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Combined Effects of Multiple Endoplasmic Reticulum Stresses on Cytokine Secretion in Macrophage

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Abstract

Cells show various stress signs when they are challenged with severe physiological problems. Majority of such cellular stresses are conveyed to endoplasmic reticulum (ER) and unfolded protein response (UPR) serves as typical defense mechanism against ER stress. This study investigated an interaction between ER stress agents using macropage cell line Raw 264.7. When activated by lipopolysaccharide (LPS), the cell lines showed typical indicators of ER stress. Along with molecular chaperones, the activation process leads to the production of additional inflammatory mediators. Following activation, the macrophage cell line was further treated with TUN and characterized in terms of chaperone expression and cytokine secretion. When treated with TUN, the activated macrophage cell leads to increased secretion of IL-6 although expression of ER stress markers, GRP94 and GRP78 increased. The secretion of cytokines continued until the addition of BFA which inhibits protein targeting from ER to Golgi. However, secretion of cytokines was ceased upon dual treatments with BFA and TG. This result strongly implies that cells may differently deal with various polypeptides depending on the urgency in cellular function under ER stress. Considering IL-6 is one of the most important signal molecules in macrophage, the molecule might be able to circumvent ER stress and UPR to reach its targeting site.

Key Words: Tunicamycin, ER stress, Apoptosis, Cytokine, Macrophage, ELISA

INTRODUCTION

The endoplasmic reticulum (ER) is an essential cellular compartment for protein synthesis and maturation. ER can result in accumulation of unfolded or misfolded proteins upon inhibition of protein glycosylation or disulfide bond formation, disruption of Ca^{2+} homeostasis, and viral infection (He, 2006; Hotamisligil, 2006). To lessen the excessive protein loading, the cells start the unfolded protein response (UPR). This response prompts a signaling cascade for transient reduction of protein translation, deletion of misfolded proteins, and induction of molecular chaperones (Ni and Lee, 2007).

During the ER stress, upregulation of ER chaperones is critical for cell survival by expediting the correct folding and assembly of ER chaperones as well as by preventing their aggregation and consequent secretion out of affected cells. Three groups of molecular chaperones exist: heat shock protein family (GRP78, GRP94 and co-chaperones), lectin-like (calnexin, and calreticulin), and substrate-specific chaperones such HSP47. There are two known chaperone system in the ER, calnexin/calreticulin and GRP78/GRP94 (Ron and Walter, 2007).

When the extent of ER stress intensifies with no solution available to alleviate the situation, cells advance to the programmed cell death, apoptosis. To date, no report is available that cells have been rescued from a severe ER stress and reversed to the normal status. Usually, apoptosis is the final destination for the cells under the significant ER stress (Schröder and Kaufman, 2005; Rutkowski *et al.*, 2008). At least three pathways are known to be involved in the apoptotic events: activation of caspase-12, transcriptional activation of the gene for C/EBP homologous protein (CHOP), and activation of the cJUN NH2-terminal kinase (JNK) pathway (Oyadomari and Mori, 2004). All of the three apoptosis pathways eventually lead to the activation of caspase-3; thus, ER stress signals are finally transmitted to the mitochondria (Senkal *et al.*, 2011).

Macrophages are phagocytes, acting in both non-specific defenses as well as to help initiate specific defense mechanisms (Zhang *et al.*, 2008). In immune system, ER stress leads to the activation of inflammatory signaling pathways and several serine/threonine kinases are activated by inflammatory or stressful stimuli, including JNK, inhibition of NF-κB kinase (IKK) (Wellen and Hotamisligil, 2005; Mosser and Edwards, 2008). JNK and IKK are activated in the innate immune

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response by Toll-like receptor (TLR) signaling in response to LPS, peptidoglycan, double-stranded RNA, and other microbial products (Kawai and Akira, 2007).

