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The ER Stress Pathway Involving CHOP Is Activated in the Lungs of LPS-Treated Mice

Motoyoshi Endo[1,2], Seiichi Oyadomari[1], Moritaka Suga[2], Masataka Mori[1] and Tomomi Gotoh[1,*]

Departments of [1]Molecular Genetics and [2]Respiratory Medicine, Graduate School of Medical Sciences, Kumamoto University, Honjo 1-1-1, Kumamoto 860-8556

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CHOP is a C/EBP family transcription factor involved in endoplasmic reticulum (ER) stress-mediated apoptosis. To determine if the ER stress pathway is involved in the pathogenesis of LPS-treated mouse lung injury, mice were given lipopolysaccharide (LPS) intraperitoneally. The mRNAs for activating transcription factor (ATF) 4 and X-box binding protein (XBP) 1, transcriptional activators of the CHOP gene, and that for CHOP were induced by or after the LPS treatment. Apoptosis induced by LPS treatment was suppressed in the lungs of *Chop*-knockout mice. Overexpression of CHOP induced apoptosis in a lung cancer–derived cell line. These results suggest that the ER stress pathway, involving CHOP, is activated and plays a role in the pathogenesis of septic shock lung.

Key words: apoptosis, ATF4, CHOP, endoplasmic reticulum stress, inflammation, lipopolysaccharide, nitric oxide, XBP1.

Abbreviations: ER, endoplasmic reticulum; CHOP, C/EBP homologous protein; NO, nitric oxide.

Septic shock syndrome induced by severe infection remains one of the major clinical problems, and the mortality rate is high (*1*, *2*). The production of huge amounts of nitric oxide (NO) in sepsis and other severe infections is thought to be one of the major causes of septic shock syndrome (*3–5*). NO is an important multifunctional biomolecule (*6–8*), however, excess amounts of NO can cause hypotension and cell damage (*9*). LPS induces iNOS (inducible form of NO synthase), and inflammatory cytokines such as interleukin (IL)-1β, IL-2, IL-6, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ in a variety of cells including vascular endothelial cells, smooth muscle cells, macrophages and parenchymal cells (*10*, *11*). NO has cytotoxic effects, including reactions with proteins and nucleic acids, and apoptosis occurs in many cell types (*12*). We reported that NO induces apoptosis through the ER stress pathway including CHOP in pancreatic β-cells, microglia and macrophages (*13–15*). It is also known that inflammatory cytokines induce apoptosis.

CHOP, also known as GADD153, is a member of the C/EBP transcription factor family that heterodimerizes with other C/EBPs (*16*). CHOP is induced in response to cellular stress, especially ER stress, and is involved in the ER stress-induced apoptosis pathway (*17*). We showed that ER stress- and CHOP-mediated apoptosis is involved in the pathogenesis of mouse hereditary diabetes mellitus caused by a point mutation of the *insulin* gene (Akita mouse) and ischemia–induced neuronal cell death *in vivo* (*18*). Recently, we found that translocation of Bax, which is a pro-apoptotic Bcl-2 family member, from the cytosol to mitochondria plays a key role in ER stress-mediated apoptosis downstream of CHOP induction (*19*). However, the precise mechanism of ER stress-induced apoptosis has remained unclear. It is also not known whether ER stress-induced apoptosis is involved in the pathogeneses of inflammatory diseases.

The lung is one of the major target organs in septic shock syndrome. Not only hypotension and cardiac failure, but also direct lung damage in sepsis cause pulmonary edema and dysfunction of gas exchange. We previously showed that iNOS is induced in the lung of a septic shock mouse model and that a large amount of NO is produced (*20*). Here we examined whether the ER stress pathway is involved in the cell injury in LPS-treated mouse lungs.

MATERIALS AND METHODS

Animals and LPS Treatment—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 described (*18*), and were provided by Shizuo Akira (Osaka University, Japan). Specific pathogen-free male C57BL/6 and *Chop* knockout mice weighing 19–25 g (6 weeks of age) were given Escherichia coli LPS (serotype 0127:B8., Sigma, St. Louis, MO, USA) intraperitoneally at 160 mg/kg of body weight and then killed at the indicated times after being anesthetized with ether.

RNA Blot Analysis—Total RNAs from mouse tissues and human lung cancer-derived RERF-LC Ad2 cells were prepared by the guanidium thiocyanate-phenol-chloroform extraction procedure (*21*). After electrophoresis in formaldehyde-containing agarose gels, the RNAs were transferred to nylon membranes. For the hybridization, we used the following probes; digoxigenin-labeled antisense RNAs for mouse IL-1β (nucleotides 78–640; GenBank, accession no. NM_008361), mouse iNOS (*22*), mouse ATF4

*To whom correspondence should be addressed. Tel: +81-96-373-5140, Fax: +81-96-373-5145, E-mail: tomomi@gpo.kumamoto-u.ac.jp

(nucleotides 221–743; GenBank, accession no. M94087), human ATF4 (nucleotides 253–1347; GenBank, accession no. BC016855), mouse XBP1 (nucleotides 269–682; GenBank, accession no. NM_013842), human XBP1 (nucleotides 612–921; GenBank, accession no. P17861), mouse CHOP (nucleotides 68–585; GenBank, accession no. X67083), human CHOP (nucleotides 190–779; GenBank, accession no. S40706), human BiP (nucleotides 463–1242; GenBank, accession no. X87949), human calreticulin (nucleotides 305–1133; GenBank, accession no. BC007911), rat β-actin (nucleotides 417–1223; GenBank, accession no. NM_031144), and human GAPDH (nucleotides 366–680; GenBank, accession no. M33197). Chemiluminescence signals derived from the hybridized probes were detected with a Las-1000 Plus bioimage analyzer (Fuji Photo Film, Tokyo, Japan) and DIG luminescence detection kits (Roche Molecular Biochemicals, Indianapolis, IN, USA).

