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Inhibition of reactive oxygen species down-regulates protein synthesis in RAW 264.7

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ABSTRACT

In order to examine the endoplasmic reticulum responses in macrophages, we stimulate macrophage cell line RAW 264.7 by LPS. We found the phosphorylation of eukaryotic initiation factor eIF2α and the expression of ATF4, GADD34, and GADD153 in RAW 264.7 cells in late time by the relatively large amount of LPS stimulation. Unexpectedly LPS in the presence of ROS inhibitor *N*-acetyl-L-cysteine rapidly induced phosphorylation of eIF2α and induction of GADD34 expression. We measured intra-cytoplasmic TNFα production in LPS stimulated RAW 264.7 cells. TNFα production induced by LPS stimulation was greatly suppressed by *N*-acetyl-L-cysteine. This suppression occurred relatively early, which correlated with early eIF2α phosphorylation indicating ER stress mediated shutoff of protein synthesis.

Immune cells are subjected to various types of stresses during their responses to infection, and these in turn can induce endoplasmic reticulum (ER) stress. The UPR in immunology has been studied mainly in B cells. XBP-1 is the first transcription factor shown to be selectively and specifically required for the terminal differentiation of B lymphocytes to plasma cells. Interleukin (IL)-4 controls the transcription of XBP-1 and XBP-1 is involved in controlling the production of IL-6 [1]. In T cells, after priming via signals through the T cell receptor (TCR), ER stress response is required for the differentiation of naive T cells into cytokine-secreting effector cells [2]. We hypothesized that ER stress responses might be concerned with LPS-induced activation of macrophages. ER is the site of synthesis and folding of secreted, membrane-bound, and some organelle-targeted proteins. Stresses reduce protein folding capacity of the ER, which results in the accumulation and aggregation of unfolded proteins, a condition referred to as ER stress. To combat the deleterious effects of ER stress, cells have evolved various protective strategies, collectively termed the "unfolded protein response" (UPR) [3]. The UPR is a pro-survival response to reduce the accumulation of unfolded proteins and restore normal ER functions. However, if protein aggregation is persistent and the stress cannot be resolved, signaling switches from pro-survival to pro-apoptotic. Among the ER stress signals, we focused on the signaling through pancreatic ER kinase (PKR)-like ER kinase (PERK). PERK is a receptor of ER stress and activated upon accumulation of improperly folded proteins [4,5]. Activated PERK phosphorylates α-subunit of eukaryotic initiation factor (eIF) 2 on Ser^{51} [6]. Phosphorylation of eIF2α is known to reduce protein synthesis at the level of initiation [7]. This prevents recycling of eIF2 into its active GTP-bound form by the nucleotide exchanging factor eIF2B, thereby transiently inhibiting general mRNA translation [8]. Phosphorylation of eIF2α enables translation of activating transcription factor 4 (ATF4) [9]. ATF4 induces the transcription of genes required to restore ER homeostasis [10,11]. LPS is known to induce phagocytes to produce ROS [12,13], which results in the imbalance of oxidants/antioxidants and oxidative stress.. In this study, we examined whether ER stress responses were induced in macrophage cell lines RAW 264.7. We stimulated RAW 264.7 by LPS and examined the phosphorylation of eIF2α and the expression of other ER related molecules.

Materials and methods

Reagents and antibodies. We purchased LPS (*Escherichia coli* 0111:B4) from Sigma–Aldrich (St. Louis, MO). *N*-Acetyl-L-cysteine (NAC) (Sigma–Aldrich) was dissolved at 1 mol/L in deionized water and neutralized by titration with NaOH. The mAb phospho-eIF2α (Ser^{51}) was purchased from Cell Signaling (Danvers, MA). Other antibodies were obtained from Santa Cruz (Santa Cruz, CA).

Cell culture. RAW 264.7 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM (Sigma–Aldrich) supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2.

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Measurement of intracellular ROS accumulation. Flow cytometry was used to measure intracellular ROS accumulation with 5-(and 6-)-chloromethyl-2′,7′-dichilorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Invitrogen, Carlsbad, CA) as described previously [14]. RAW 264.7 cells were stimulated with LPS for the indicated time periods with and without pretreatment with 20 mM or 100 mM NAC for 2 h. CM-H_2DCFDA was added at 5 μM during the last 30 min at 37 °C in the dark. Fluorescence was analyzed by flow cytometry using a FACS Calibur (Becton Dickinson, Franklin Lakes, NJ) using excitation and emission wavelengths of 485 and 535 nm, respectively. Data were processed by using Cell Quest program (Becton Dickinson).