ER stress is chemically induced using drugs such as tunicamycin (TUN), brefeldin A (BFA), and thapsigargin (TG). TUN induces ER stress inhibiting the N-glycosylation of newly biosynthesized proteins. BFA blocks membrane export out of the ER in vivo and inhibits vesicle formation both in vivo. TG, an inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA), also induces ER stress by disrupting the homeostatic balance of the Ca^{2+} concentration in the ER (Shimoke *et al.*, 2005). In addition to the effect on the calcium mobilization, TG was shown to promote transcriptional machinery and factors to bind to the murine *ifnb1* (Martinon *et al.*, 2010). The ER contains a number of molecular chaperones involved in the post-translational modification, disulfide bond formation, folding, assembly and quality control of newly synthesized proteins to conserve cellular homeostasis (Oyadomari *et al.*, 2006).

No study has been carried out focusing upon the combined effect of ER stresses originating from different sources to date. Under circumstances, the present study is to characterize an interaction between jointly applied ER stresses on the cells according to quantitation of cytokines as secretory proteins. Using Raw 264.7 murine macrophage cells, we studied the effects of the three ER stress agents above on activated macrophage cell on the bases of expression of ER stress markers and secretion of cytokines (IL-6 and TNF-alpha). The three agents of ER stress used are TUN, BFA and TG. Their different effects on the activated macrophage were compared among the three ER stress agents concerning the level of ER stress and IL-6 secretion.

MATERIALS AND METHODS

Cell culture and induction of ER stress

Raw 264.7 cell, murine macrophage cell, was cultured in DMEM medium (Welgene. Inc) supplemented with 10% heat-inactivated FBS (Fetal Bovine Serum) and 5% penicillin-streptomycin (5 μg/ml). All cells were incubated in humidified air containing 5% CO_2 at 37°C. Endoplasmic reticulum stress inducers tunicamycin was purchased from Sigma. ER stress was induced by applying 2.5 μg/ml TUN solution. To prepare TUN stock solution, TUN was dissolved in DMSO at room temperature. Brefeldin A (BFA) was purchased from Sigma and used at the final concentration of 10 μg/ml as dissolved in a 95% ethanol. Thapsigargin was purchased from Assay Design and used at the concentration of 2 μM as delivered in DMSO. The macrophage was activated by treating the cells up to 100 ng/ml of lipopolysaccharide (LPS, Sigma). LPS was dissolved in PBS to make 1 mg/ml at room temperature and the solution was stored in aliquot at -20°C until use.

Western blotting

Protein samples were separated on a 12% SDS-PAGE and transferred onto nitrocellulose membrane. The membranes were blocked in a 5% non-fat milk with 0.1% Tween TBS (TBS-T) buffer for 1 h and incubated with the appropriate primary antibody overnight: anti-GADD153, anti-GRP94 (Santa Cruz), anti-GRP78 (Stressgen), anti-β-actin. After four cycles of washing with TBS-T buffer, the membranes were incubated with secondary antibody. Following four rounds of washing with TBS-T buffer, the immunoblot was visualized using an ECL system (Roche).

ELISA

After each sample was fixed in the plate, mouse IL-6 ELISAset (eBioscience) and anti-mouse IL-6 was sequentially added up to 100 μl/well, followed by incubation overnight at 4°C. Plates were then washed three times with PBST before addition of 1X Assay diluents and the diluted samples were further incubated for 1 h. At the termination of incubation, plates were washed three times with PBST and incubated with streptavidin-HRP diluted in 1X Assay diluents. When the plates were thoroughly washed again, TMB peroxidase substrate and 50 μl of 2N sulfuric acid were added to the wells to stop the enzyme reaction. The enzyme activity was detected

