CHOP at an earlier period than LPS treatment. The depletion of NO retards CHOP induction and prevents apoptosis in LPS plus IFN-γ-treated cells. We concluded that apoptosis is prevented in LPS-treated macrophages, because the ER function-protective mechanisms are induced before CHOP expression, and induction level of CHOP is low.**

*Keywords*: CHOP/ER stress/PERK/XBP1/LPS.

*Abbreviations*: BiP, IgH chain binding protein; CHOP, C/EBP homologous protein; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERSE, ER stress response element; FCS, fetal calf serum; GADD34, growth arrest and DNA damage-inducible gene 34; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; NO, nitric oxide; PDI, protein-disulfide isomerase; PERK, PKR–like ER kinase; RT–PCR, reverse transcription polymerase chain reaction; TG, thapsigargin; TM, tunicamycin; XBP1, X-box binding protein 1.

Protein modification and folding of newly synthesized secretory and cell membrane proteins in the endoplasmic reticulum (ER) is impaired under various physiological or pathological conditions (*1*, *2*). The perturbation of the ER functions, which is called ER stress, activates the ER stress pathways, including translational attenuation, induction of ER chaperones, such as IgH chain binding protein (BiP), and degradation of unfolded proteins by a system known as (ERAD, ER-associated degradation), to restore ER functions and protect cells (*1*, *2*). However, when the ER functions are severely impaired, apoptosis occurs to remove the damaged cells. This apoptosis is mediated by factors including C/EBP homologous protein (CHOP)/GADD153, ASK1 and caspase-12 (*3*–*7*). CHOP is a transcriptional factor that belongs to the C/EBP family (*4*, *8*, *9*). CHOP heterodimerizes with other C/EBP family members, and CHOP-C/EBP dimer binds to the CHOP site, which is distinct from the C/EBP site, and thus induces apoptosis. However, the apoptosis pathway downstream of CHOP has not yet been fully elucidated. Wang *et al.* (*10*) identified candidate target genes of the CHOP protein by using a representational difference analysis. However, these genes are distinct from the known factors involved in the ER stress response and apoptosis. McCullough *et al.* (*11*) reported that CHOP expression results in a down-regulation of the anti-apoptotic molecule Bcl-2 expression, the depletion of cellular glutathione and an exaggerated production of reactive oxygen species. Marciniak *et al.* (*12*) reported that CHOP inhibits ER stress-induced attenuation of protein synthesis by the dephosphorylation of the α-subunit of translation initiation factor 2 (eIF2α) through the induction of growth arrest and DNA damage-inducible gene 34 (GADD34). They also reported that CHOP induces Ero1α, which activates disulfide bond formation in ER proteins. They thus showed that expression of CHOP leads to the accumulation of high-molecular weight protein complexes in the ER, and it thus impairs the ER function. The apoptosis signal induced by CHOP is transmitted to mitochondria through the translocation of pro-apoptotic molecule Bax from cytosol to mitochondria (*13*). However, the mechanism by which CHOP induces the translocation of Bax is unknown. Recently, Puthalakath *et al.* (*14*) showed that Bim, a pro-apoptotic BH3-only type Bcl-2 family member, is induced by ER stress in a CHOP-dependent

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manner. They also showed that Bim is essential for ER stress-CHOP pathway-dependent apoptosis. This suggests that Bim is induced by ER stress-CHOP pathway and induces activation and translocation of Bax, and then the apoptosis signal is transmitted to mitochondria. CHOP is induced at the transcription level in response to ER stress. The ER stress pathway was first identified as a cellular response pathway, induced by the accumulation of unfolded proteins in the ER to preserve ER functions. The processes of protein maturation in the ER are complex and easily disturbed by various stresses, such as ischaemia, oxidative stress, disturbance of $Ca^{2+}$ homoeostasis and accumulation of abnormal proteins in the ER (*2*). Therefore, the ER stress pathway is also activated by various cellular stresses, but when the stress is severe, the apoptosis pathway, including CHOP, is induced to remove any damaged cells. When a large amount of cells are lost as a result of apoptosis, the functions of tissues or organs are impaired. Therefore, the ER stress-CHOP pathway is involved in the pathogenesis of various diseases (*2*, *6*). The ER stress-CHOP pathway is involved in nitric oxide (NO)-induced apoptosis in pancreatic β-cells (*15*), microglias (*16*) and macrophages (*17*). In addition, the ER stress-CHOP pathway is crucial in hereditary mouse diabetes mellitus (*18*), non-steroidal anti-inflammatory drug-induced apoptosis in gastric mucosal cells (*19*), brain ischaemia–reperfusion injury (*20*), neuronal apoptosis induced by neurotrophic factor deprivation or stimulation with excess amounts of neurotransmitter (*21*, *22*) and experimental mouse pancreatitis (*23*).

The ER stress-CHOP pathway is also involved in the pathogenesis of inflammation (*24*, *25*). When mice are given lipopolysaccharide (LPS) intratracheally, inflammation of the lung is induced (*25*). In this situation, the ER stress pathway, including CHOP, was induced. However, apoptotic cells were barely detected in the lung tissue in this condition. In addition, the LPS-induced lung inflammation, including the IL-1β activity in bronchoalveolar lavage fluid, is attenuated in *Chop*-knockout mice. Caspase-11, which is required for the activation of procaspase-1 and pro-IL-1β (*26*), is induced by LPS treatment in the lung and primary cultured macrophages in a CHOP-dependent manner. IL-1β is secreted from activated macrophages and plays a central role at an early stage of the inflammatory response. IL-1β also activates other inflammatory cells including macrophages themselves. In addition, the induction of caspase-11 has also been reported to be required for the activation of IL-18, which is involved in inflammatory and allergic responses (*27*). Therefore, CHOP plays a crucial role in the pathogenesis of inflammation through the induction of caspase-11.

As mentioned above, the expression of CHOP induces apoptosis when cells are treated with typical ER stress inducers, such as thapsigargin (TG) or tunicamycin (TM). When macrophages are treated with LPS plus IFN-γ, CHOP-mediated apoptosis is also induced in a NO-dependent manner. However, even though CHOP is induced in inflammatory lesions and LPS-treated macrophages, apoptotic cells are rarely observed in those conditions (*25*). The molecular mechanisms by which the induction of the ER stress-CHOP pathway in inflammation does not induce apoptosis remain unknown. Therefore, the difference between the precise induction manner of the ER stress-CHOP pathway by inflammatory stimuli and the typical ER stress-induction stimulus was investigated using macrophage-derived RAW 264.7 cell lines.

This study demonstrated that the induction of CHOP by LPS is delayed in comparison with that induced by the ER stress inducer, TG, even though ER function-protective molecules, such as BiP, are induced at an early stage. In fact, pretreatment with LPS prevented TG-induced apoptosis, and overexpression of BiP prevents CHOP-induced apoptosis. TG-induced apoptosis is CHOP dependent. In addition, activation of PERK, which is one of the ER stress sensors and crucial for CHOP induction, is suppressed in the LPS-induced ER stress pathway. Furthermore, treatment with a NO donor induces CHOP at an earlier period than LPS treatment, and the depletion of NO retards CHOP induction and prevents apoptosis in LPS plus IFN-γ-treated cells. These results show that treatment with LPS specifically activates part of the ER stress response pathway; therefore, ER function-protective molecules are induced before CHOP expression and induction level of CHOP is low. As a result, LPS-induced CHOP expression does not induce apoptosis, but induces IL-1β activation pathway.

## Experimental procedures

### *Antibodies*

Polyclonal antibodies against CHOP and BiP were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and polyclonal antibody against PERK was obtained from ABGENT (San Diego, CA, USA). Monoclonal antibodies against phospho-PERK (activated form), Hsc70 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and CHEMICON International, Inc. (Temecula, CA, USA), respectively.

### *Plasmids*

pEGFP-C1, a mammalian expression plasmid for enhanced GFP, was obtained from Clontech Laboratories, Inc. (Palo Alto, CA, USA). A mammalian CHOP expression plasmid, pOPRSVI-CHOP, was described (*17*). pcDNA3.1-BiP, a mammalian expression plasmid for mouse BiP, was constructed by inserting the full-length mouse BiP cDNA into just downstream of the CMV promoter of a mammalian expression plasmid pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA) after linker attachment.