RT-PCR Analysis—Total RNA from mouse lungs was prepared by the guanidium thiocyanate-phenol-chloroform extract procedure (*21*). cDNA was synthesized with a Superscript one-Step RT-PCR system (Invitrogen, Carlsbad, CA, USA). The primers used for detection of XBP1 mRNA were as follows: sense primer, 5′-AAACAGAGTAGCAGCGCAGACTGC-3′ and antisense primer, 5′-GGATCTCTAAAACTAGAGGCTTGGTG-3′. These primers are expected to give PCR products with sizes of 600 bp for unspliced form and 574 bp for spliced (active) form XBP1, respectively. PCR consisted of an initial denaturation cycle of 94°C for 2 min, followed by 25 cycles consisting of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and elongation at 68°C for 1 min. An additional cycle at 72°C for 7 min completed the amplification process. The amplified PCR products were separated by 3% agarose gel electrophoresis and visualized by ethidium bromide staining.

Immunohistochemical Staining—Lungs were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4), and the excised tissues were immersed in a fixative solution for 3 h at 4°C, washed in PBS for 15 min, dehydrated through a graded series of ethanol and xylene, and then embedded in a paraffin block. Sections (5 μm thick) were cut, air-dried, deparaffinized and autoclaved (121°C, 15 min) in citrate buffer to enhance the penetration of antibodies. Then, the sections were pretreated with 5 mM periodic acid for 10 min at room temperature to inhibit endogenous peroxidase activity. For the immunostaining of CHOP, the specimens were incubated for 1 h with 100-fold diluted polyclonal antibodies against mouse CHOP (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and then washed 3 times with PBS for 5 min. Then, the sections were incubated for 1 h with 500-fold diluted second antibodies conjugated with peroxidase, and visualized by incubation with a 3,3′-diaminobenzidine solution. For immunostaining of the active form (p17) of caspase-3, the specimens were incubated for 1 h with 125-fold diluted polyclonal antibodies against active form caspase-3 (Promega Inc., Madison, WI, USA), and washed 3 times with PBS for 5 min. Then the sections were incubated for 1 h with 500-fold diluted second antibodies conjugated with peroxidase, and visualized by incubation with a 3,3′-diaminobenzidine solution.

Detection of Apoptosis—Apoptosis was detected by the terminal transferase-mediated dUTP-biotin nick end labeling (TUNEL) method. After sections (5 μm) had been cut, air-dried, and deparaffinized, DNA fragmentation was detected with an *in situ* apoptosis detection kit (Takara, Otsu, Japan) according to the protocol recommended by the manufacturer.

Plasmids—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 (GenBank accession no. X67083). The product was inserted into the EcoRV site of pcDNA3.1(+) (Invitrogen), yielding pcDNA3.1-mCHOP. pEGFP-C1, a mammalian expression plasmid for enhanced GFP, was obtained from CLONTECH Laboratories, Inc. (Palo Alto, CA, USA).

Cell Culture, Treatment of Cells and Transfection—Human lung cancer-derived RERF-LC Ad2 cells were provided by the Health Science Research Resources Bank (Osaka, Japan). Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and treated with E. coli LPS (serotype 0127:B8., Sigma, 80 μg/ml) plus recombinant human interferon-γ (IFN-γ) (PEPROTECH EC, London, UK., 100 U/ml) for the indicated periods. Transfection of cells with plasmids was carried out using Lipofectamine 2000 (Invitrogen) according to the protocol recommended by the manufacturer. In each experiment, the same total amounts of plasmids were transfected by adding insert-less expression plasmids.

RESULTS

Induction of mRNAs for Inflammation and ER Stress-Related Proteins in the Lungs of LPS-Treated Mice—Inflammatory stimuli, such as acute lung injury in sepsis, induce the production of a huge amount of NO and may cause activation of the ER stress pathway (Fig. 1). ER stress sensor proteins, PERK, IRE1 and ATF6, are activated in the ER stress response. ATF4 and XBP1, ER stress-associated transcriptional enhancers, are induced, as are cell-protecting mechanisms. However, when ER functions are severely impaired, CHOP is induced by ATF4 and XBP1, and apoptosis follows. Whether apoptosis is positive or negative as to the pathogenesis of acute lung injury has remained controversial. Using the LPS-treated mouse lung as a model, we performed several experiments.

