Tumor Necrosis Factor α (TNFα) Induces the Unfolded Protein Response (UPR) in a Reactive Oxygen Species (ROS)-dependent Fashion, and the UPR Counteracts ROS Accumulation by TNFα*  

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Accumulation of unfolded proteins in the endoplasmic reticulum (ER) causes ER overload, resulting in ER stress. To cope with ER stress, mammalian cells trigger a specific response known as the unfolded protein response (UPR). Although recent studies have indicated cross-talk between ER stress and oxidative stress, the mechanistic link is not fully understood. By using murine fibrosarcoma L929 cells, in which tumor necrosis factor (TNF) α induces accumulation of reactive oxygen species (ROS) and cell death, we show that TNFα induces the UPR in a ROS-dependent fashion. In contrast to TNFα, oxidative stresses by H2O2 or arsenite only induce eukaryotic initiation factor 2α phosphorylation, but not activation of PERK- or IRE1-dependent pathways, indicating the specificity of downstream signaling induced by various oxidative stresses. Conversely, the UPR induced by tunicamycin substantially suppresses TNFα-induced ROS accumulation and cell death by inhibiting reduction of cellular glutathione levels. Collectively, some, but not all, oxidative stresses induce the UPR, and pre-emptive UPR counteracts TNFα-induced ROS accumulation.

Newly synthesized secretory and membrane-associated proteins are correctly folded and assembled in the endoplasmic reticulum (ER).2 Once ER function is perturbed by various pathological conditions, newly synthesized unfolded proteins accumulate in the ER, resulting in ER stress. To cope with accumulated unfolded ER proteins, mammalian cells trigger a specific response termed the unfolded protein response (UPR) (1–3). There are three distinct signaling pathways that are triggered in response to ER stress, mediated by PERK, ATF6, and IRE1. Under non-pathological conditions, all three components associate with the abundant luminal chaperon Bip (also known as glucose-regulated protein 78) and this interaction keeps these signaling molecules in an inactive state (4, 5). Once unfolded proteins accumulate in the ER, Bip preferentially associates with the unfolded proteins instead of PERK, ATF6, and IRE1, resulting in activation of their downstream signaling molecules. PERK is an ER-resident serine/threonine protein kinase that phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2α) (6). Phosphorylation of eIF2α subsequently inhibits protein synthesis to prevent further influx of nascent proteins into an already saturated ER lumen. Paradoxically, eIF2α phosphorylation induces translation of a transcription factor ATF4 and subsequent expression of the ATF4 target genes, GADD34 and CHOP (7). The second signaling pathway is mediated by the basic leucine zipper-type transcription factor, ATF6. ATF6 is synthesized as a type II transmembrane precursor protein with a molecular mass with 90 kDa (p90 ATF6), and anchored to the ER membrane where it is retained by Bip. In response to ER stress, ATF6 is released from Bip and transported to the Golgi complex (8), where ATF6 undergoes sequential cleavages by two proteases, S1P and S2P (9). The processed form of ATF6 (p50ATF6) translocates to the nucleus and binds to the ER stress responsive element (ERSE), and subsequently activates target genes. Many ER chaperons, including Bip, glucose-regulated protein 94, and calreticulin, contain an ERSE in their promoter regions and are induced by ATF6 (10). The third signaling pathway is composed of IRE1 and XBP1. IRE1 is a type I transmembrane protein containing a serine threonine kinase and ribonuclease domains. Under normal conditions, only the unspliced form of XBP1[XBPI(U)] mRNA is translated, but its product is a weak transcriptional activator with a short protein half-life. Upon ER stress, IRE1 is activated and cuts 26 nucleotides out from XBP1(U) mRNA to generate spliced XBP1[XBP1(S)] mRNA, which encodes the more stable and transcriptionally active XBP1(S) protein. XBP1(S) binds to the unfolded protein response element (UPRE) and activates target genes (11–13).