### *Cell culture and treatment of cells*

Mouse macrophage-like RAW 264.7 cells were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum. Cells were treated with various combinations of *Escherichia coli* LPS (serotype 0127: B8, Sigma, St. Louis, MO, USA), mouse IFN-γ, TG, a sarcoplasmic/ER $Ca^{2+}$ ATPase (SERCA) inhibitor (TG), TM, an N-linked glycosylation inhibitor, or an NO donor *S*-nitro-*N*-acetyl-DL-penicillamine (SNAP) for the indicated periods. An NO scavenger, carboxy-PTIO, (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide), was added to the medium concomitantly with the addition of LPS or LPS plus IFN-γ. COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfection of COS-7 cells with plasmids



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was carried out using Lipofectamine 2000 (Invitrogen), according to the protocol provided by the manufacturer. In each experiment, the same total amount of plasmids were transfected by adding insert-less expression plasmids.

#### Isolation and treatment of peritoneal macrophages

All procedures were approved by the Animal Care and Use Committee of Kumamoto University. Mice lacking the *Chop* gene (C57BL/6 background) were generated as previously described (*15*). Mice peritoneal macrophages, derived from wild-type and *Chop*-knockout mice, were prepared and grown in RPMI 1640 medium supplemented with 10% FCS, as described earlier (*17*). After being cultured for 3 days, non-adherent cells were removed, and the adherent cells were treated with TG or an NO donor SNAP for the indicated periods.

#### Detection of apoptosis

The disruption of mitochondrial membrane potential is a characteristic of apoptotic cells. To analyse mitochondrial membrane depolarization, cells were stained with a mitochondrial membrane potential-indicating dye DePsipher (Trevigen, Inc., Gaitherburg, MD, USA) and observed by fluorescence microscopy, as previously described (*17*). The red-orange fluorescence indicates that the dye was imported to the mitochondria, depending on the intact mitochondrial membrane potential. If apoptosis is induced, then the potential is disrupted, and the dye remains in its green fluorescent form in the cytosol. To analyse morphological changes of nuclei, culture medium was removed, and the cells were stained with phosphate-buffered saline (PBS) containing 8 μg/ml of the Hoechst dye 33258 for 5 min and washed with PBS. The stained cells were observed under a fluorescence microscope. Release of lactate dehydrogenase (LDH) from damaged cells was measured as an index of cell destruction, using a LDH cell damage assay kit (Wako Pure Chemical Industries, Osaka, Japan).

#### RNA blot analysis

Total RNA was prepared from cells using a guanidium thiocyanate–phenol–chloroform extraction procedure (*28*). After electrophoresis in formaldehyde-containing agarose gels, the RNA was transferred to nylon membranes. The membranes were then hybridized with digoxigenin-labelled anti-sense RNA probes for the following transcripts: mouse BiP/GRP78 (nt 276–982: GenBank accession number AJ002387), mouse CHOP (nt 68–585: GenBank accession number X67083), mouse ATF4 (nt 221–743: GenBank accession number M94087), mouse $p58^{IPK}$ (nt 268–818: GenBank accession number BC013766), mouse EDEM (nt 497–1893: GenBank accession number BC023237), mouse Derlin-1 (nt 93–675: GenBank accession number NM_024207), mouse Derlin-2 (nt 672–2999: GenBank accession number NM_033562) and rat GAPDH (nt 238–1042: GenBank accession number M17701). Signals derived from the hybridized probes were detected using a DIG luminescence detection kit (Roche Molecular Biochemicals, Indianapolis, IN, USA), and the intensity of chemiluminescence was quantified using a LAS1000plus chemiluminescence imager (Fuji Photo Film Co Ltd, Tokyo, Japan).

#### RT-PCR analysis

Total RNA was prepared from the cells as described above (*28*). cDNA was synthesized using the Superscript One-Step RT-PCR System (Invitrogen, Life Technologies, Carlsbad, CA, USA). The primers used for PCR were: sense primer, 5′-GAAAGCGCTGCG GAGGAAAC-3′ and antisense primer, 5′-GAGGGGATCTCTAA AACTAGAGGC-3′ for mouse X-box binding protein 1 (XBP1) (GenBank accession number NM_013842); sense primer, 5′-TGGC ACAGTCAAGGCTGAGA-3′ and antisense primer, 5′-CTTCTGA GTGGCAGTGATGG-3′ for mouse GAPDH (GenBank accession number BC083149); sense primer, 5′-GAAAGGATGGTTAATGA TGCTGAG-3′ and antisense primer, 5′-GTCTTCAATGTCCGCA TCCTG-3′ for BiP (GenBank, accession number AJ002387); sense primer, 5′-CATACACCACCACACCTGAAAG-3′ and antisense primer, 5′-CCGTTTCCTAGTTCTTCCTTGC-3′ for CHOP (GenBank, accession number X67083). PCR was performed with an initial denaturation cycle at 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 1 min. An additional 72°C for 5 min completed the amplification process. The amplified PCR products were separated by electrophoresis on 1% agarose gels and then visualized with ethidium bromide staining, and the intensity of bands was quantified using ImageGauge System (Fuji Photo Film Co Ltd). When ER stress-inducing stimuli are given to cells and ER stress-sensor IRE1 is activated, XBP1 mRNA is spliced and activated by IRE1 active form, then XBP1 active form protein is produced (*29–31*). The primer sets for XBP1 were expected to give PCR products with a size of 629 bp for the unspliced (inactive) form and 603 bp for the spliced (active) form, respectively. To clearly distinguish the active form from the inactive form of XBP1 mRNA, the RT-PCR products were digested with *Pst*I. The RT-PCR product from the XBP1 inactive form contains one *Pst*I site. Since the *Pst*I site of XBP1 unspliced form mRNA straddles the boarder between the spliced out region and the active form of XBP1 mRNA, the RT-PCR product from the XBP1 active form is not digested with *Pst*I. Therefore, subsequent electrophoresis revealed the inactive form as two cleaved fragments (316 and 313 bp) and the active form as a non-cleaved fragment (603 bp).

#### Immunoblot analysis

RAW 264.7 or COS-7 cells were homogenized in lysis buffer [50 mM Tris–HCl, 300 mM NaCl, 1% Triton X-100 (pH 7.2)]. After centrifugation, the supernatants were used for an immunoblot analysis. Immunodetection was performed as previously described (*32*).

#### Measurement of NO production

The concentration of $NO_2^-$ plus $NO_3^-$ in culture supernatants was measured using the Griess reagent with the $NO_2/NO_3$ assay kit-C (Dojindo, Kumamoto, Japan) after a reduction of $NO_2^-$ to $NO_3^-$, according to the protocol provided by the manufacturer.

## Results

### Induction of CHOP, but not apoptosis, by treatment with LPS in RAW 264.7 cells

LPS treatment induces inflammation and the ER stress-CHOP pathway in the mouse lung. CHOP is thought to be an apoptosis-inducing molecule (*4*). However, a very few apoptotic cells are observed in the LPS-treated mouse lung tissue, when mice were given LPS intratracheally (*25*). Therefore, the effect of LPS treatment on apoptosis was investigated using mouse macrophage-like RAW 264.7 cells (Fig. 1A–C). When cells were treated with the typical ER stress-inducing reagent, TG (2 μM), almost all cells showed apoptotic changes, such as loss of mitochondrial membrane potential (Fig. 1A), chromatin condensation and nucleus fragmentation (Fig. 1B), detachment from the culture dish bottom (Fig. 1A and B) and LDH release (Fig. 1C). These apoptotic changes were diminished when treated with lower concentrations of TG (Fig. 1A–C). Figure 1D shows that CHOP is involved in TG-induced apoptosis in macrophages. Apoptosis induced by TG (2 μM) was suppressed in primary cultured macrophages from *Chop*-knockout mice. In contrast to TG treatment, when RAW 264.7 cells were treated with high concentration of LPS (150 μg/ml), apoptotic cells were rarely observed (Fig. 1A–C), even though inflammatory genes including IL-1β and TNF-α are rapidly induced in this condition (data not shown). These results were consistent with those of LPS-treated mouse lung (*25*). Next, cells were treated with LPS or TG, and mRNAs for ER stress-related genes were analysed by the northern blot analysis (Fig. 2A and B). The induction of BiP, a major ER chaperone, mRNA, was detected at 3 h, maximally induced ~6 h and gradually decreased thereafter, with both LPS and TG. CHOP is involved