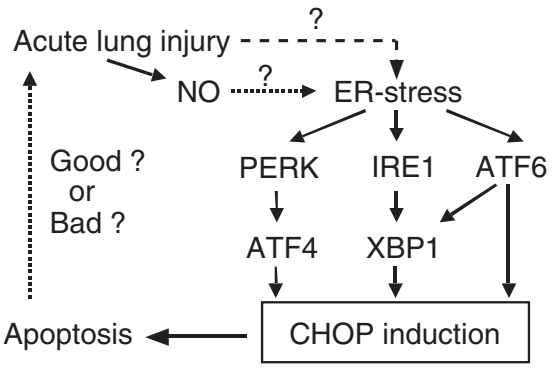

Fig. 1. **Hypothesis for the involvement of the ER stress pathway in acute lung injury induced in mice by LPS.**

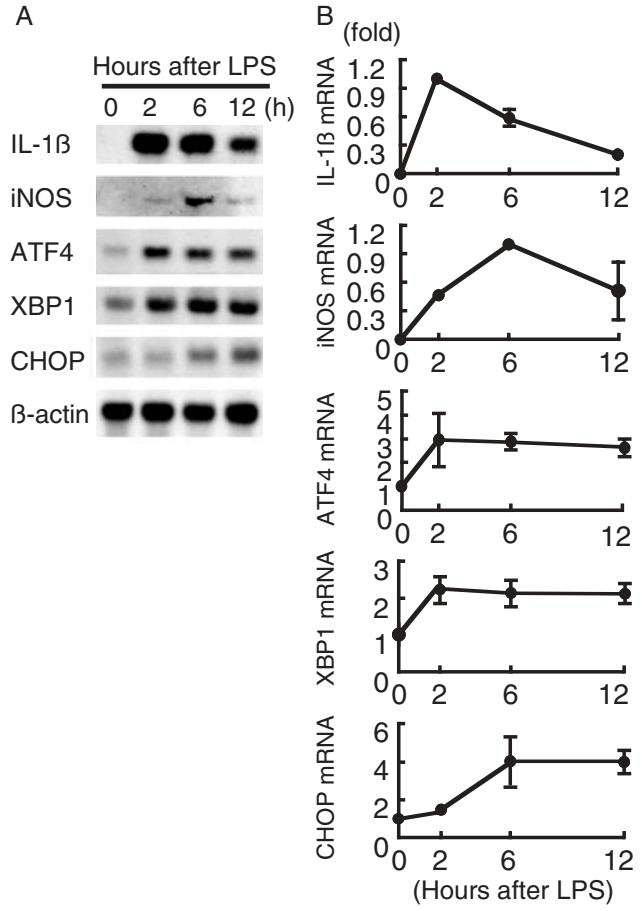

Fig. 2. **Effects of LPS treatment on mRNAs for IL-1β, iNOS, ATF4, XBP1, CHOP, and β-actin in the mouse lung.** (A) LPS was given to mice intraperitoneally. After the indicated times, total RNAs were isolated from lungs. The RNAs (2.2 μg) were subjected to blot analysis for IL-1β, iNOS, ATF4, XBP1, CHOP, and β-actin. (B) The results in A were quantified and are shown as means ± ranges (n = 2). The maximal values were set at 1.0 for IL-1β and iNOS mRNAs, and the control values prior to treatment were set at 1.0 for ATF4, XBP1, and CHOP mRNAs.

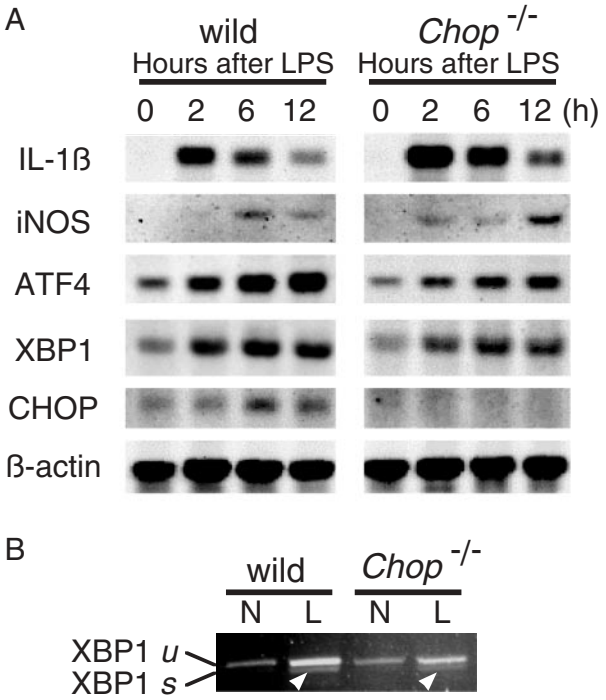

Fig. 3. **Effects of LPS treatment on mRNAs for IL-1β, iNOS, ATF4, XBP1, CHOP, and β-actin in the lungs of wild type and *Chop* knockout mice.** (A) LPS was given to mice intraperitoneally. After the indicated times, total RNAs were isolated from the lungs of wild type and *Chop* knockout mice. The RNAs (2.2 μg) were subjected to blot analysis for IL-1β, iNOS, ATF4, XBP1, CHOP, and β-actin. (B) Induction and splicing of XBP1 mRNA in the lungs of wild and *Chop* knockout mice. RT-PCR analysis was performed using total RNAs from lungs at 0 and 2 h after LPS treatment with a primer set encompassing the spliced-out region in XBP1 mRNA. The PCR products were resolved on 3% agarose gels to separate unspliced (XBP1*u*) and spliced (XBP1*s*, arrowheads) XBP1 mRNAs.