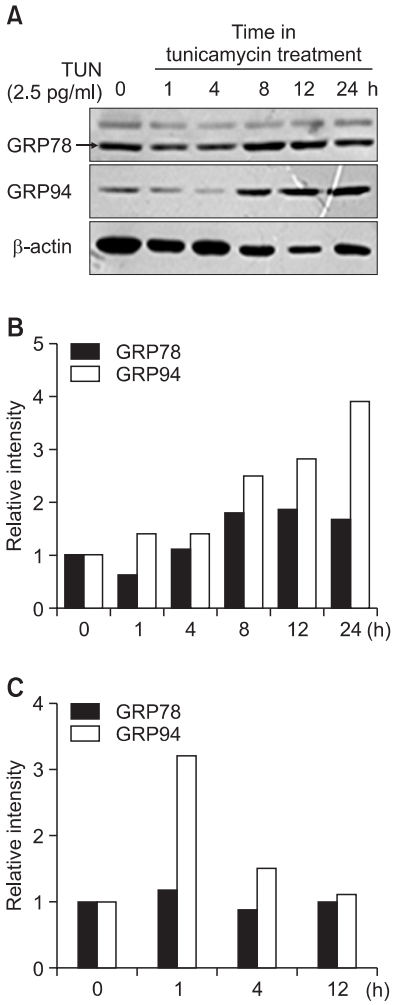

Fig. 1. Expression of molecular chaperones in macrophages treated with tunicamycin or LPS. Raw 264.7 cells were treated with tunicamycin for 0-24 h. Cell lysates were analyzed for the expression of GRP94 and GRP78 by western blotting (A). The expression of molecular chaperones was quantitated in (B). Relative intensity refers to the converted value of protein expression assuming the band intensity at time 0 as 1.0. (C) Raw 264.7 cells treated with LPS (100 ng/ml) for 0-12 h. The level of molecular chaperones was analyzed as in Fig. 1-(B).

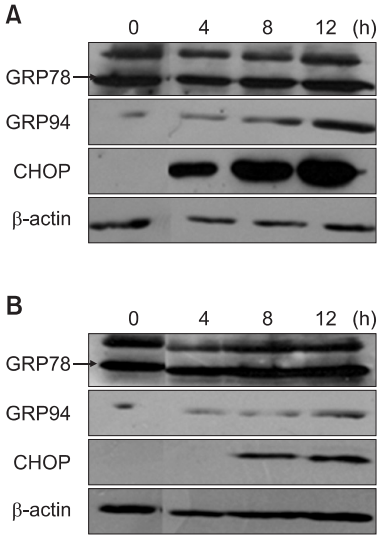

Fig. 2. Increased expression of GRP94 and CHOP in activated macrophages following tunicamycin treatment. Macrophages, activated by LPS, were treated by tunicamycin or BFA. The expression of molecular chaperones was detected by western blotting. (A) Raw 264.7 cells were pretreated with 100 ng/ml LPS for 1 h and further treated with 2.5 μg/ml tunicamycin from 1 to 12 h. Cell extracts were analyzed with western blotting using antibody to GRP94 and CHOP. (B) Raw 264.7 cells were pretreated with 100 ng/ml LPS for 1 h, and treated with 10 μg/ml BFA from 1 to 12 h. Cell extracts were analyzed with western blotting using antibody to GRP94 or CHOP, respectively.

at 450 nm using an ELISA reader (MQX200 μQunat, Bio-Tech Instruments, Inc.).

RESULTS

Endoplasmic reticulum stress in macrophage induced by TUN or LPS treatment

Raw 264.7 were treated with TUN at 2.5 μg/ml for 0-12 h. The cell lysates were analyzed for the expression of GRP94 and GRP78 via western blotting by antisera against each molecular chaperone. After the cell line was treated with TUN, GRP78 and GRP94 showed a similar expression pattern (Figs. 1A and 1B). The expression of GRP78 markedly increased after 8 h of treatment. Also, GRP94, anti-apoptotic factor and ER stress marker, expressed higher when the incubation period prolonged up to 8 h. The expression of CHOP was also measured; however, no significant level of expression was apparent after the treatment (data not shown). When cells were treated with LPS, the expression of molecular chaperones significantly increased (Fig. 1C). These results indicate that LPS and TUN cause ER stress when the two agents singularly applied to the macrophage cell. In other words, LPS-activated macrophages virtually experience a type of ER stress considering expression of ER stress markers induced by TUN.

TUN and BFA have different effect on macrophage cells in CHOP expression

Following activation by LPS, Macrophages were additionally treated by TUN or BFA. The expression of molecular chaperones was detected by western blotting. Fig. 2A shows