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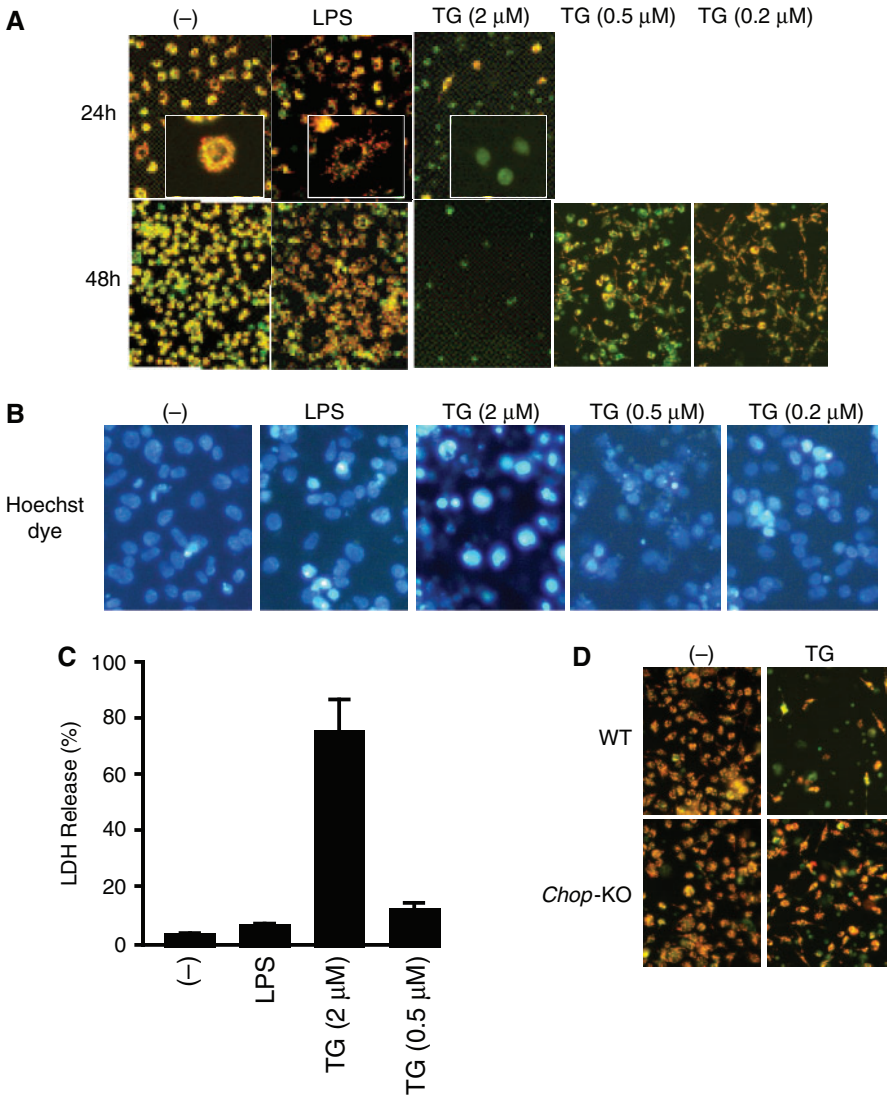


**Fig. 1 An analysis of apoptosis induction in RAW 264.7 cells.** (A) RAW 264.7 cells were treated with LPS (150 μg/ml) or indicated concentrations of TG, for the indicated periods, and cells were stained with a mitochondrial membrane potential-indicating dye, DePsipher, as described in the Experimental Procedures section. Original magnifications: ×400. Magnified images of the cells treated for 24 h are also shown. (B) RAW 264.7 cells were treated with LPS (150 μg/ml) or indicated concentrations of TG, for 24 h, and cells were stained with a DNA-specific fluorochrome Hoechst dye 33258. Original magnifications: ×400. (C) RAW 264.7 cells were treated with LPS (150 μg/ml) or indicated concentrations of TG, for 24 h. The LDH activity of the culture medium was measured, and the results are shown as mean ± SD ($n = 4$). The LDH activity of the cell extract before treatment is set at 100%. (D) Peritoneal macrophages from wild-type and *Chop*-knockout mice were treated with TG (2 μM) for 24 h, and cells were stained with a mitochondrial membrane potential-indicating dye, DePsipher, as described in the Experimental Procedures section. Original magnifications: ×400.

in the ER stress-mediated apoptosis, and also in the activation of IL-1β (*25*). ATF4 is one of the transcriptional activator of the *Chop* gene (*4*). In contrast to BiP mRNA, mRNAs for CHOP and ATF4 were detected at low level before treatment, down-regulated at early times and then induced after 12 h with LPS treatment. When cells were treated with TG, mRNAs for CHOP and ATF4 were almost maximally induced at 3 h and were maintained at high level thereafter. The maximal induction level of CHOP by LPS was lower than that by TG. $p58^{IPK}$ is an ER-resident Hsp40 family molecule and is an inhibitor of PERK (*33*). EDEM, Derlin-1 and -2 are involved in ERAD, through which unfolded proteins in ER are retro-transported to cytosol and degraded in ubiquitin-proteasome system in cytosol (*1*, *2*, *34*). Therefore, BiP, $p58^{IPK}$, EDEM, Derlin-1 and -2 function as guardians of ER functions under ER stress. mRNAs for $p58^{IPK}$, EDEM, Derlin-1 and -2 were detected at low levels before treatment. mRNAs for $p58^{IPK}$ and Derlin-2 mRNAs were induced at 3 h and gradually increased thereafter, following both LPS and TG. The maximum induction levels of both $p58^{IPK}$ and Derlin-2 mRNAs were higher with TG treatment in comparison with those induced by LPS treatment. In case of EDEM and Derlin-1 mRNAs, induction by LPS treatment was detected a little later in comparison with that by the treatment with TG. However, induction of those mRNAs by LPS treatment was obviously earlier than the induction of CHOP mRNA by LPS. CHOP enhances the accumulation of unfolded proteins in ER, through activation of GADD34 and Ero1α,



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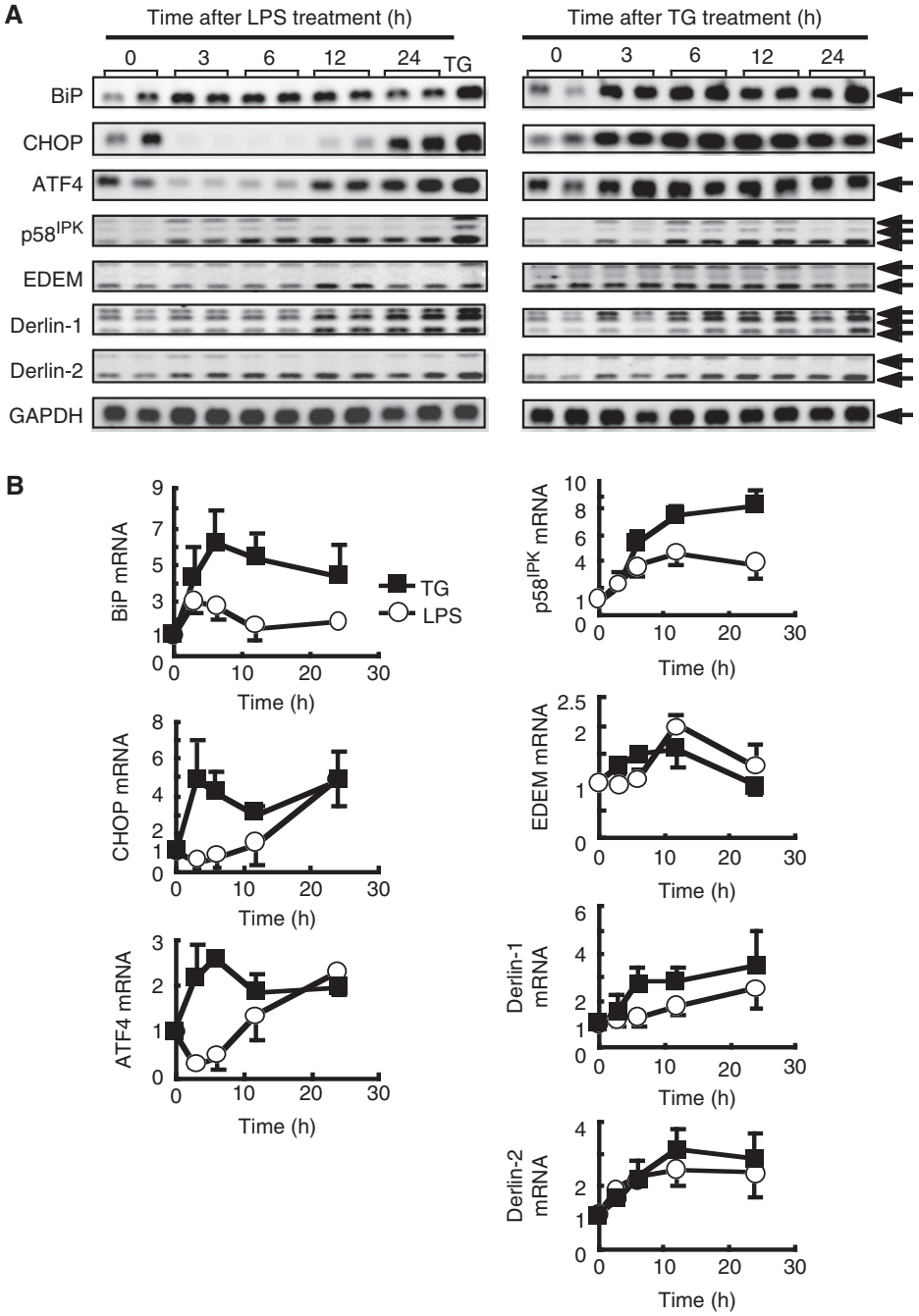