Mice were given LPS intraperitoneally, and the mRNAs for IL-1β and iNOS were then analyzed as inflammation markers (Fig. 2). IL-1β mRNA, which was not detected in untreated mice, was dramatically induced, reaching a maximum 2 h after injection and decreasing thereafter. iNOS mRNA, which was not detected in untreated mice, began to increase at 2 h after treatment, reached a maximum at 6 h, and had decreased at 12 h. Thus, LPS treatment induces inflammation in the mouse lung. Then we examined whether the ER stress pathway is activated in the lung inflammation induced by LPS treatment (Fig. 2). ATF4 and XBP1 are transcriptional enhancers of the genes for CHOP and other ER stress-related genes (Fig. 1). ATF4 and XBP1 mRNAs present at low levels in untreated mice were induced and reached a maximum at 2 h after treatment, and remained little changed thereafter. CHOP mRNA, which was present at a low level in untreated mice, was induced somewhat later than the ATF4 and XBP1 mRNAs, reached a maximum at 6 h, and was little changed at 12 h. These results suggest that CHOP mRNA was probably induced by XBP1 and ATF4. β-actin mRNA remained little changed with the treatment.

Involvement of CHOP-Mediated Apoptosis in LPS-Induced Lung Damage—As mentioned above, immunostimulation induces ER stress- and CHOP-mediated apoptosis in RAW 264.7 cells (*15*). Therefore, we examined whether LPS treatment would induce CHOP-mediated apoptosis in the lung *in vivo*. When *Chop* knockout mice were treated with LPS, the mRNAs for IL-1β, ATF4, and XBP1 were induced, as found in wild type mice (Fig. 3A). iNOS mRNA was induced a little later and reached a maximum at 12 h after treatment in *Chop* knockout mice. Even in wild type mice, induction of iNOS mRNA did not precede that of the ATF4 and XBP1 mRNA. This suggests that NO produced by iNOS does not play a major role in LPS-induced activation of the ER stress pathway *in vivo*. However, involvement of NO produced by other types of NOS remains to be examined. Upon ER stress, XBP1 mRNA is induced, and then subjected to unique and specific splicing by activated IRE1, one of the ER stress sensors. Then the active form XBP1 is produced, and which induces the expression of ER stress-response genes (*23–25*). Therefore, induction of XBP1 mRNA and its splicing are good markers for activation of the ER stress pathway. Figure 3B shows the

unspliced form XBP1 mRNA was expressed at a low level and that the spliced form was not expressed in either wild type or *Chop* knockout mice lungs before LPS treatment. In contrast, the unspliced form XBP1 mRNA was markedly induced, and the spliced form XBP1 mRNA was a little induced in both LPS treated-wild type and *Chop* knockout mice lungs. These results show that LPS treatment activates the ER stress pathway upstream of CHOP in both wild type and *Chop* knockout mice lungs. The induction of the unspliced form XBP1 mRNA by LPS was lower in *Chop* knockout mice lungs for an unknown reason. The delay in iNOS induction in *Chop* knockout mice suggests that the CHOP-mediated pathway is involved in the enhancement of iNOS induction. Then we determined if LPS treatment induces CHOP-mediated apoptosis in the lung. Apoptosis was analyzed by the TUNEL method at 12 h after LPS treatment. Figure 4 (A and B) shows that apoptosis is induced in the LPS-treated mouse lung, and that this apoptosis is suppressed in *Chop* knockout mice. The number of TUNEL-positive cells per area in *Chop* knockout mice was about one-third of that seen in wild type mice. The thickness of the alveolar wall reflects the inflammatory edema in pneumonia. The thickness of the alveolar wall that was seen in wild type mice after LPS treatment was suppressed in *Chop* knockout mice. This finding suggests that inflammation induced by LPS is suppressed in *Chop* knockout mice. Activation of caspase-3 occurs downstream of the apoptosis signal cascade, and is the point of no return in the apoptosis pathway. Therefore, expression of the active form of caspase-3 is a marker of apoptotic cells. Immunostaining of the active form of caspase-3 in the lungs of LPS-treated mice is shown in Fig. 4C. In the wild-type mouse lung, cells positive for active caspase-3 were observed. In contrast, the number of positive cells was greatly decreased in *Chop* knockout mice. Therefore, CHOP plays an important role in the process of LPS-induced apoptosis in the mouse lung.

Identification of CHOP-Induced Cells in LPS-Treated Mouse Lungs—To identify cells in which CHOP is induced by LPS treatment, immunohistochemical analysis of the lung was performed (Fig. 5). CHOP-positive cells were undetectable in the control lung (Fig. 5A, PBS). In contrast, strong immunoreactivity was detected in the lungs of LPS-treated mice (Fig. 5A, LPS; Fig. 5B). Various cells including respiratory epithelial cells (arrowhead in a), vascular endothelial cells (arrow in a), alveolar type I epithelial cells (arrow in b), and alveolar type II epithelial cells (arrowhead in b) were positive for CHOP, while other cells, which appeared to be alveolar macrophages and neutrophils, were also positive. Apoptosis of vascular endothelial cells and alveolar epithelial cells may possibly be the cause of lung edema in septic shock lung.

Induction of mRNAs for ER Stress-Related Genes in Human Lung Cancer-Derived RERF-LC Ad2 Cells Treated with LPS plus IFN-γ—We next examined whether inflammatory stimuli activate the ER stress pathway in a lung-derived cell line. RERF-LC Ad2 cells were treated with LPS plus IFN-γ, and then the expression of mRNAs for ER stress-related genes was analyzed (Fig. 6). In our previous work, treatment with LPS alone was not enough to induce ER stress-mediated apoptosis in RAW 264.7 macrophages (*26*), but treatment with LPS plus IFN-γ induced apoptosis. Therefore, we treated RERF-LC Ad2 cells with LPS plus