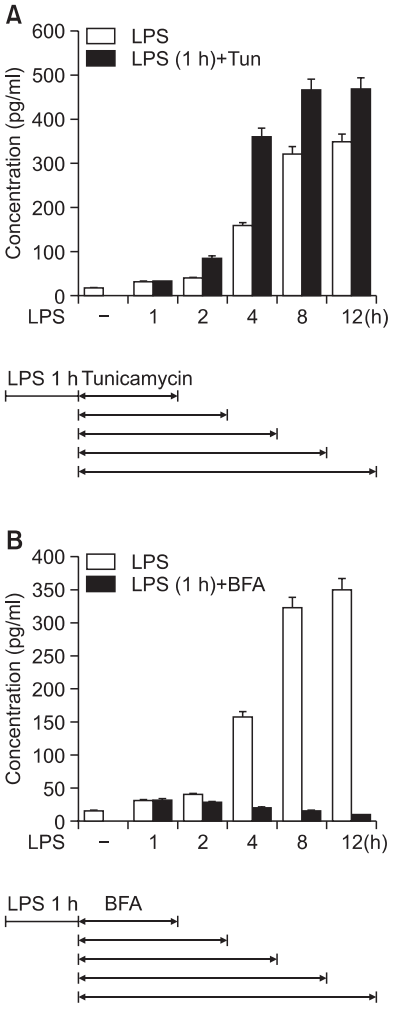

Fig. 3. IL-6 expression from macrophages treated with ER stress agents. Raw 264.7 cells were treated with LPS (100 ng/ml) for 1 h and further treated with 2.5 μg/ml tunicamycin or BFA for 1-12 h. After each treatment, protein samples were analyzed by ELISA for IL-6 expression. (A) Under the tunicamycin treatment, secretion of IL-6 was not affected; rather, the significantly increased. (B) This is different from other ER stress agents such as BFA.

that Raw 264.7 cells, treated with 100 ng/ml LPS for 1 h, were further treated with 2.5 μg/ml TUN up to 12 h. Cell extracts were analyzed by western blotting against GRP94 and CHOP. In Fig. 1, CHOP expression was not detected; however, a time-dependent expression of CHOP was obvious after TUN treatment of the activated macrophage. In Fig. 2B, a similar expression pattern was apparent in the LPS-activated Raw 264.7 cells which were further treated with BFA up to 12 h at 10 μg/ml. Expression of CHOP appeared lower than TUN-treated macrophage. Compared to TUN, BFA exerts a lower level of ER stress to the activated macrophage following the treatment. Although BFA is a strong inhibitor of protein trafficking in the endomembrane system of mammalian cells, it affects the activated macrophage to a less extent with respect to ER stress. As ER stress agent, TUN has a strong effect on the activated macrophage cells in terms of CHOP expression and significance of ER stress.

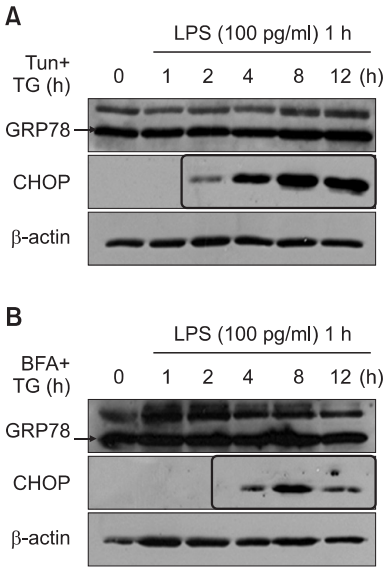

Fig. 4. Joint application of ER stress agent to activated macrophage. Pair of ER stress agents were applied to LPS-activated macrophage. (A) Macrophage cells were pretreated with 100 ng/ml LPS for 1 h, and treated with 2.5 μg/ml tunicamycin along with 2 μM TG from 1 to 12 h. Protein samples (40 μg/ml) after the joint treatment were analyzed by western blotting for CHOP expression (boxed in the middle panel). (B) the cells were activated with 100 ng/ml LPS for 1 h, and treated with 10 μg/ml BFA along with 2 μM TG from 1 to 12 h. Following the BFA treatment, protein samples (40 μg) were analyzed by western blotting especially for CHOP expression. Both in (A) and (B), the expression of CHOP appeared to be stimulated since the CHOP appeared as early as 2 hr of the joint treatment, different from treatment with a single agent shown in Fig. 2.