There are four known kinases, including PERK, PKR, GCN2, and HRI, that phosphorylate eIF2α (2, 14). PKR is activated by double-stranded RNA and phosphorylates eIF2α during viral infection. GCN2 and HRI play the equivalent role in amino acid-starved cells and heme-deprived reticulocytes, respectively. However, the kinase(s) responsible for phosphorylating eIF2α in response to oxidative stress remain to be identified (15). Interestingly, a recent study has revealed an intimate correlation between ER stress and accumulation of reactive oxygen spe-
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cies (ROS) (16). Whereas tunicamycin, an inhibitor of protein N-linked glycosylation, only weakly induced accumulation of ROS in wild-type cells, the same treatment induced marked accumulation of ROS in cells lacking PERK or ATF4. Furthermore, microarray analysis revealed that the PERK- and ATF4-dependent pathways induce genes associated with amino acid transport and reduced glutathione (GSH) biosynthesis, which might be responsible for elimination of ROS.

Although accumulating evidence has indicated cross-talk between oxidative stress and ER stress response pathways, its detailed molecular mechanism remains to be elucidated. By using murine fibrosarcoma L929 cells, in which TNFα induces ROS accumulation, we have investigated whether TNFα induces the UPR, and whether protective activation of the UPR induced by tunicamycin could affect ROS accumulation induced by TNFα. Here we show that TNFα, but not H2O2 or arsenite, induces the UPR in a ROS-dependent fashion, revealing the specificity of downstream signaling components induced by various oxidative stresses. Moreover, the UPR induction by pretreatment with tunicamycin substantially inhibits TNFα-induced ROS accumulation and cell death. Given that ER stress induces accumulation of ROS in PERK or ATF4 knockout (KO) cells to greater levels than wild-type cells (16), the UPR normally functions to counteract ROS accumulation induced by various stresses, including ER stress itself and TNFα, to protect cells from cell death.

EXPERIMENTAL PROCEDURES

Reagents and Cell Culture—Recombinant murine and human TNFα were purchased from BD Pharmingen. Antibodies (Abs) specific for phospho-eIF2α, total eIF2α, XBP1, Bip, and α-tubulin were purchased from Cell Signaling, Santa Cruz Biotechnology, Stressgen, and Sigma. Anti-PERK and anti-ATF6 Abs were described as previously (6, 10). Tunicamycin, butylated hydroxyanisole (BHA), sodium arsenite, 5- and 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA), and dihydroethidium (DHE) were purchased from Calbiochem, Wako Pure Chemicals, and Molecular Probes. L929 cells were cultured in RPMI 1640 containing 10% fetal calf serum. HeLa cells, wild-type, RelA KO, and TNF receptor-associated factor (TRAF)2 and TRAF5 double KO (DKO) murine embryonic fibroblasts (MEF) were cultured in high-glucose Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. The cells were plated in 6-well plates and stimulated with TNFα, H2O2, or arsenite for the indicated time periods. After stimulation, the cells were incubated with phenol red-free medium (Opti-MEM) containing 20 mM Hepes (pH 7.4), 120 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. The suspensions were collected by centrifugation at 15,000 × g for 5 min, and the supernatants were used as detergent-insoluble fractions. The equal amounts of protein (50 μg for the detergent-soluble fractions, and 100 μg for detergent-insoluble fractions) were subjected to SDS-PAGE and probed with anti-Bip Ab.

Northern Blot Analysis—Northern blot analysis was performed as described previously (17). L929 cells and wild-type MEFs were stimulated with TNFα or tunicamycin for the indicated time periods in the absence or presence of BHA. cDNA probes for GADD34, XBP1, and β-actin were prepared by RT-PCR, and 32P-labeled with Rediprime™ kit (Amersham Biosciences). After hybridization, radioactive signals were analyzed on BAS2500 (Fuji Photo Film) using Image Gauge software (Fuji) and normalized based on the signal of β-actin.

Analysis of XBP1 mRNA Splicing by IRE1—Recombinant human IRE1α and human IRE1β were transfected into L929 cells (3.5 × 105) or tunicamycin-stimulated L929 cells (4 × 105) by electroporation (Gene Pulsar II, Bio-Rad) at 300 V and 975 μF (18). After transfection, 12 h later, the cells were lysed in a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin. After centrifugation, cell lysates were subjected to 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were probed with Abs to phospho-eIF2α, total eIF2α, and XBP1. To confirm the equal loading of the lysates, the membranes were reprobed with anti-α-tubulin Ab. The membranes were developed with Enhanced Chemiluminescence (ECL) Western blotting Detection System Plus (Amersham Biosciences).