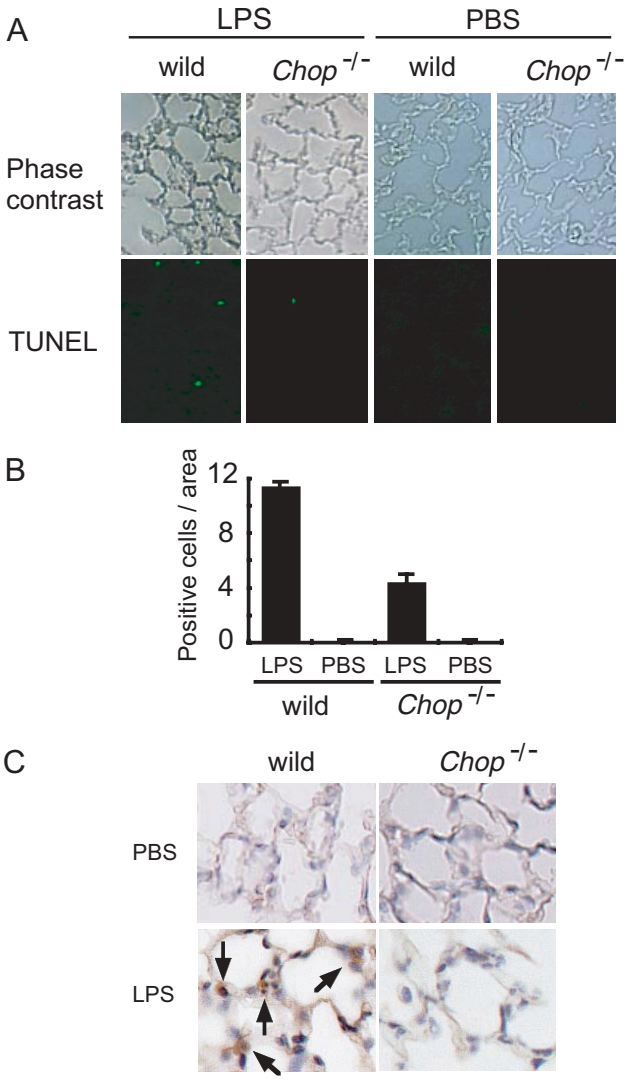

Fig. 4. **Induction of CHOP-mediated apoptosis in LPS-treated mouse lung.** (A) LPS was given to wild type and *Chop* knockout mice intraperitoneally. The same volume of PBS was given intraperitoneally as a negative control. Twelve hours after injection, the lungs of wild type and *Chop* knockout mice were analyzed for apoptosis by the terminal transferase-mediated dUTP-biotin nick end labeling (TUNEL) method. Phase-contrast images and fluorescence images of the same fields are shown. Original magnification: ×200. (B) The results in A were quantified by calculating the number of TUNEL-positive cells per one scope area (×200), and are shown as means ± SD ($n = 5$). (C) Immunostaining of the lungs of wild type and *Chop* knockout mice with an antibody against the active form of caspase-3. Arrows indicate positive cells for the active form of caspase-3. Original magnification: ×200.

IFN-γ, instead with LPS alone. ATF4 and XBP1 are transcriptional enhancer proteins of the *Chop* gene. ATF4 mRNA, which was present in untreated cells, remained little changed up to 12 h, and had increased markedly at 24 h. XBP1 mRNA, which was also present in untreated cells, increased gradually up to 24 h. BiP and calreticulin are induced in ER stress. BiP and calreticulin mRNAs, which were present in untreated cells, had increased at 12 h, and then had decreased to control levels at 24 h. CHOP mRNA, which was not detected in control cells,

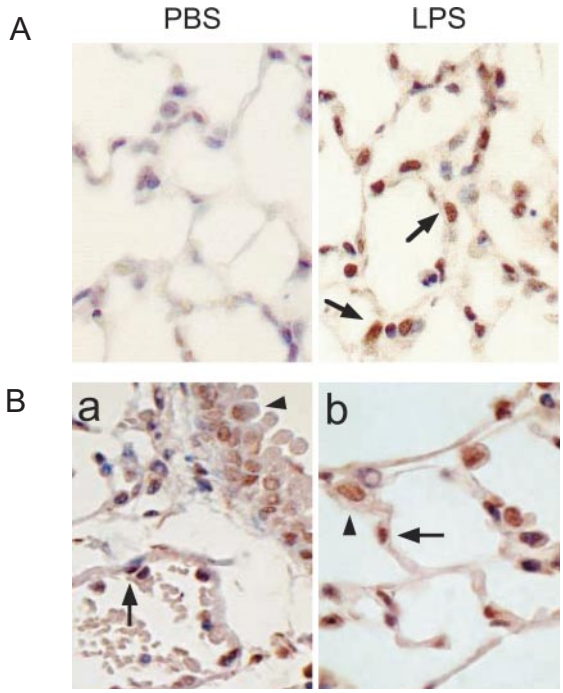

Fig. 5. **Induction of CHOP in LPS-treated mouse lung.** (A) Lungs of control (PBS) and LPS-treated mice (12 h) were immunostained with an antibody against CHOP (diluted 100-fold). Arrows indicate alveolar type II epithelial cells positive for CHOP. Original magnification: ×200. (B) Lungs of LPS-treated wild type mice (12 h) were immunostained with an antibody against CHOP as in A. Many CHOP-positive cells such as respiratory epithelial cells (arrowhead in a), vascular epithelium endothelial cells (arrow in a), alveolar type II epithelial cells (arrowhead in b), and alveolar type I epithelial cells (arrow in b) were detected. Original magnification: ×400.