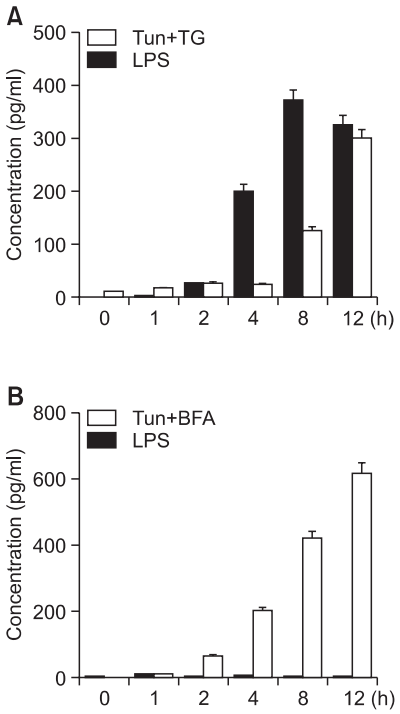

Fig. 5. Continued secretion of IL-6 from macrophage further treated with thapsigargin. After addition of additional ER stress agents, the secretion of IL-6 was monitored from the previously ER stressed macrophages. (A) Raw 264.7 cells were treated with 100 ng/ml LPS and with 2.5 μg/ml tunicamycin for 1 h. The cells were further treated with 2 μM TG for 1-12 h. Protein samples after TG treatment were analyzed by ELISA for IL-6 expression. Even in the presence of TG, IL-6 continues to secret more in quantity than LPS-only treatment. (B) The cells were treated with LPS and tunicamycin similarly as in (A). They were additionally treated with BFA from 1 h to 12 h. IL-6 secretion was effectively inhibited under BFA treatment.

TUN enhances secretion of IL-6 from macrophage cells

This segment of experiment focused on the effects of TUN on the activation of Raw 264.7 cells and production of IL-6 in Raw 264.7 activated by LPS. The cells were treated with 100 ng/ml LPS and half of the activated cells were treated further with TUN at 2.5 μg/ml concentration or BFA (10 μg/ml) for 1 to 12 h. When Raw 264.7 cells were pretreated with LPS, prior to TUN treatment, interleukin-6 (IL-6) expression was augmented according to the ELISA (Fig. 3A). In contrast, BFA was shown to inhibit the secretion of IL-6 from the activated Raw 264.7 cells (Fig. 3B). Considering the difference in IL-6 secretion, ER stress agents have different effects on macrophage cells.

In the presence of BFA, most IL-6 is retained in the cells without secretion. However, IL-6 was still detected in the supernatant (Fig. 3B). The secretion of IL-6 may be due to delayed onset of BFA effect for the short BFA treatments (e.g., 1-2 hr). For 4-12 hr treatment, however, the presence of IL-6 might result from cells which underwent membrane disintegration possibly because of apoptosis due to prolonged treatment with BFA.

Intense ER stress with further application of induction agents

Another ER stress agent was applied to LPS-activated macrophage which is also treated with TUN for ER stress. Macrophage cells were pretreated with 100 ng/ml LPS for 1 h, and treated with 2.5 μg/ml TUN along with 2 μM thapsigargin (TG) from 1 to 12 h. After the additional treatment with TG (40 μg/ml), samples were analyzed by western blotting for CHOP expression (*boxed in the middle panel, Fig. 4A*). Following treatment with LPS and BFA, the cells were treated with TG for 1-12 h. Following the BFA treatment, CHOP expression was analyzed by western blotting. Both in (A) and (B), the expression of CHOP appeared as early as 2 h of the joint treatment, different from treatment with a single agent shown in Fig. 2, where CHOP expression was evident only after 4 h of incubation with the two ER stress inducer.

Continued secretion of IL-6 from macrophage despite further treatment with TG

After Raw 264.7 cells were treated with LPS and TUN, the cell lines were additionally treated with 2 μM TG for 1-12 h. When IL-6 expression was analyzed by ELISA, TG was shown not to inhibit the release of IL-6 from the cell lines (Fig. 5A). The section of IL-6 was finally shown repressed within 2 hours of BFA treatment (Fig. 5B).

DISCUSSION

The present study characterized the effects of ER stress on activation of macrophage cell and on the activated macrophage cells. When treated with LPS or TUN, Raw 264.7 induces ER stress in each treatment and GRP78 and GRP94 show a similar expression pattern in macrophage cells. Raw 264.7 cells were treated with LPS and TUN, the activated macrophage cells shows a significant inflammatory response with upregulation of IL-6.