Measurement of ROS Accumulation—L929 (4 × 105) or tunicamycin-stimulated L929 cells (2 × 105) were plated in 6-well plates and stimulated with TNFα, H2O2, or arsenite for the indicated time periods. After stimulation, the cells were incubated with phenol red-free medium (Opti-MEM) containing 20 mM Hepes (pH 7.4), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 17.5 mM β-glycerophosphate, 20 mM NaF, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin, and 1 μg/ml leupeptin. The suspensions were collected by centrifugation at 15,000 × g for 5 min, and the supernatants were used as detergent-insoluble fractions. The equal amounts of protein (50 μg for the detergent-soluble fractions, and 100 μg for detergent-insoluble fractions) were subjected to SDS-PAGE and probed with anti-Bip Ab. cDNA probes for GADD34 and 32P-labeled with Rediprime™ kit (Amersham Biosciences). After hybridization, radioactive signals were analyzed on BAS2500 using Image Gauge software (Fuji) and normalized based on the signal of β-actin.

RNA Interference—Duplex siRNAs with two nucleotides overhang at the 3′-end of the sequence were purchased from Polyplus Transfection. The siRNAs were transfected into L929 cells (2 × 105) with Lipofectamine 2000 (Invitrogen) or Lipofectamine RNAiMax (Invitrogen) according to the manufacturer’s instructions. The siRNAs were transfected into L929 cells (2 × 105) with Lipofectamine RNAiMax (Invitrogen) according to the manufacturer’s instructions.
Qiagen. The target sequences were as follows: murine PERK, 5′-GGUAUGCCGGAAAGUUAA-3′; green fluorescent protein (GFP), 5′-GGCUAGCUCCAGGAGCACC-3′. L929 cells (3 × 10⁶) were transfected with the indicated siRNAs using a Nucleofector according to a manufacturer’s instructions (Amaxa). Transfection efficiency was determined by counting GFP-positive cells using flow cytometry after transfection with an expression vector for GFP.

Electron Microscopy—L929 cells (4 × 10⁵) were unstimulated or stimulated with TNFα or tunicamycin in the presence or absence of BHA for 6 h, and then serially fixed with 2% glutaraldehyde in phosphate-buffered saline for 2 h and then with 2% OsO₄ for 2 h before embedding in Epon 812. Thin sections were prepared using a MT-5000 ultramicrotome (Dupont Pharmaceuticals), stained with uranyl acetate followed by lead citrate, and then observed (×5,000 or 15,000) on a JEM1230 electron microscope (JEOL).

Measurement of GSH—Cellular GSH levels were measured by using a colorimetric assay kit (OxisResearch™) as previously described (19).

WST Assay—L929 (1 × 10⁶), RelA KO (5 × 10⁵), or DKO (5 × 10⁵) cells were plated in 96-well plates and untreated or pretreated with tunicamycin for 12 h. The cells were washed to remove residual tunicamycin, then stimulated with TNFα for an additional 16 h. Cell viability was determined by WST assay using a Cell Counting kit (Dojindo) as previously described (19).

Statistical Analysis—Statistical analysis was performed by Student’s t test. p value < 0.05 was considered to be significant.