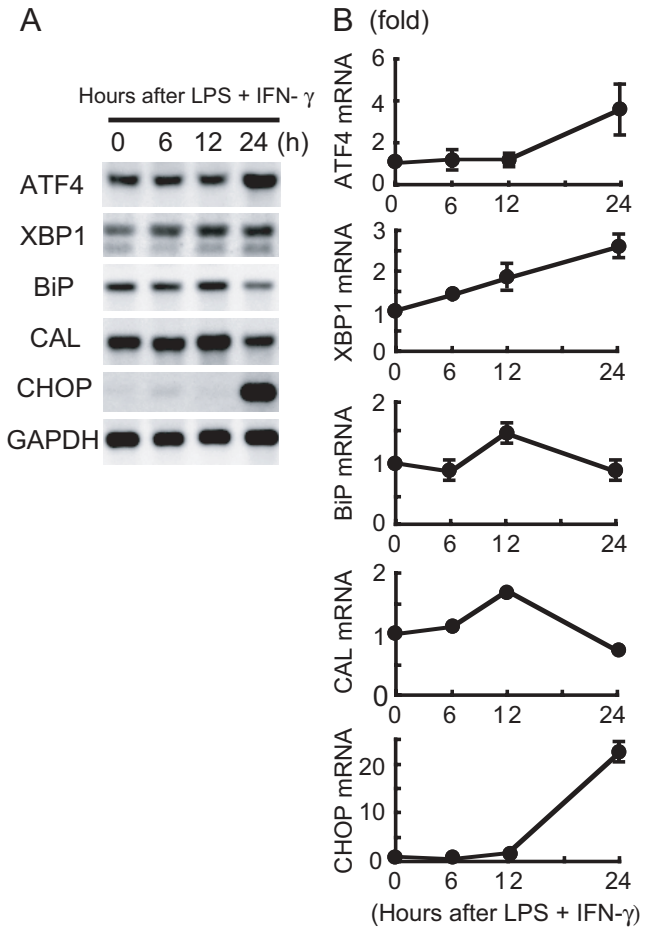

Fig. 6. **Effects of LPS and IFN-γ treatment on mRNAs for ATF4, XBP1, BiP, calreticulin (CAL), CHOP, and β-actin in human lung-cancer derived cells.** (A) RERF-LC Ad2 cells were treated with LPS (80 μg/ml) and IFN-γ (100 U/ml). After the indicated times, total RNAs were isolated from the cells. The RNAs (2.2 μg) were subjected to blot analysis for ATF4, XBP1, BiP, calreticulin, CHOP, and GAPDH. (B) The results in A were quantified and are shown as means ± ranges ($n = 2$). The control values prior to treatment were set at 1.0.

remained barely detectable up to 12 h, but was markedly increased at 24 h. Induction of CHOP mRNA was delayed compared to that of XBP1 mRNA. These results show the ER stress pathway including CHOP is induced in immunostimulated lung-derived cells, and suggest that the induction of CHOP mRNA is mediated by induction of XBP1. Though XBP1 and ATF4 mRNAs are already expressed in untreated cells, and XBP1 mRNA is already induced at 6 h, the induction of CHOP is abrupt at 24 h. These results suggest that other ER stress-induced factor(s) are also involved in CHOP induction. Though NO may not be involved in the early stage of the ER stress response including the induction of XBP1 and ATF4, it remains possible that NO accumulation due to LPS plus IFN-γ treatment is involved in the late stage of the ER stress response including CHOP induction.

Expression of CHOP Induces Apoptosis in RERF-LC Ad2 Cells—We then examined whether expression of CHOP induces apoptosis in RERF-LC Ad2 cells (Fig. 7). Cells were cotransfected with expression plasmids for EGFP and CHOP. Twenty-four h after transfection, cells were stained with Hoechst dye and then observed by fluorescence microscopy. When CHOP was coexpressed with EGFP, about 60% of the transfected EGFP-positive cells were condensed and showed chromatin condensation, reflecting apoptosis. In contrast, when EGFP alone was expressed, an apoptotic change was barely observed (about 5%) in EGFP-positive cells. These results show that CHOP induces apoptosis in lung-derived cells.

DISCUSSION

Recently, ER stress-mediated apoptosis was reported to be involved in the pathogeneses of several diseases, including diabetes mellitus and neurodegenerative diseases (*18*, *27*). CHOP is involved in the ER stress-mediated apoptosis pathway. Yet, little is known of the involvement of ER stress- and CHOP-mediated apoptosis in lung diseases. Cystic fibrosis is a hereditary disorder of the exocrine glands, its major symptom being chronic obstructive pulmonary disorders. It was reported that misfolded ΔF508 CFTR protein accumulates in the ER and thereby causes ER stress (*28*). However, the involvement of CHOP in this condition remains unknown. Because lung epithelial cells secrete a large amount of proteins such as surfactants, it can be speculated that these cells are prone to ER stress.