**Fig. 2 A northern blot analysis of mRNAs for ER stress-related genes in RAW 264.7 cells, treated with LPS or TG, and an analysis of the anti-apoptotic effect of LPS pretreatment and BiP.** (A) RAW 264.7 cells were treated with LPS (150 μg/ml) or TG (2 μM) for the indicated hours, and total RNA (2.5 μg) was subjected to a northern blot analysis for BiP, CHOP, ATF4, p58$^{IPK}$, EDEM, Derlin-1, -2 and GAPDH. The total RNA from RAW 264.7 cells, treated with TG (2 μM) for 12 h, was used as a control in the left panels (TG). Northern blots from two dishes for each condition are shown. The arrows indicate the positions of each detected mRNA band. (B) The results of (A) and two other independent experiments were calculated. The results at 0 h are set as 1.0, and are shown as mean ± SD ($n = 4$). Closed boxes show results from TG-treated cells, and open circles show results from LPS-treated cells. (C) RAW 264.7 cells were treated with LPS (150 μg/ml) or TG (2 μM) for 24 h, and cells were stained with a mitochondrial membrane potential-indicating dye, DePsipher, as described in the Experimental Procedures section. In case of TG treatment, the cells were pretreated with LPS (150 μg/ml) for 5 h or 24 h, or not pretreated, then medium was changed and TG was added to the medium. Original magnification: ×400. (D) RAW 264.7 cells were treated with the indicated concentrations of TG for the indicated hours, and total RNA (2.5 μg) was subjected to a northern blot analysis for BiP, CHOP and GAPDH. Northern blots from two dishes for each condition are shown. (E) COS-7 cells were transfected with expression plasmids for the various combinations of EGFP, BiP and CHOP, as indicated on the top. Twenty-four hours after transfection, the cells were stained with a DNA-specific fluorochrome Hoechst dye 33258 and were observed by fluorescence microscopy. EGFP images (upper panels) and Hoechst dye images (lower panels) of the same fields are shown. Original magnification: ×400. (F) COS-7 cells were treated as in (E), and cell extracts (20 μg of protein) were subjected to immunoblot analysis for GFP and GAPDH. (G) COS-7 cells were transfected with expression plasmids for BiP or CHOP or BiP plus CHOP, as indicated on the top. Twenty-four hours after transfection, cell extracts (20 μg of protein) were subjected to immunoblot analysis for BiP and CHOP.

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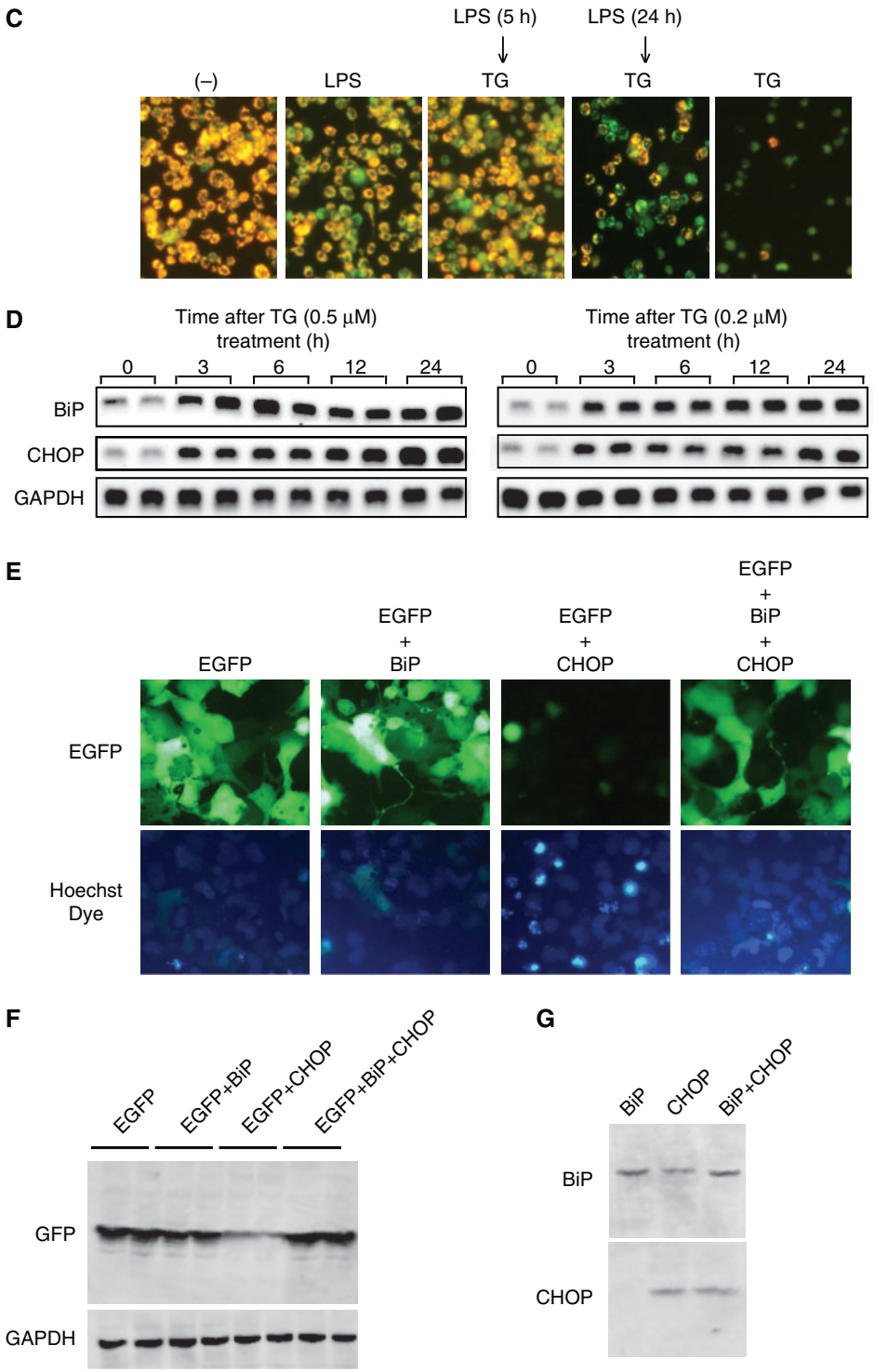


**Fig. 2** Continued.

decreasing ER function (*12*). The results in Fig. 2A and B show that ER stress-mediated cellular protective pathways were induced before the induction of CHOP, following LPS treatment. Therefore, it can be speculated that CHOP expression does not induce apoptosis in the LPS-induced ER stress response, because defensive mechanisms were induced before CHOP expression. In contrast, defensive mechanisms were not sufficiently induced before CHOP expression following TG treatment. Therefore, treatment with TG induces CHOP-mediated apoptosis. To confirm this conclusion, we studied whether pretreatment with LPS prevents TG -induced apoptosis in RAW 264.7 cells (Fig. 2C). As expected, when cells were treated with LPS for 5 h before TG treatment, apoptosis was prevented. When cells were treated with LPS for 24 h before TG treatment, apoptosis was partially prevented, probably because induction of BiP and