Measurement of intracellular tumor necrosis factor α (TNFα). Intracellular TNFα was stained using Intracellular Cytokine Staining Starter Kit-Mouse (BD Bioscience) according to the manufacturer's instruction. Briefly, RAW 264.7 cells were stimulated with LPS for the indicated periods with or without pretreatment of NAC, then the cells were harvested and washed in PBS, and resuspended in Stain buffer. The cells were fixed, permeabilized with Cytofix/Cytoperm buffer and then labeled with PE-conjugated TNFα Ab and isotype control Ab for 30 min at room temperature in the dark. Then, the cells were washed twice in Perm/Wash buffer, and resuspended in 500 μL of Stain buffer. Fluorescence was analyzed with the FACS Calibur and data were processed by using Cell Quest program.

Immunoblotting. Cells were lysed in sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 5% bromophenol blue). Then, 50 μg of total protein was subjected to 10% SDS–PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA) using Trans-Blot Semi-Dry (BIO RAD, Hercules, CA). Immunoblotting was performed with the appropriate antibody, developed by Enhanced Chemiluminescence (ECL) Western blotting Detection System Plus (GE Healthcare, Tokyo, Japan) as described previously [15].. Some blots were reproved with other antibodies after stripping in 62.5 mM Tris–HCl (pH 6.8)/100 mM β-mercaptoethanol/2% SDS.

Results

Induction of ER stresses by LPS stimulation to RAW 264 cells

We stimulated RAW 264.7 cells with LPS. We could detect phosphorylation of eIF2α by LPS stimulation in late time. Although 1 μg/ml LPS could induce phosphorylation of eIF2α significantly higher amount of LPS (100 μg/ml LPS) induced clear phosphorylation of eIF2α in RAW 264.7 cells in late time (Fig. 1). Thus in order to show clear results, we show here the results of the experiments using 100 μg/ml of LPS. Phosphorylation of eIF2α enables translation of activating transcription factor 4 (ATF4) [4,9]. ATF4 controls transcriptions of proteins, which are, contribute to preserve ER homeostasis in stress conditions [10,11]. We could detect ATF4, GADD34 and GADD153 expression (Fig. 1).

ROS inhibition induced ER stresses in early time

LPS induced ROS production in RAW 264.7 cells and the production of ROS was inhibited by NAC treatment (Fig. 2). On the contrary to our intensition, ROS inhibition induced ER stress. When we added NAC with the stimulation of RAW 264.7 by LPS, we found the rapidly induced phosphorylation of eIF2α. The phosphorylation of eIF2α lasted until 12 h, although the addition of NAC without LPS had no effects on eIF2α phosphorylation (Fig. 3A). The expression of GADD34 was induced by LPS in RAW 264.7 cells. When NAC was included with LPS, GADD34 expression was shifted to an earlier time (Fig. 3B).

ER stresses induced by ROS inhibition induced shutoff of TNFα synthesis

Because phosphorylation of eIF2α induces shutoff of protein synthesis, we measured intra-cytoplasmic TNF production in LPS stimulated RAW 264.7 cells. As shown in Fig. 4, TNFα production induced by LPS stimulation was greatly suppressed by NAC. This suppression occurred relatively early, which correlated with early eIF2α phosphorylation.

Discussion

We have shown in this study that high dosage of LPS induces ROS production and eIF2α phosphorylation (an indicator of ER stress) in RAW 264.7 cells at a late time (12–18 h) (Fig. 1). The induction of eIF2α phosphorylation requires a relatively large amount of LPS (>1 μg/mL). Lower amounts of LPS (100 ng/mL) induced only low levels of eIF2α phosphorylation (data not shown). It has been shown that LPS (150 μg/mL) plus interferon- (IFN-)γ (100 U/mL) induces GADD153 (CHOP) and ATF6 expression in RAW 264.7 cells [3]. LPS-induced phosphorylation of eIF2α is a sig-