This study used three different lines of ER stress inducers. TUN interferes with protein N-glycosylation. BFA and TG inhibit the membrane development in ER to Golgi transport and the sarcoplasmic/endoplasmic Ca^{2+}-ATPase (SERCA) which regulates calcium influx from cytosol to ER, respectively (Shimoke *et al.*, 2005; Andreas *et al.*, 2002). Despite different induction mechanisms, the three agents caused a comparable level of ER stress as evidenced by the expression of ER stress markers. As a downstream response to the ER stress, the levels of IL-6 secretion are very different among the ER stress agents used in this study. Different from TUN, TG and BFA markedly decreased the secretion of IL-6. Even for combined use with TG, the secretion of IL-6 sustains comparable to that achieved by single treatment with TUN. This observation strongly implies the ER stress may exert a heterogeneous effect to the cells depending upon the source of stress.

Although a direct comparison cannot be made between TUN-only and TUN plus TG treatment, significant difference still exists in terms of IL-6 secretion especially at 4 and 12 h (Figs. 3A and 4A). Also, TG exerted a complete inhibition of IL-6 secretion following BFA treatment of the activated macrophage. Most likely, the difference might result from calcium deficiency in the lumen of ER, caused by TG. The effect of luminal calcium might be further characterized with a standardized quantitation of IL-6 secretion such as per cell number or protein mass.

The preventive action of TUN on LPS-induced macrophage activation might not be related to protein glycoslyation. BFA inhibited LPS-induced activation through preventing the secretion of inflammatory cytokines. In fact, this idea is supported by the result that BFA inhibits LPS-induced IL-6 expression. ER stress agents have different effect to macrophage cells in IL-6 and CHOP protein expression.

IL-6 is produced by a wide variety of cell types that includes fibroblasts, endothelial cells, monocytes/macrophages, endometrial stromal cells keratinocytes, and vascular smooth muscle cells. Under denaturing conditions, IL-6 is separated as various phosphoglycoproteins with molecular mass of 23-30 kDa (May *et al.*, 1991). Considering TUN specifically blocks N-glycosylation, the 25 kDa species may evade the TUN's inhibitory action and exit the ER with proper conformation. Among these polypeptides, the O-glycosylated species of IL-6 is the detected one in this study. This strongly indicates that protein with proper conformation may still exit the ER and eventually secret from the cell despite ER stress conditions. Also, the UPR response is not indiscriminative mode of action to the nascent polypeptides in the ER. The response may exert a selective effect of the peptides under biosynthesis in the lumen of the ER.

The levels of IL-6 secretion greatly varied among the ER stress agents used in this study. Activated macrophage cells still secreted IL-6 in the presence of TUN which had caused a significant level of ER stress. Different from TUN, TG and BFA strongly inhibited IL-6 secretion into the media. Inhibition by TG or BFA, however, could be negated by supplementing TUN. Under circumstances, ER stress agents may enhance IL-6 secretion from macrophage activated with LPS. ER stress agent can be applied as new immunologic adjuvants for vaccination.

When TG was supplemented to the LPS→BFA or LPS→TUN experiment, the CHOP was obviously induced more after short incubation periods (e.g., 1-2 hours). This suggests that TG may play a synergistic role in ER stress induction. Over the treatment with LPS and TUN, however, TG appeared to reduce CHOP expression for 4-12 hrs. Indeed, CHOP expression was much lower in LPS→TUN→TG than LPS→TUN treatment (Figs. 4A and 2A, respectively). TG may add a different level of stress depending on the length of incubation. Recent studies indicate that TG enhanced transcriptional activators to bind to IFNb-1 cis-elements despite its induction of ER stress due to the inhibition of calcium utilization (Martinon *et al.*, 2010; Zeng *et al.*, 2010). The twofold role of TG might exert different level of ER stress, as seen in this study, according to the length of incubation period.

In conclusion, the results in the present study strongly imply the ER stress agents may exert a different effect on intracellular trafficking of protein depending upon the type and duration of pre-existing stress. Further study is necessary to elucidate whether ER stress affects protein targeting machinery and secretion of nascent polypeptides on a priority basis.

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