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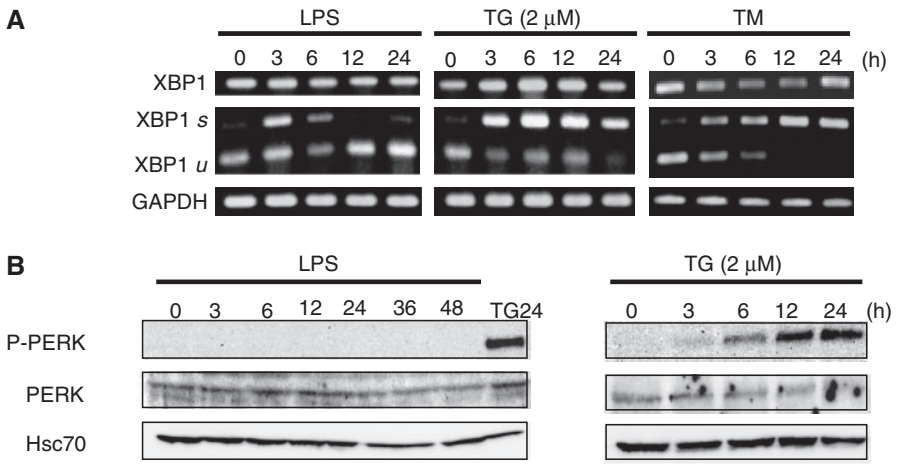


**Fig. 3 An analysis of the activation of XBP1 and PERK by the treatment with LPS or TG in RAW 264.7 cells.** (A) RAW 264.7 cells were treated with LPS (150 μg/ml) or TG (2 μM) or TM (1 μg/ml) for the indicated periods, then total RNA was prepared. RT-PCR analysis was performed using total RNA to detect XBP1 and GAPDH mRNAs. In case of XBP1, a RT-PCR analysis was performed using a primer set, including the spliced-out region in XBP1 mRNA. The PCR products were directly subjected to agarose gel electrophoresis (upper panels) or were subjected to agarose gel electrophoresis after digestion with *Pst*I to distinguish between the spliced form (active form, XBP1s) and unspliced form (inactive form, XBP1u; middle panels), as described in the Experimental Procedures section. Agarose gel electrophoresis of XBP1 and XBP1u shows two bands, because their sizes were almost same and were not separated. In case of GAPDH, the PCR products were directly subjected to agarose gel electrophoresis (lower panels). (B) RAW 264.7 cells were treated with LPS (150 μg/ml) or TG (2 μM) for the indicated periods, then whole cell extracts were subjected to immunoblot analysis using antibodies against phosphorylated (activated) PERK (P-PERK), PERK and Hsc70. Whole cell extracts of RAW 264.7 cells, treated with TG (2 μM) for 24 h, were used as a positive control in the left panels (TG24).

other ER function-protective molecules by LPS had already decreased at 24 h. In Fig. 1, apoptosis, induced by the treatment with lower concentrations of TG (0.5 or 0.2 μM), was obviously reduced in comparison with those with high concentration of TG (2 μM). Difference in the time course of CHOP mRNA induction between LPS and TG treatment can be due to high concentration of TG. Therefore, we investigated whether the induction of CHOP mRNA is delayed, in the case of treatment with lower concentration of TG. Figure 2D showed CHOP and BiP mRNAs to be rapidly induced, even when cells were treated with lower concentration of TG. Therefore, we can consider that apoptotic cells were reduced in the case of the treatment with lower concentration of TG, because the activation level of the death pathway, such as CHOP induction, is low (data not shown). These results showed that ER stress pathways, induced by LPS treatment, are fundamentally different from those by the TG treatment. To examine whether overexpression of BiP prevents CHOP-induced apoptosis, COS-7 cells were co-transfected with expression plasmids for EGFP, BiP and CHOP, and apoptosis was detected with Hoechst dye nuclear staining and EGFP expression (Fig. 2E–G). Expression of BiP alone gave no obvious effects on the expression of EGFP in COS-7 cells. GFP-positive transfected cells were decreased when CHOP was co-expressed, and many nuclear shrunk and chromatin condensed apoptotic cells were detected in this condition (Fig. 2E and F). In contrast, when both BiP and CHOP are co-expressed, GFP-positive cells were increased and chromatin-condensed apoptotic cells were decreased, compared with the condition without BiP. In our previous report (*15*), we also showed that overexpression of calretuculin, one of the major ER chaperones, prevents ER stress-CHOP-mediated apoptosis. These results directly show that induction of ER function-preventive molecules prevent ER stress-CHOP-mediated apoptosis.

### *Treatment with LPS does not induce PERK activation in RAW 264.7 cells*

Next, the molecular mechanisms associated with the delayed induction of CHOP mRNA by LPS in comparison with the ER function-protective molecule genes were investigated (Fig. 3). There are three major ER stress sensors (PERK, IRE1 and ATF6) on the ER membrane. The transcription of the *Chop* gene is regulated through all three ER stress sensor-signalling pathways (*4*). However, it is thought that the PERK-ATF4-signalling pathway plays a dominant role in the induction of CHOP over that of the ATF6- and IRE1-signalling pathways (*35*). When ER stress-inducing stresses were given to cells, ER stress sensors were activated (*1*, *2*). Among them, ATF6 is transported to the Golgi complex, and is activated by sequential cleavage by Site-1 and -2 proteases (*36*); then, the ATF6 active form is transported to the nucleus and it functions as transcriptional activator for ER stress-related genes, including *Chop*. The transcription of transcription factor XBP1 is activated by the ATF6 active form. Next, XBP1 mRNA is transported to cytosol. A small fragment of XBP1 mRNA is spliced out by activated IRE1 followed by translation of XBP1 active form from this XBP1 mRNA spliced form. Therefore, induction of the XBP1 active form by ER stress depends both on ATF6 and IRE1. XBP1 plays a crucial role in the induction of the protective factors for ER functions, including BiP, EDEM and Derlin (*34*, *37*, *38*). Figure 2 shows that induction of CHOP by LPS treatment was delayed in comparison

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with the ER function-protective factors. Therefore, we examined whether the delay of CHOP induction in LPS treatment depends on the difference in the time course of activation among three ER stress sensors (Fig. 3). The inactive and active forms of XBP1 mRNA can be distinguished by means of *Pst*I digestion. Figure 3A shows that XBP1 mRNA active form (XBP1*s*) was detected at 3 h when cells were treated with LPS, TG or TM. With LPS treatment, activation of XBP1 mRNA was suppressed thereafter. In contrast, the activation of XBP1 mRNA was detected until 24 h, when RAW 264.7 cells were treated with TG or TM. The ER stress sensor PERK is activated by auto-phosphorylation. Figure 3B shows that PERK is barely activated by the treatment with LPS. On the other hand, active form of PERK was slightly detected at 3 h and increased thereafter when RAW 264.7 cells were treated with TG. These results show that LPS treatment rapidly activates the IRE1-XBP1 pathway but does not activate PERK pathway. This suggests that the delay of CHOP induction in RAW 264.7 cells treated with LPS is due to the lack of PERK activation, because the PERK pathway contributes most to the induction of CHOP. Activation by splicing of XBP1 mRNA is completely dependent on ER stress sensor IRE1 active form and is a specific hallmark of ER stress. Therefore, even though PERK is not activated by LPS treatment, LPS-induced cellular response process can be regarded as a kind of ER stress response. We previously reported that ER stress response elements (ERSEs) of *Chop* gene promoter region are involved in the ER stress-mediated CHOP induction (*17*). ERSEs are the binding sites of ATF6 and XBP-1 active forms. ERSE is distinct from ATF4-responsive elements (amino acid-regulatory element; AARE), which are activated through PERK-oriented signal pathway. Therefore, we assume that CHOP is induced by LPS treatment through ERSE. In contrast, treatment with the typical ER stress inducer, TG, rapidly activates both the IRE1-XBP and PERK pathways. In this condition, transcription of *Chop* gene is activated through both ERSE and AARE. Therefore, both ER function-protective factors and CHOP are rapidly induced in TG-treated cells.