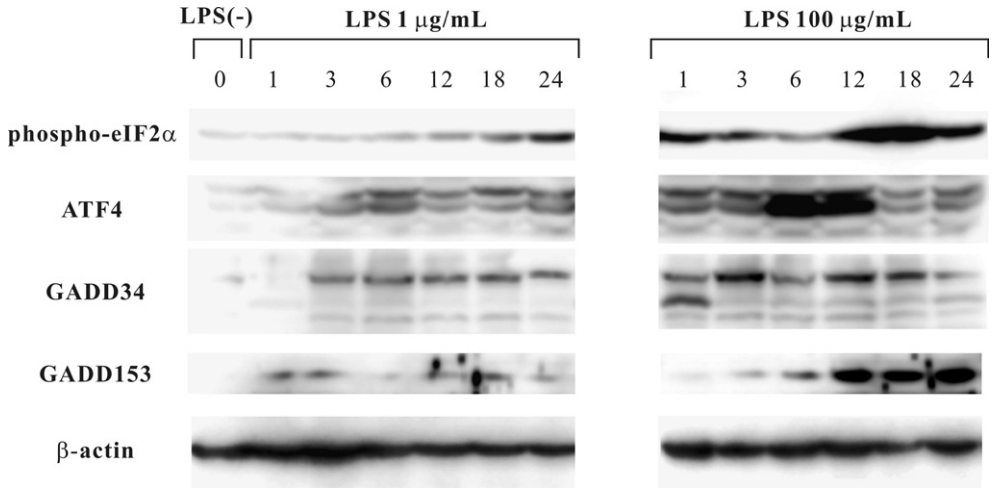

Fig. 1. The effects of LPS dosage on ER stress response in RAW 264.7 cells. RAW 264.7 cells were stimulated with LPS (1 μg/mL or 100 μg/mL) for the indicated time periods. Whole cell lysates were probed with the Ab specific for a phosphorylated form of eIF2α (Ser^{51}), ATF4, GADD34 or GADD153 and analyzed by Western blotting.

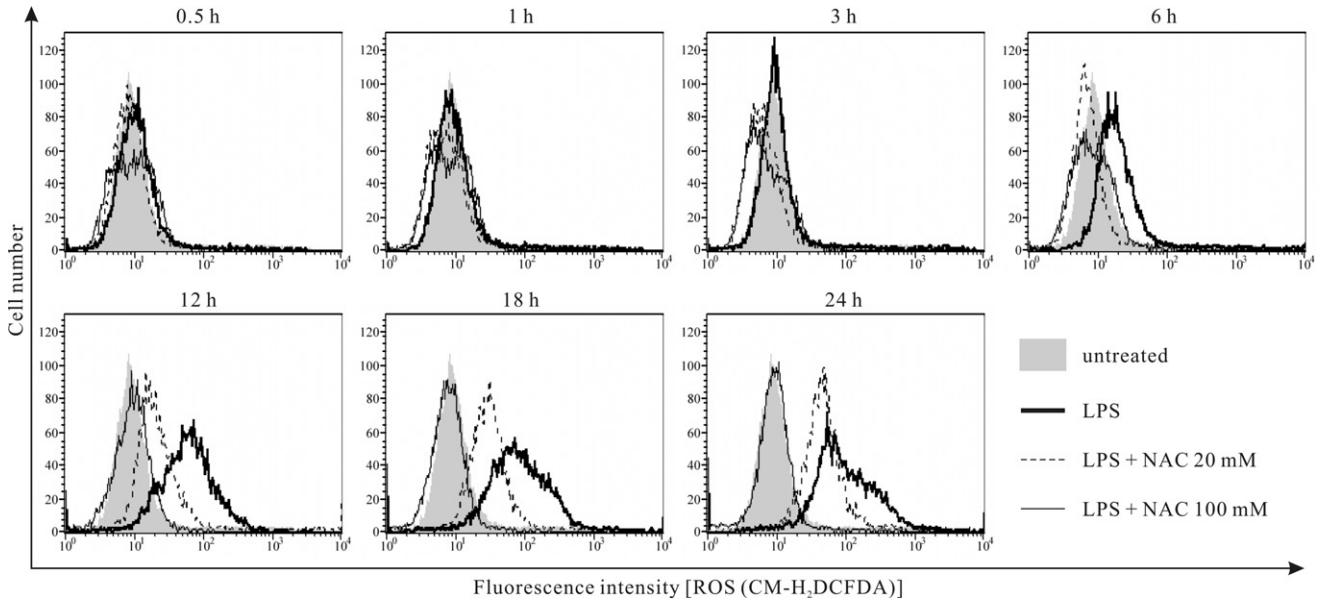

Fig. 2. ROS production induced by LPS in RAW 264.7 cells. RAW 264.7 cells were stimulated with LPS (100 μg/mL) for the indicated time periods with and without pretreatment of 20 mM or 100 mM NAC for 2 h, labeled with CM-H_2DCFDA for the last 30 min, and ROS accumulation was analyzed by flow cytometry and compared with unstimulated cells.