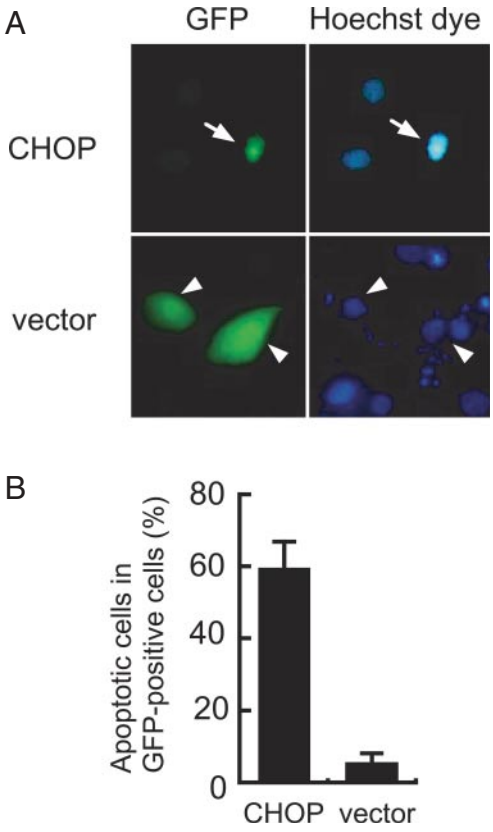

Fig. 7. **Induction of apoptosis in RERF-LC Ad2 cells transfected with CHOP.** (A) Cells were cotransfected with expression plasmids for EGFP and CHOP. Twenty-four hours after transfection, cells were stained with Hoechst dye 33258. EGFP fluorescence images and Hoechst dye images of the same fields are shown. Arrows indicate transfected cells that are positive for GFP and apoptotic. Arrowheads indicate transfected cells that are positive for GFP but not apoptotic. Original magnification: ×200. (B) Experiments were performed as in A, and the percentages of apoptotic cells are shown as means ± SD for three dishes. More than 100 GFP positive cells in each dish were analyzed.

Inflammatory stimuli induce apoptosis through various pathways (*29*). We previously reported that the ER stress pathway involving CHOP is activated in immunostimulated RAW 264.7 macrophages (*15*). In this study, we found that the ER stress pathway, including CHOP, is activated in the lungs of LPS-treated mice. We also showed that the lung damage induced by LPS treatment is attenuated in *Chop* knockout mice. These results show that CHOP plays a key role in LPS-induced lung damage.

NO is one of the candidates by which the ER stress pathway is activated in this model. Xu *et al*. (*30*) reported that NO induces the coupling of mitochondrial signaling with the ER stress response. Oyadomari *et al*. (*13*) showed that NO activates the ER stress pathway, probably by depleting the ER Ca^{2+} store. However, induction of XBP1 and ATF4 mRNA was not proceded by that of iNOS. Therefore, NO produced by iNOS does not play a key role in the activation of the ER stress pathway, at least in our model. We need to investigate how LPS treatment activates the ER stress pathway *in vivo*.

While apoptosis in the lung was observed in our mouse model, the question of whether apoptosis is positive or negative as to the pathogenesis of acute lung injury remains unknown. Matute-Bello *et al*. (*29*) suggested that the rate of clearance of apoptotic neutrophils is associated with the resolution of acute inflammation in the lung. In our LPS-treated mouse model, LPS-induced apoptosis was prevented in *Chop* knockout mice. Wild type mice died within 24 h after LPS treatment, whereas *Chop* knockout mice were alive at that point (unpublished results). Therefore, apoptosis mediated by CHOP may accelarate the process of acute lung injury.

In this study, we showed that CHOP is induced in various cells in LPS-treated mouse lung. However, the number of apoptotic cells was much lower than that of CHOP-expressing cells. There are at least three possible reasons why only a small portion of the CHOP-positive cells died. The first is the lack of activation of other molecules that activate CHOP to induce apoptosis. Phosphorylation by p38 MAP kinase is known to induce the DNA binding activity of CHOP (*31*). Other unknown factor(s) may also activate CHOP. The second is the lack of signal transducing molecule(s) downstream of CHOP. CHOP induces apoptosis in some but not all cell types (*17*). CHOP induces apoptosis through the mitochondrial pathway, but the precise apoptosis cascade downstream of CHOP is unknown. Therefore, why some cells are resistant to CHOP-induced apoptosis remains unknown. The third is the clearance of apoptotic cells by macrophages. When apoptotic cells are rapidly engulfed, only a small portion of the apoptotic cells is detectable.

As far as we know, this is the first report showing that the ER stress pathway plays an important role in inflammation *in vivo*. Severe inflammatory diseases of the lungs can be fatal. Prevention of ER stress-induced lung injury may become a new target for therapy.

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REFERENCES

1. Brigham, K.L. and Meyrick, B. (1986) Endotoxin and lung injury. *Am. Rev. Respir. Dis.* **133**, 913–927
2. Cope, D.K., Grimbert, F., Downey, J.M., and Taylor, A.E. (1992) Pulmonary capillary pressure: a review. *Crit. Care Med.* **20**, 1043–1056
3. Nava, E., Palmer, R.M., and Moncada, S. (1992) The role of nitric oxide in endotoxic shock: effects of NG-monomethyl-L-arginine. *J. Cardiovasc. Pharmacol.* **20**, S132–S134
4. Evans, T., Carpenter, A., Silva, A., and Cohen, J. (1994) Inhibition of nitric oxide synthase in experimental gram-negative sepsis. *J. Infect. Dis.* **169**, 343–349
5. Tracey, W.R., Tse, J., and Carter, G. (1995) Lipopolysaccharide-induced changes in plasma nitrite and nitrate concentrations in rats and mice: pharmacological evaluation of nitric oxide synthase inhibitors. *J. Pharmacol. Exp. Ther.* **272**, 1011–1015
6. Calver, A., Collier, J., and Vallance, P. (1993) Nitric oxide and cardiovascular control. *Exp. Physiol.* **78**, 303–326