### *Production of an excessive amount of NO strongly contributes to the rapid induction of CHOP in LPS plus IFN-γ-treated RAW 264.7 cells*

In the previous report, we showed that excess of both exogenous and endogenous NO induces ER stress- and CHOP-mediated apoptosis in RAW 264.7 cells (*17*, *39*). Therefore, we adopted endogenously produced NO-induced apoptosis as a physiological model of ER stress-mediated apoptosis in the following experiments, to compare with LPS-induced ER stress response (Fig. 4). Figure 4A shows that apoptosis was induced in RAW 264.7 cells by the treatment with LPS plus IFN-γ, but not by the treatment with LPS or IFN-γ alone. Therefore, the effect of IFN-γ treatment itself is not the direct cause of apoptosis induction in the case of LPS plus IFN-γ treatment. Treatment with NO scavenger PTIO suppressed apoptosis induced by LPS plus IFN-γ treatment. This result shows that apoptosis induced by LPS plus IFN-γ treatment is dependent on NO production, as we already reported (*39*). To support this conclusion, the amount of NO production in RAW 264.7 cells, stimulated by various ER stress-inducing reagents, was measured (Fig. 4B). NO production, measured by $NO_2$ plus $NO_3$ in the culture medium, was barely in either untreated or TG-treated cells. When cells were treated with LPS or IFN-γ alone, production of NO (∼40 μM in both conditions) was detected, and when cells were treated with LPS plus IFN-γ, NO production was markedly increased (∼110 μM). A large amount of NO induces apoptosis, but this apoptosis is suppressed with the pretreatment with small amount of NO (*6*, *32*). These results clearly show that apoptosis is induced in LPS plus IFN-γ-treated RAW 264.7 cells, because of the higher level of NO production in comparison with cells treated with LPS or IFN-γ alone. In addition, treatment with IFN-γ alone induced BiP mRNA and XBP1 mRNA active form (XBP1*s*) at an early stage, as in the treatment with LPS alone or LPS plus IFN-γ (Fig. 4C and D). In contrast to the treatment with LPS plus IFN-γ, induction of CHOP was barely detected at an early stage in the case of IFN-γ treatment, as that of LPS treatment. Therefore, the effect of IFN-γ treatment itself is not the direct cause of CHOP induction at an early stage in the case of LPS plus IFN-γ treatment. In addition, treatment with IFN-γ alone induced BiP mRNA and XBP1 mRNA active form (XBP1s) at an early stage, as the treatment with LPS alone or LPS plus IFN-γ (Fig. 4C and D). These results show that apoptosis is induced in LPS plus IFN-γ-treated RAW 264.7 cells, because of the rapid induction of CHOP in comparison with cells treated with LPS or IFN-γ alone. Next, the differences in the time course of BiP and CHOP mRNAs induction between cells treated with LPS and cells treated with NO donor SNAP were investigated to show directly the effects of large amount of NO (Fig. 5A and B). BiP mRNA was similarly induced by LPS or SNAP. In contrast, CHOP was induced in SNAP-treated cells already at 3 h, even though CHOP was not induced in LPS-treated cells at the same time. Next, the contribution of NO in ER stress activation was examined from the opposite direction (Fig. 5C–F). Induction level of CHOP by LPS plus IFN-γ treatment was obviously higher than that by LPS treatment. As shown in Fig. 4A, carboxy-PTIO depletes NO, and addition of PTIO prevents apoptosis of the RAW 264.7 cells treated with LPS plus IFN-γ. In order to investigate the effects of endogenously produced NO to ER stress response pathway, we examined whether depletion of NO by the PTIO changes the pattern of the activation of ER stress pathway in RAW 264.7 cells treated with LPS plus IFN-γ (Fig. 5C–F). Addition of PTIO had almost no influence on the expression of BiP, CHOP and XBP1, induced by LPS treatment. In contrast, the expression induced by LPS plus IFN-γ treatment was somewhat suppressed by the addition of PTIO. The induction of CHOP at 6 h was markedly suppressed, even though suppression at 12 h was mild. Induction of CHOP by LPS plus IFN-γ treatment at 12 h was partially suppressed by the treatment with PKR inhibitor,



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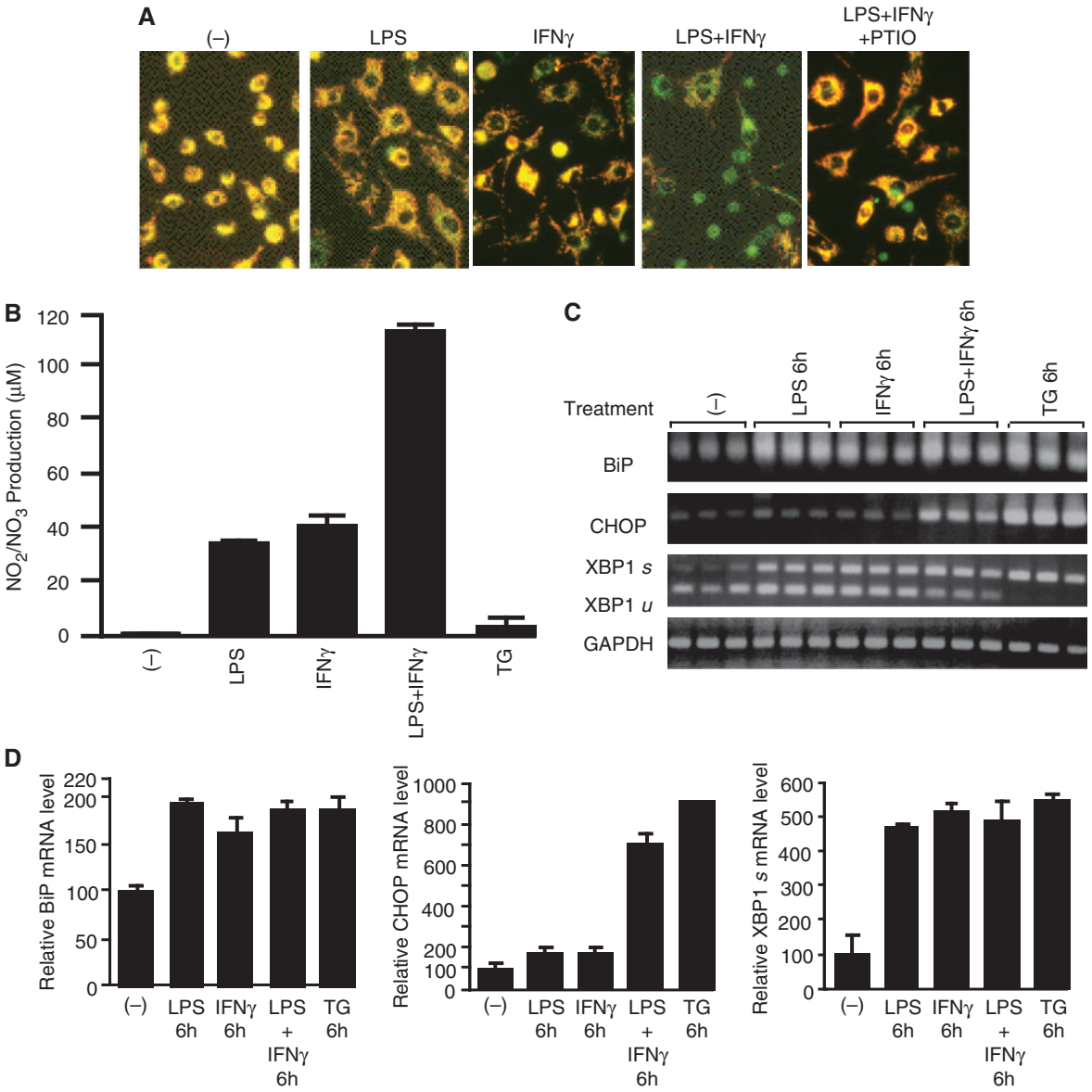


**Fig. 4 An analysis of apoptosis induction, NO production and ER stress activation in RAW 264.7 cells, treated with LPS or IFN-γ or LPS plus IFN-γ.** (A) RAW 264.7 cells were treated with the various combinations of LPS (150 μg/ml), IFN-γ (100 U/ml) and NO scavenger carboxy-PTIO (PTIO, 300 μM) for 24 h, as indicated on the top, then cells were stained with a mitochondrial membrane potential-indicating dye, DePsipher, as described in the Experimental Procedures section. Original magnification: ×400. (B) RAW 264.7 cells were treated with LPS (150 μg/ml) or IFN-γ (100 U/ml) or LPS (150 μg/ml) plus IFN-γ (100 U/ml) or TG (2 μM), for 24 h or untreated. $NO_2^-$ plus $NO_3^-$ in the medium was measured as described in the Experimental Procedures section and the results are shown as mean + SD ($n = 3$). (C) RAW 264.7 cells were treated with LPS (150 μg/ml) or IFN-γ (100 U/ml) or LPS (150 μg/ml) plus IFN-γ (100 U/ml) or TG (2 μM) for 6 h, as indicated on the top; then total RNA was subjected to RT-PCR analysis for BiP, CHOP, XBP1 and GAPDH mRNAs, as described in the Experimental Procedures section. In case of XBP1, the PCR products were subjected to agarose gel electrophoresis after digestion with *Pst*I to distinguish between the spliced form (active form, XBP1s) and the unspliced form (inactive form, XBP1u), as mentioned in Experimental Procedures section. Agarose gel electrophoresis of XBP1u consists of two bands, because their sizes were almost the same and were not separated. RT-PCR analyses from three dishes for each condition are shown. (D) The results for BiP, CHOP and XBP1s in (C) were calculated and are indicated as mean + SD ($n = 3$). The value of the each untreated sample is set at 100.