RESULTS

TNFα Induces the UPR in L929 Cells—Treatment of cells with oxidative agents such as arsenite and heavy metal ions has been shown to induce oxidative stress and eIF2α phosphorylation (20). However, it is still controversial whether oxidative stresses induced by various agents elicit the UPR. Given that TNFα induces ROS accumulation in murine fibrosarcoma L929 cells (21), we first investigated whether TNFα induces eIF2α phosphorylation in L929 cells. We stimulated L929 cells with TNFα or tunicamycin for 2–10 h and examined eIF2α phosphorylation using Ab specific for the phosphorylated form of eIF2α. Phosphorylation of eIF2α was detected at 4 h and persisted up to 10 h after tunicamycin stimulation (Fig. 1A). Interestingly, eIF2α phosphorylation was also induced at 2 h and persisted up to 10 h after TNFα stimulation. Because eIF2α phosphorylation subsequently induces ATF4 and GADD34 (2), we next examined the induction of GADD34 mRNA after TNFα stimulation by Northern blot analysis. TNFα, as well as tunicamycin, induced GADD34 mRNA in L929 cells (Fig. 1B). Notably, eIF2α phosphorylation and the induction of GADD34 mRNAs after TNFα treatment were faster than those after tunicamycin treatment (Fig. 1, A and B). These results indicate that TNFα induces a pathway initiated by phosphorylation of eIF2α and followed by the up-regulation of GADD34.

In addition to eIF2α phosphorylation, ER stress activates the ATF6-dependent pathway that subsequently induces the expression of many genes containing the ERSE in the promoter regions, including Bip, calnexin, calreticulin, and XBP1 (10, 22). Upon ER stress, ATF6 is converted from a 90-kDa protein (p90ATF6) to a 50-kDa protein (p50ATF6), resulting in nuclear translocation. Thus, we examined whether TNFα induces p50ATF6 by Western blotting using anti-ATF6 Ab (22). Although the induction levels of p50ATF6 by TNFα were weaker than tunicamycin, TNFα substantially induced p50ATF6 with a peak at 4 h after stimulation (Fig. 1A). We then examined whether TNFα induces the expression of XBP1 mRNA by Northern blot analysis. As shown in Fig. 1B, TNFα as well as tunicamycin markedly increased the expression of XBP1 mRNA, indicating that TNFα also activates the ATF6-dependent pathway.

ER stress also activates the IRE1-dependent pathway (11–13). Activated IRE1 cuts out 26 nucleotides of the unspliced XBP1(U) mRNA to generate the spliced XBP1(S) mRNA, which encodes transcriptionally active XBP1(S) with a molecular mass of 50 kDa. The RT-PCR products produced from the spliced and unspliced XBP1 mRNA can be easily detected following PstI digestion of PCR products under “Experimental Procedures.” After digestion with PstI, the PCR products were subjected to 2% agarose gel electrophoresis. The PCR products of XBP1(S) mRNA remained intact (454 bp), whereas the PCR products of XBP1(U) mRNA were cut into two fragments of 289 and 191 bp, as indicated by the arrows.
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induces XBP1(S) protein expression by Western blotting using anti-XBP1 Ab. Consistent with the RT-PCR analysis, the kinetics of TNFα-induced expression of XBP1(S) were faster than those of tunicamycin induction (Fig. 1A). Combined together, these data indicate that TNFα treatment induces the full UPR in L929 cells.

The TNFα-induced UPR Is ROS-dependent—To investigate the contribution of ROS to TNFα-induced UPR in L929 cells, we examined the inhibitory effect of the antioxidant, BHA, on the TNFα-induced UPR. We previously demonstrated that BHA has a stronger antioxidant activity than N-acetyl-L-cysteine (19, 24). Indeed, BHA completely inhibited TNFα-induced ROS accumulation in L929 cells (Fig. 2A). We then examined the effect of BHA on TNFα-induced UPR in L929 cells. As shown in Fig. 2B, BHA significantly inhibited eIF2α phosphorylation and the up-regulation of XBP1(S). Moreover, BHA almost completely inhibited induction of GADD34 and XBP1 mRNAs (Fig. 2C). We next tested the inhibitory effect of BHA on the splicing of XBP1 mRNA. As shown in Fig. 2D, BHA significantly inhibited TNFα-induced disappearance of the unspliced XBP1 mRNA. These results indicate that TNFα-induced UPR in L929 cells is mediated by ROS.

In contrast to TNFα-induced UPR, the tunicamycin-induced UPR has both ROS-dependent and -independent components. Tunicamycin-induced eIF2α phosphorylation and induction of GADD34 mRNA were not significantly inhibited by BHA (Fig. 2, B and C). However, BHA weakly and transiently inhibited induction of XBP1 mRNA and protein expression, but not splicing of XBP1 mRNA (Fig. 2, B–D). These results suggest that antioxidants have little effect on preventing PERK and IRE1 activation, and a modest effect on inhibiting ATF6-dependent up-regulation of XBP1 mRNA.