7. Davies, M.G., Fulton, G.J., and Hagen, P.O. (1995) Clinical biology of nitric oxide. *Br. J. Surg.* **82**, 1598–1610
8. Moncada, S., Palmer, R.M., and Higgs, E.A. (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43**, 109–142
9. Meyer, J., Lentz, C.W., Stothert, J.C., Jr., Traber, L.D., Herndon, D.N., and Traber, D.L. (1994) Effects of nitric oxide synthesis inhibition in hyperdynamic endotoxemia. *Crit. Care Med.* **22**, 306–312
10. Nathan, C. and Xie, Q.W. (1994) Nitric oxide synthases: roles, tolls, and controls. *Cell* **78**, 915–918
11. Wang, D., Wei, J., Hsu, K., Jau, J., Lieu, M.W., Chao, T.J., and Chen, H.I. (1999) Effects of nitric oxide synthase inhibitors on systemic hypotension, cytokines and inducible nitric oxide synthase expression and lung injury following endotoxin administration in rats. *J. Biomed. Sci.* **6**, 28–35
12. Brüne, B., von Knethen, A., and Sandau, K.B. (1999) Nitric oxide (NO): an effector of apoptosis. *Cell Death Differ.* **6**, 969–975
13. Oyadomari, S., Takeda, K., Takiguchi, M., Gotoh, T., Matsumoto, M., Wada, I., Akira, S., Araki, E., and Mori, M. (2001) Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the endoplasmic reticulum stress pathway. *Proc. Natl Acad. Sci. USA* **98**, 10845–10850
14. Kawahara, K., Oyadomari, S., Gotoh, T., Kohsaka, S., Nakayama, H., and Mori, M. (2001) Induction of CHOP and apoptosis by nitric oxide in p53-deficient microglial cells. *FEBS Lett.* **506**, 135–139
15. Gotoh, T., Oyadomari, S., Mori, K., and Mori, M. (2002) Nitric oxide-induced apoptosis in RAW 264.7 macrophages is mediated by endoplasmic reticulum stress pathway involving ATF6 and CHOP. *J. Biol. Chem.* **277**, 12343–12350
16. Ron, D. and Habener, J.F. (1992) CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes Dev.* **6**, 439–453
17. Zinszner, H., Kuroda, M., Wang, X., Batchvarova, N., Lightfoot, R.T., Remotti, H., Stevens, J.L., and Ron, D. (1998) CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev.* **12**, 982–995
18. Oyadomari, S., Koizumi, A., Takeda, K., Gotoh, T., Akira, S., Araki, E., and Mori, M. (2002) Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *J. Clin. Invest.* **109**, 525–532
19. Gotoh, T., Terada, K., Oyadomari, S., and Mori, M. (2004) hsp70-DnaJ chaperone pair prevents nitric oxide- and CHOP-induced apoptosis by inhibiting translocation of Bax to mitochondria. *Cell Death Differ.* **11**, 390–402
20. Salimuddin, Nagasaki, A., Gotoh, T., Isobe, H., and Mori, M. (1999) Regulation of the genes for arginase isoforms and related enzymes in mouse macrophages by lipopolysaccharide. *Am. J. Physiol.* **277**, E110–E117
21. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159
22. Nagasaki, A., Gotoh, T., Takeya, M., Yu, Y., Takiguchi, M., Matsuzaki, H., Takatsuki, K., and Mori, M. (1996) Coinduction of nitric oxide synthase, argininosuccinate synthetase, and argininosuccinate lyase in lipopolysaccharide-treated rats. RNA blot, immunoblot, and immunohistochemical analyses. *J. Biol. Chem.* **271**, 2658–2662
23. Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., and Mori, K. (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* **107**, 881–891
24. Lee, K., Tirasophon, W., Shen, X., Michalak, M., Prywes, R., Okada, T., Yoshida, H., Mori, K., and Kaufman, R.J. (2002) IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. *Genes Dev.* **16**, 452–466
25. Calfon, M., Zeng, H., Urano, F., Till, J.H., Hubbard, S.R., Harding, H.P., Clark, S.G., and Ron, D. (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* **415**, 92–96
26. Gotoh, T. and Mori, M. (1999) Arginase II downregulates nitric oxide (NO) production and prevents NO-mediated apoptosis in murine macrophage-derived RAW 264.7 cells. *J. Cell Biol.* **144**, 427–434
27. Milhavet, O., Martindale, J.L., Camandola, S., Chan, S.L., Gary, D.S., Cheng, A., Holbrook, N.J., and Mattson, M.P. (2002) Involvement of Gadd153 in the pathogenic action of presenilin-1 mutations. *J. Neurochem.* **83**, 673–681
28. Okiyoneda, T., Harada, K., Takeya, M., Yamahira, K., Wada, I., Shuto, T., Suico, M.A., Hashimoto, Y., and Kai, H. (2003) ΔF508 CFTR pool in the ER is increased by calnexin overexpression. *Mol. Biol. Cell* **31**, 31
29. Matute-Bello, G. and Martin, T.R. (2003) Science review: apoptosis in acute lung injury. *Crit. Care* **7**, 355–358
30. Xu, W., Liu, L., Charles, I.G., and Moncada, S. (2004) Nitric oxide induces coupling of mitochondrial signalling with the endoplasmic reticulum stress response. *Nat. Cell Biol.* **6**, 1129–1134
31. Wang, X.Z. and Ron, D. (1996) Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP Kinase. *Science* **272**, 1347–1349