2-aminopurine (data not shown). PKR is one of the eIF2α kinases as PERK, and induces CHOP (*40*). Therefore, we can speculate that induction of CHOP at 12 h by the treatment with LPS plus IFN-γ is partly dependent on NO-ER stress pathway and partly dependent on PKR. Induction of BiP by the treatment with LPS plus IFN-γ was slightly suppressed both at 6 h and 12 h, probably because both LPS and IFN-γ can activate ER function-protective pathway, including BiP through NO-independent mechanisms.

In Fig. 6, we directly examined whether CHOP is crucial in NO-induced macrophage apoptosis, using *Chop*-knockout primary cultured peritoneal macrophages. When wild-type macrophages were treated with 1.5 mM SNAP for 12 h, most cells lost mitochondrial membrane potential. In contrast, more than half of macrophages from *Chop*-knockout mice retained membrane potential after SNAP treatment. These results indicate that CHOP is crucial in NO-induced macrophage apoptosis, as we previously reported (*17*).

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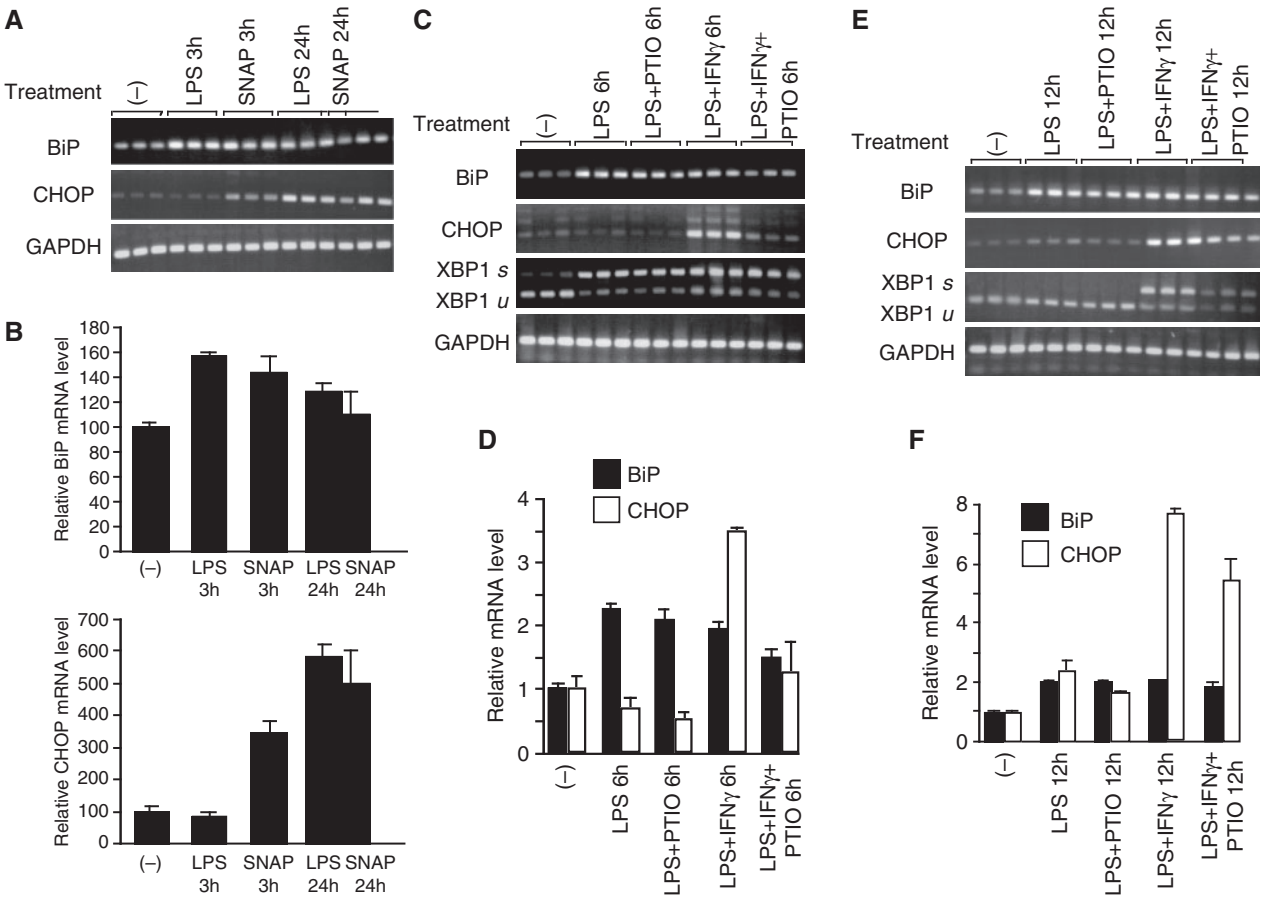


**Fig. 5 An analysis of the involvement of NO in the activation of ER stress pathway in RAW 264.7 cells.** (A) RAW 264.7 cells were treated with LPS (150 μg/ml) or NO donor SNAP (1.5 mM) for the indicated periods or untreated, then total RNA was subjected to RT-PCR analysis for BiP, CHOP and GAPDH mRNAs, as described in the Experimental Procedures section. RT-PCR analyses from three dishes for each condition are shown. (B) The results for BiP and CHOP in (A) were calculated and are indicated as mean + SD ($n = 3$). The value of the each untreated sample is set at 100. (C) RAW 264.7 cells were treated with the various combinations of LPS (150 μg/ml), IFN-γ (100 U/ml) and NO scavenger carboxy-PTIO (PTIO, 300 μM) for 6 h, as indicated on the top, then total RNA was subjected to RT-PCR analysis for BiP, CHOP, XBP1 and GAPDH mRNAs, as described in the Experimental Procedures section. In case of XBP1, the PCR products were subjected to agarose gel electrophoresis after digestion with *Pst*I to distinguish between the spliced form (active form, XBP1s) and the unspliced form (inactive form, XBP1u), as described in the Experimental Procedures section. Agarose gel electrophoresis of XBP1u consists of two bands, because their sizes were almost the same and were not separated. RT-PCR analyses from three dishes for each condition are shown. (D) The results for BiP (black bars) and CHOP (white bars) in (C) were calculated and are indicated as mean + SD ($n = 3$). The value of the each untreated sample is set at 1. (E) RAW 264.7 cells were treated with the various combinations of LPS (150 μg/ml), IFN-γ (100 U/ml) and NO scavenger carboxy-PTIO (PTIO, 300 μM) for 12 h, as indicated on the top, then the total RNA was subjected to RT-PCR analysis for BiP, CHOP, XBP1 and GAPDH mRNAs, as described in Experimental Procedures section. In case of XBP1, the PCR products were subjected to agarose gel electrophoresis after digestion with *Pst*I to distinguish between the spliced (active form, XBP1s) and unspliced form (inactive form, XBP1u). Agarose gel electrophoresis of XBP1u consists of two bands, because their sizes were almost same and were not separated. RT-PCR analyses from three dishes for each condition are shown. (F) The results for BiP (black bars) and CHOP (white bars) in (E) were calculated and are indicated as mean + SD ($n = 3$). The value of the each untreated sample is set at 1.

From the results shown in this article, we conclude that the process of ER stress response, induced by LPS, is clearly different from typical ER stress response, in which apoptosis is induced. Especially, induction of CHOP, which is crucial in ER stress-induced apoptosis, is retarded and weak in the case of LPS treatment, compared with typical ER stress activator including endogenously produced NO.