We have previously shown that TNFα does not induce ROS accumulation in wild-type MEFs (19). Then, we therefore examined whether TNFα induces the UPR in wild-type MEFs. Whereas tunicamycin-induced phosphorylation of eIF2α (Fig. 2G), the expression of GADD34 and XBP1 mRNAs (Fig. 2E), splicing of XBP1 mRNA (Fig. 2F), XBP1(S) (Fig. 2G), and TNFα did not up-regulate any of these molecules in wild-type MEFs. These results substantiate that ROS play an essential role in inducing the UPR by TNFα.

TNFα Activates Promoters Containing the UPRE and ERSE in a ROS-dependent Fashion—Taken that TNFα induces the UPR in L929 cells in a ROS-dependent fashion, we next examined whether TNFα activates the promoter activities of ER stress-responsive genes. Overexpression of truncated XBP1(S) or p50ATF6 activates promoters containing an ER stress responsive element termed the UPRE, which was originally identified as a binding sequence of ATF6 in vitro (18). To examine whether TNFα up-regulates transcriptional activity of a promoter containing the UPRE, we transiently transfected L929 cells with the pGL3–5 × ATF6 plasmid containing 5 copies of the UPRE, and stimulated the cells with TNFα or tunicamycin. Consistent with the induction of XBP1(S) by TNFα (Fig. 1A), TNFα increased the reporter activity to 2.5-fold compared with unstimulated cells (Fig. 3A). Importantly, BHA treatment completely inhibited TNFα-induced 5 × ATF6 reporter gene inducers...
activity, indicating that ROS were essential for the UPRE-dependent transcriptional activation by TNFα. Interestingly, tunicamycin-induced 5 × ATF6 reporter gene activity was also partially inhibited by BHA (Fig. 3B), which is consistent with its partial inhibitory effect on the induction of XBP1S (Fig. 2B). We next examined whether TNFα activates the 5 × ATF6 reporter gene activity in other types of cells. Consistent with the finding that TNFα does not induce ROS accumulation in wild-type MEFs (19) or HeLa cells (data not shown), TNFα did not induce the 5 × ATF6 reporter gene activation in either wild-type MEFs (Fig. 3C) or HeLa cells (Fig. 3D). Furthermore, BHA treatment only marginally inhibited tunicamycin-induced 5 × ATF6 reporter gene activation in wild-type MEFs (Fig. 3C) and HeLa cells (Fig. 3D), indicating that the contribution of ROS to tunicamycin-induced UPR activation is variable among cell types.

In addition to the UPRE, expression of ER stress responsive genes, such as Bip and calreticulin, is regulated by another element, termed the ERSE. Thus, we next examined whether TNFα also activates the promoter activity of Bip (10). To this end, we transiently transfected L929 cells with pGL3-Bip (−132/+7) containing three ERSE sites and stimulated the cells with TNFα or tunicamycin. TNFα weakly, but substantially activated the Bip reporter gene activity, and this activation was inhibited by BHA (Fig. 3E). Similarly, tunicamycin-induced Bip reporter gene activity was also partially inhibited by BHA (Fig. 3E). In contrast, TNFα did not activate the Bip reporter gene activity and BHA did not inhibit tunicamycin-induced Bip reporter gene activity in HeLa cells (Fig. 3F). Collectively, these results reveal an intimate correlation between TNFα-induced ROS accumulation and activation of UPRE- and ERSE-dependent reporter gene expression.