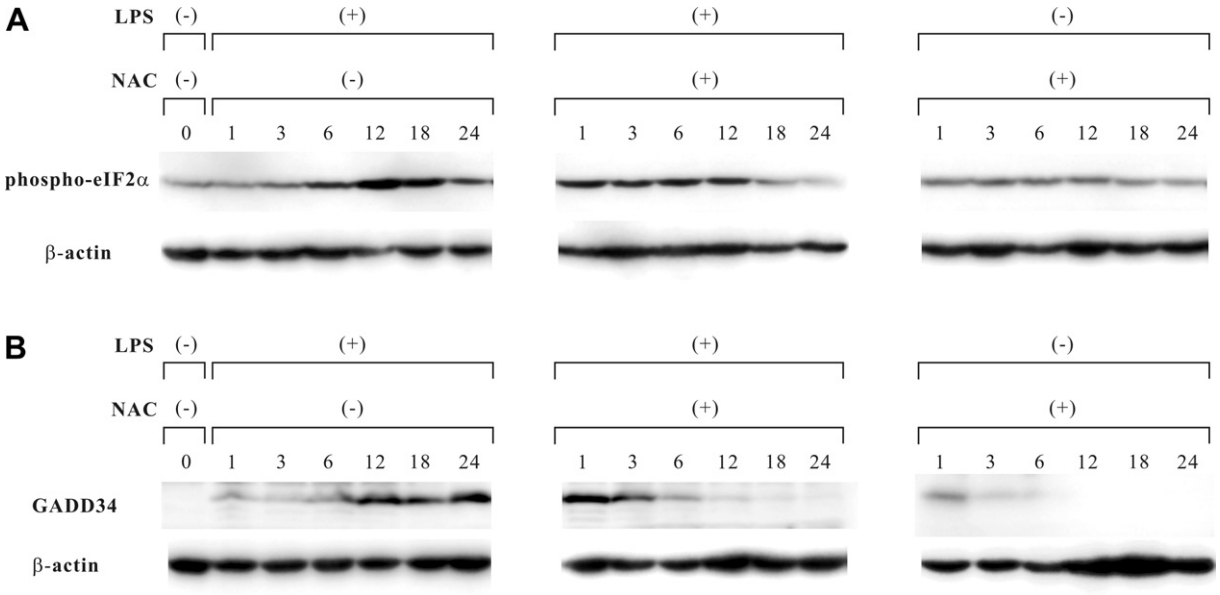

Fig. 3. Inhibition of ROS induces eIF2α phosphorylation in RAW 264.7 cells. (A) RAW 264.7 cells were stimulated with LPS (100 μg/mL) for the indicated time periods with and without pretreatment of 100 mM NAC for 2 h. Whole cell lysates were probed with the antibody specific for a phosphorylated form of eIF2α (Ser^{51}) and analyzed by Western blotting. To verify equal loading, the membranes were reprobed with anti-β-actin antibody. (B) RAW 264.7 cells were stimulated as described in (A). Whole cell lysates were probed with an antibody specific for GADD34, and analyzed as described in (A).

nificant observation because downstream molecules such as ATF4 and GADD34 were strongly induced at a late time after LPS stimulation of RAW 264.7 cells (Fig. 1).

Our primary finding is that inhibition of ROS production accelerates phosphorylation of eIF2α (Fig. 3A). Inhibition required LPS stimulation, because NAC administration alone failed to induce eIF2α phosphorylation (Fig. 3A).

We investigated the alteration of protein synthesis by ROS inhibition, focusing on expression of TNFα. Intracellular TNFα production was attenuated by NAC in RAW 264.7 cells (Fig. 4). Furthermore, production of ROS may evade eIF2α phosphorylation and thereby circumvent down-regulation of protein synthesis, permitting production of inflammatory cytokines. In T cells, effector cytokines such as IL-4 and IFN-γ are produced at very low levels after initial priming of naive T cells but are abundantly secreted after a second T cell receptor (TCR) stimulation. These phenomena were explained by ER stress response. It has been shown that primed T cells undergo phosphorylation of eIF2α, increased expression of stress-response genes and accumulation of cytoplasmic granules associated with RNA-binding proteins. Restimulation of the cells resulted in rapid eIF2α dephosphorylation, ribosomal mRNA loading and cytokine secretion [10].