## Discussion

CHOP induces caspase-11 and functions as an activator of the process of IL-1β maturation (*25*). Therefore, CHOP plays a crucial role in the early stage of inflammation, and apoptosis is not involved in this process. However, the molecular mechanisms, by which the expression of CHOP induced by inflammatory stimuli does not induce apoptosis, were not elucidated. This report showed that treatment with LPS activates the ER stress pathway, including CHOP in cultured macrophage cell lines; however, the induction of CHOP was delayed in comparison with the induction of protective factors, such as BiP and EDEM. In addition, the maximal induction level of CHOP by LPS was lower than that by TG. There are some reports that address the molecular mechanisms of CHOP-mediated apoptosis. Marciniak *et al.* (*12*) reported that CHOP inhibits ER stress-induced attenuation of protein synthesis by the dephosphorylation of the α-subunit of translation initiation factor 2 (eIF2α)



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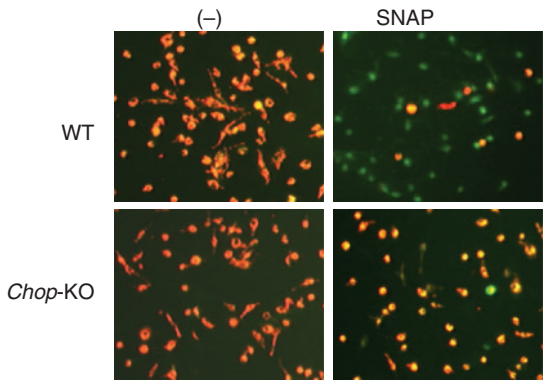


**Fig. 6 Analysis of the involvement of CHOP in NO-induced apoptosis, using primary cultured peritoneal macrophages from *Chop*-knockout mice.** Primary cultured peritoneal macrophages were prepared from wild-type (WT) and *Chop*-knockout mice (*Chop*-KO). Cells were treated with 1.5 mM NO donor SNAP for 12 h, and cells were stained with a mitochondrial membrane potential-indicating dye, DePsipher, as described in the Experimental Procedures section. Original magnification: ×400.

through the induction of GADD34. They also reported that CHOP induces Ero1α, which is localized in ER lumen. Ero1α oxidizes protein disulfide isomerases (PDIs), which then transfer their disulfide bonds to ER client proteins. Therefore, induction of CHOP enhances accumulation of misfolded proteins in the ER under ER stress conditions, decreases ER functions, which is followed by the activation of ER stress pathway. When RAW 264.7 cells were treated with the typical ER stress inducer TG or NO, CHOP and ER function-protective factor mRNAs were promptly induced, and apoptosis was induced. In contrast, when RAW 264.7 cells were treated with LPS, the induction of CHOP was delayed and weak in comparison with that of ER function-protective factors, and apoptosis was not induced. BiP assists protein folding in the ER, and activation of ERAD by the induction of ERAD-associated molecules, such as EDEM and Derlin, prevents the accumulation of unfolded or misfolded proteins in the ER. Therefore, it can be deduced that the ER stress-enhancing and apoptosis-inducing effect of CHOP is suppressed in LPS-treated RAW 264.7 cells, because the ER function-protective system is enhanced before CHOP expression, and the induction level of CHOP is not enough to overcome such already induced ER function-protective system. In fact, ER stress- and CHOP-mediated apoptosis, induced by TG, was suppressed by the pretreatment with LPS, and apoptosis induced by CHOP expression was prevented by BiP overexpression.

Next, we examined the mechanism by which the induction of CHOP was delayed in LPS-treated RAW 264.7 cells, even though ER stress-protective factors are promptly induced. When ER stress-inducing stimuli are given to cells and ER functions are disturbed, ER stress sensors on the ER membrane sense the accumulation of abnormal structured proteins, and then those sensors are activated. It is generally believed that ER stress sensors bind with BiP at their ER luminal portion under unstressed conditions and are inactivated (*1*, *2*). Under ER stress conditions, BiP dissociates from the ER stress sensors, and the ER stress sensors are activated. Though the IRE1-XBP1 pathway was promptly activated, the PERK pathway was not activated in LPS-treated RAW 264.7 cells. In contrast, both sensor systems were promptly activated in TG-treated RAW 264.7 cells. Induction of CHOP depends strongly on the PERK system and weakly on other two sensor systems (*4*, *17*, *35*). Therefore, the induction of CHOP by LPS treatment was delayed, because CHOP was induced through the IRE1 and ATF6 systems, not the PERK system in this condition. If all three ER stress sensors are regulated equally just by BiP binding and dissociation, it is highly likely that all three ER stress sensors are similarly activated under ER stress conditions. The results presented in this study strongly suggest that the regulatory mechanisms of ER stress sensors are more complex. Kimata *et al*. (*41*) reported that IRE1 is activated via two steps, both of which are regulated by ER stress, albeit in different ways. In the first step, BiP dissociation from IRE1 leads to cluster formation. In the second step, direct interaction of unfolded proteins with the intraluminal portion of IRE1 orients the cytosolic effector domains of clustered IRE1 molecules, then IRE1 is activated. There are moderate amino acid sequence similarities between the luminal domain of IRE1 and that of PERK. Therefore, it is possible that both IRE1 and PERK require association with unfolded proteins in activation, but, there are some differences in their affinity for unfolded proteins. This suggests that the types of accumulated unfolded ER proteins vary depending on the nature of the stress, and IRE1 and PERK are differentially activated depending on the nature of the ER stress-inducing stimuli. More detailed molecular analysis of ER stress sensors are needed to verify this hypothesis.

Treatment with LPS or IFN-γ alone does not induce apoptosis, but treatment with LPS plus IFN-γ induces CHOP-mediated apoptosis. Apoptosis induced by the treatment with LPS plus IFN-γ depends on the production of a large amount of NO (*17*, *39*). Therefore, we examined LPS plus IFN-γ treatment-induced apoptosis as the endogenously induced ER stress-mediated apoptosis. NO production is much larger when the cells were treated with LPS plus IFN-γ in comparison with the cells treated with either LPS or IFN-γ alone. Treatment with LPS plus IFN-γ promptly induced BiP and CHOP as the treatment with TG, in RAW 264.7 cells. Furthermore, the depletion of NO delayed the induction of CHOP in the LPS plus IFN-γ-treated RAW 264.7 cells. However, the induction of BiP was barely influenced by NO depletion. These results suggest that endogenously produced NO is associated with the activation of the PERK-ATF4-CHOP pathway in LPS plus IFN-γ-treated RAW 264.7 cells. Treatment with large amount of NO causes tyrosine nitration of the sarcoplasmic/ER $Ca^{2+}$-ATPase (SERCA), *S*-nitrosylation of ryanodine receptor and *S*-nitrosylation of PDI in ER. Through these modifications, SERCA and PDI were inhibited (*42–44*), whereas ryanodine receptor was activated (*45*). SERCA functions as a $Ca^{2+}$ uptake pump from the

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cytosol to the ER. Ryanodine receptor functions in $Ca^{2+}$ release from the ER to the cytosol, and PDI catalyses thiol-disulfide exchange to facilitate disulfide bond formation. High concentrations of ER $Ca^{2+}$ are required for the proper function of many ER proteins, including ER chaperones, such as calreticulin and calnexin (*46*). Therefore, excess NO disturbs $Ca^{2+}$ homoeostasis and formation of proper disulfide bonds in the ER and induces the accumulation of misfolded proteins in the ER, thus activating the ER stress pathway (*6*). In contrast, pretreatment with a small amount of NO prevents subsequent apoptosis induced by the treatment with large amount of NO, through the induction of cytosolic Hsp70-Hsp40 chaperone systems (*32*). On the other hand, the molecular mechanisms by which LPS treatment activates the ER stress pathway remain to be elucidated. One possible mechanism is that LPS treatment induces many kinds of secretory proteins such as cytokines, and places a burden on the ER protein quality control system. Thereafter, the ER stress pathway is activated to enhance the folding activity in the ER. Therefore, difference in the accumulated proteins in the ER causes the differential activation of ER stress sensors. When RAW 264.7 cells were treated with lower concentrations of TG, the maximal induction levels of BiP and CHOP mRNAs decreased in comparison with those with 2 μM TG (data not shown), but the time course of induction was almost identical. This suggests that the molecular mechanism of ER stress sensors-activation by LPS is essentially different from that by TG, and is probably also different from that by NO. This report showed that the cellular responses to ER stress are variable, depending on the difference in stimuli. Therefore, a more detailed study should be conducted that addresses the pathological roles of the ER stress pathway.

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### Conflict of interest

None declared.

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