**TNFα and Tunicamycin Induce Dilatation of the ER**—Given that TNFα induced the UPR in a ROS-dependent fashion, it was intriguing to examine whether TNFα induces morphological changes of the ER in L929 cells using electron microscopy. We treated L929 cells with TNFα or tunicamycin in the absence or presence of BHA for 6 h. In unstimulated cells, endoplasmic reticulum was recognized as long and thin cisternae surrounded by ribosomes observed as electron-dense dots (Fig. 4, B and C). TNFα induced prominent vacuolization in the cytoplasm without affecting nuclear morphology, consistent with necrotic cell death (Fig. 4, D–I). Taken that the dilated lumen was surrounded by electron-dense dots, the dilated organelles appeared to be the ER (Fig. 4, F and I). Similarly, tunicamycin also induced a modest dilatation of the ER (Fig. 4, M–O). Importantly, BHA treatment significantly inhibited dilatation of the ER in TNFα- and tunicamycin-treated cells (Fig. 4, J–L and P–R), indicating that ROS contribute to both TNFα- and tunicamycin-induced dilatation of the ER in L929 cells.

**PERK is an eIF2α Kinase That Responds to Oxidative Stress Induced by TNFα**—Although oxidative stresses induce eIF2α phosphorylation, the kinase(s) that phosphorylate eIF2α remain to be identified (15). Because PERK has been implicated in UPR-mediated eIF2α phosphorylation (6), we examined whether PERK is activated by TNFα in a ROS-dependent fashion in L929 cells. We previously showed that phosphorylated PERK (activated PERK) migrates more slowly compared with non-phosphorylated PERK in SDS-PAGE, thereby phosphorylated PERK can be discriminated from non-phosphorylated PERK by Western blotting (6). As shown in Fig. 5A, phosphorylation of PERK was progressively induced at 4–8 h after tunicamycin stimulation. Upon TNFα stimulation, phosphorylation of PERK was similarly induced at 2 h, peaked at 4 h, and then gradually decreased (Fig. 5A). Importantly, BHA treatment substantially inhibited TNFα-, but not tunicamycin-induced phosphorylation of PERK (Fig. 5B). These results suggest that TNFα induces a ROS-dependent ER stress that activates PERK.

To investigate whether PERK is indispensable for TNFα-induced phosphorylation of eIF2α, we knocked down expression of PERK by using siRNA against PERK. As shown in Fig. 5C, transfection efficiency was almost 90% based on the percentages of GFP-positive cells after transfection with a GFP expression vector. Under this condition, transfection of PERK siRNA, but not control GFP siRNA significantly reduced endogenous PERK protein levels (Fig. 5D). We then tested whether knockdown of PERK abolishes eIF2α phosphorylation induced by TNFα. As expected, tunicamycin-induced eIF2α phosphorylation was significantly reduced in PERK knockdown cells (Fig. 5E). However, TNFα-induced eIF2α phosphorylation was not impaired. This indicates that a kinase other than PERK is also involved in TNFα-induced eIF2α phosphorylation.

**TNFα Induces an Increase in Detergent-insoluble Bip**—A recent study showed that Bip accumulates in the detergent-insoluble complex in tunicamycin-treated cells and this accumulation is enhanced under oxidized cellular conditions (25). This suggests that oxidized cellular conditions enhance accumulation of Bip in...
The figure shows the effects of TNFα and tunicamycin on the ER of L929 cells. The panels depict the time course of PERK phosphorylation and Bip accumulation in detergent-soluble and -insoluble fractions. Sections A and D illustrate the increases in PERK phosphorylation and Bip accumulation, respectively, following stimulation with TNFα and tunicamycin. Panels B and C display the time course of Bip accumulation in detergent-soluble and -insoluble fractions, respectively, indicating the delay in Bip accumulation in the detergent-insoluble fraction compared to the detergent-soluble fraction. Panels E and F show the effects of BHA on PERK phosphorylation and Bip accumulation, demonstrating the inhibitory effect of BHA on the UPR. The scale bars represent 1 μm. The arrows indicate ER cisternae.
DHE, which are mainly oxidized to increase fluorescence by H$_2$O$_2$ and superoxide anions, respectively. We stimulated L929 cells with TNFα, H$_2$O$_2$, or arsenite, and analyzed the labeling with CM-H$_2$DCFDA or DHE by flow cytometry. As shown in Fig. 6F, TNFα, H$_2$O$_2$, and arsenite induced a substantial increase in fluorescent intensity of CM-H$_2$DCFDA. The increase of the fluorescent intensity of CM-H$_2$DCFDA induced by TNFα was significantly higher than those induced by H$_2$O$_2$ or arsenite. Notably, H$_2$O$_2$ and arsenite, but not TNFα, induced a substantial increase in fluorescent intensity of DHE (Fig. 6G). These results demonstrate both qualitative and quantitative differences of accumulated ROS induced by TNFα, H$_2$O$_2$, and arsenite, which might be responsible for the differential responses to these agents in L929 cells.