We [16] and other group [17] have shown that GADD34 induces shutoff of protein synthesis, The ‘growth arrest and DNA

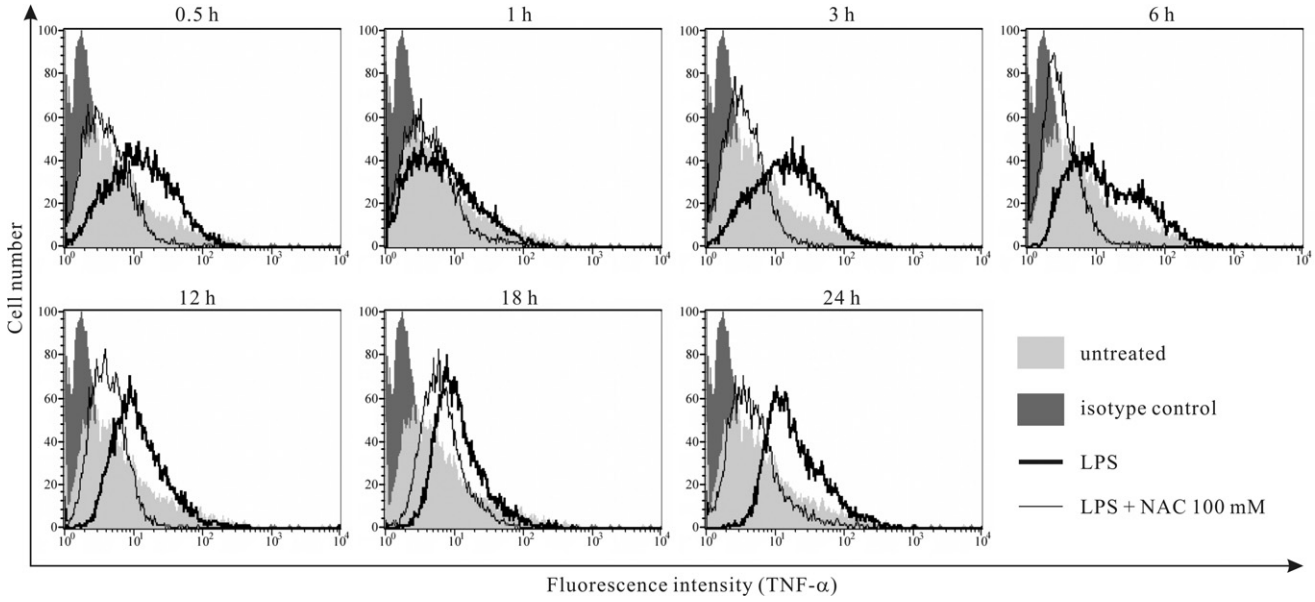

Fig. 4. Intracellular TNFα production in RAW 264.7 cells. RAW 264.7 cells were treated with LPS (100 μg/mL) for the indicated time periods with and without pretreatment with 100 mM NAC for 2 h. Collected cells were fixed, permeabilized, and then labeled with PE-conjugated TNFα antibody and isotype control antibody, and intracellular TNFα was analyzed by flow cytometry and compared with unstimulated cells.

damage' protein GADD34 (A002850) was initially reported to be induced by various types of cellular stress and DNA damage such as ultraviolet irradiation and unfolded proteins [18]. Here we showed that LPS induced GADD34 expression (Fig. 1). It is now known that GADD34 is a regulatory subunit of the protein phosphatase 1 (PP1) holoenzyme [19,20]. GADD34 together with PP1 has been shown to dephosphorylate eIF2α [21]. Recently, GADD34, which binds PP1, together with CUE domain-containing 2 (CUEDC2), dephosphorylates IKK and repressed activation of the transcription factor NF-κB. LPS stimulation of RAW 264.7 cells reduced the expression of NF-κB target gene [22]. Thus GADD34 may influence ER stress responses by affecting several points of signaling pathways.

Here we showed that bacterial product LPS can stimulate macrophages by producing ROS. Because inhibition of ROS by NAC down-regulate TNFα production by ER stress, ROS may be important for the production of inflammatory cytokines. Further works using macrophages or dendritic cells are needed to elucidate the role of ER stress or ROS in innate immunity.

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