**UPR Activation Inhibits TNFα-induced ROS Accumulation and Cell Death**—To investigate the mechanism by which TNFα induces ROS accumulation in L929 cells, we examined cellular levels of the major antioxidants, GSH, before and after TNFα stimulation in wild-type MEFs and L929 cells. Whereas GSH levels were not significantly altered before and after TNFα stimulation in wild-type MEFs as we previously reported (19), TNFα induced substantial decreases in GSH levels in L929 cells (Fig. 7A). We previously showed that the UPR up-regulates genes associated with amino acid transport and GSH biosynthesis, which may be responsible for elimination of ROS under normal conditions (16). Thus, we next examined whether pretreatment of L929 cells with tunicamycin counteracts the TNFα-induced decrease in GSH levels. As expected, pretreatment of tunicamycin for 12 h almost completely inhibited the TNFα-induced decrease in GSH. These results prompted us to investigate whether tunicamycin pretreatment inhibits TNFα-induced ROS accumulation in L929 cells. After pretreatment of L929 cells with tunicamycin, the cells were then stimulated with TNFα for 4–12 h and ROS accumulation was analyzed by flow cytometry. As shown in Fig. 7B, accumulation of ROS was progressively observed at 4–12 h after TNFα stimulation in untreated L929 cells. Interestingly, pretreatment with tunicamycin substantially inhibited TNFα-induced ROS accumulation. Furthermore, tunicamycin pretreatment significantly increased the viability of L929 cells after TNFα stimulation compared with untreated cells (Fig. 7C). Collectively, these results indicate that the tunicamycin-induced UPR inhibits TNFα-induced ROS accumulation and cell death.

We previously showed that TNFα induces accumulation of ROS in NF-κB activation-deficient cells, such as RelA (a major component of the NF-κB complex), KO, or DKO MEFs (19). Although this NF-κB function is presumably mediated by up-regulating antioxidant enzymes (26–28), the detailed molecular mechanism is not fully understood. Given that pretreatment with tunicamycin inhibited TNFα-induced accumulation of ROS in L929 cells (Fig. 7B), it is interesting to test whether preemptive UPR also suppresses TNFα-induced ROS accumulation in RelA KO and DKO MEFs. Interestingly, pretreatment with tunicamycin substantially inhibited TNFα-induced ROS accumulation (Fig. 7D), and cell death also in these cells (Fig. 7E and F).

**DISCUSSION**

In the present study, we have demonstrated that TNFα induces the UPR, including PERK-mediated eIF2α phosphorylation, and ATF6- and IRE1-mediated induction of XBP1(S), in a ROS-dependent fashion. In contrast to TNFα, oxidative stresses by H$_2$O$_2$ and arsenite induced only
Although oxidative stresses induced by arsenite and heavy metal ions have been shown to induce eIF2α phosphorylation (20), it remained to be solved which of these oxidative stresses may induce the UPR. Moreover, the molecular link between ER stress and oxidative stress is largely unknown. We have shown that TNFα activates PERK- and IRE1-dependent pathways in a ROS-dependent fashion in L929 cells (Figs. 2 and 5). Activation of the three known signaling components of the UPR, including PERK, ATF6, and IRE1, depends on their dissociation of each molecule from the abundant ER chaperone Bip. It is likely that ROS may induce accumulation of malfolded proteins that subsequently form the complex with Bip, resulting in translocation of the Bip-containing complex in the detergent-insoluble fractions (Fig. 5F). This translocation of Bip induces the dissociation of Bip from the PERK, ATF6, and IRE1, and ultimately elicits the UPR.

It is noteworthy that H2O2 or arsenite did not induce similar responses despite inducing eIF2α phosphorylation. Together, these results indicate that qualitative difference exists in the signaling cascades induced by various oxidative stresses. Given that H2O2 and arsenite, but not TNFα, induced accumulation of superoxide anions (Fig. 6G), accumulated superoxide anions might directly activate another eIF2α kinase other than PERK. Alternatively, endogenously and exogenously produced ROS might be qualitatively different. For example, ROS generated in the cells may come from mitochondria, ER, or peroxisome and specifically damage proteins in those subcellular compartments. However, there is currently no reliable way to determine the subcellular location of ROS generation using oxidation-sensitive dyes. Thus it is crucial to develop such detection systems for better understanding of the signaling specificity induced by various oxidative stresses.

Although TNFα-induced phosphorylation of PERK in a ROS-dependent fashion (Fig. 5, A and B), knockdown of endogenous PERK using siRNA did not inhibit TNFα-induced eIF2α phosphorylation (Fig. 5E). This indicates that a kinase other than PERK also phosphorylates eIF2α in response to TNFα-induced oxidative stress. Interestingly, a previous study showed that PKR responds to TNFα and phosphorylates eIF2α in NIH3T3 cells (29). Therefore, PERK and PKR may act redundantly as eIF2α kinases in response to TNFα-induced oxidative stress. Nevertheless, it is still possible that a kinase other than PERK or PKR also phosphorylates eIF2α. A further study will be required to address this issue.

Our present electron microscopic analysis has shown that TNFα induces dilatation of the ER in L929 cells (20). These results are reminiscent of a previous study in which ER dilatation was observed in pancreatic β cells in PERK knock-out mice (30). Similarly, tunicamycin induced mild dilatation of the ER (Fig. 4, M–O). In contrast to TNFα, which induced marked accumulation of ROS in L929 cells (Fig. 2A), tunicamycin (2 μg/ml) did not induce apparent accumulation of ROS (data not shown). However, a higher dose of tunicamycin (10 μg/ml) did induce accumulation of ROS at a detectable level (data not shown). These results suggest that the UPR normally functions to prevent ROS accumulation, however, the antioxidant activity induced by the UPR is overwhelmed upon severe ER stress, resulting in ROS accumulation. These data suggest that a small amount of ROS under detection levels may contribute to tunicamycin-induced dilatation of ER in L929 cells.

We previously showed that TNFα induces a reduction of cellular GSH levels, which might be responsible for accumulation of ROS, in RelA KO and DKO MEFs (19). A similar reduction of GSH levels was observed in L929 cells upon TNFα stimulation (Fig. 7A). Given that TNFα-induced NF-κB activation is impaired in RelA KO and DKO MEFs, these results indicate that some NF-κB-dependent pathways

eIF2α phosphorylation, but not activation of PERK- or IRE1-dependent pathways. Collectively, these results indicate that different oxidative stresses specify activation of different downstream signaling pathways. Furthermore, pretreatment with tunicamycin significantly inhibited TNFα-induced ROS accumulation and cell death in L929 cells, RelA KO, and DKO MEFs, indicating that gene(s) induced by the UPR also prevent ROS accumulation by TNFα.
prevent ROS accumulation. Consistent with this notion, we recently showed that TNFα induces various antioxidants in an NF-κB-dependent fashion (31). On the other hand, we showed that the PERK- and ATF4-dependent pathways induce several genes associated with amino acid transport and glutathione biosynthesis, which are responsible for elimination of ROS (16). Intriguingly, pretreatment with tunicamycin substantially inhibited ROS accumulation induced by TNFα in L929 cells, RelA KO, and DKO MEFs (Fig. 7, B and D). Consequently, ROS-mediated cell death was significantly inhibited by tunicamycin pretreatment in these cells (Fig. 7, C, E, and F). Collectively, these results suggest that the NF-κB- and UPR-dependent pathways have a similar function to suppress ROS accumulation. It remains to be determined whether the target genes are overlapped between these two pathways. Identification of the molecule(s) induced by NF-κB or the UPR that inhibit ROS accumulation is crucial for understanding and manipulating the pathological conditions, in which ROS are critically involved.

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