Lead Induces the Expression of Endoplasmic Reticulum Chaperones GRP78 and GRP94 in Vascular Endothelial Cells via the JNK-AP-1 Pathway

Yasuhiro Shinkai,* Chika Yamamoto,*† and Toshiyuki Kaji*††
*Organization for Frontier Research in Preventive Pharmaceutical Sciences and †Department of Environmental Health, Faculty of Pharmaceutical Sciences, Hokuriku University, Kanazawa, Ishikawa 920-1181, Japan

1 To whom correspondence should be addressed at Department of Environmental Health, Faculty of Pharmaceutical Sciences, Hokuriku University, Ho-3 Kanagawa-machi, Kanazawa, Ishikawa 920-1181, Japan. Fax: +81-76-229-6208. E-mail: t-kaji@hokuriku-u.ac.jp.

Received September 4, 2009; accepted January 5, 2010

Lead, a ubiquitous heavy metal, is an important industrial and environmental pollutant that can target the vascular endothelium. To clarify the effects of lead on the unfolded protein response (UPR) and their significance in cytotoxicity, we examined the expression and function of endoplasmic reticulum (ER) chaperones glucose-regulated protein 78 (GRP78) and glucose-regulated protein 94 (GRP94) in vascular endothelial cells. We used bovine aortic endothelial cells as an in vitro model of the vascular endothelium. Exposure of vascular endothelial cells to lead nitrate resulted in a marked induction of GRP78 and GRP94 messenger RNA levels. In response to lead, the expression of GRP78 and GRP94 proteins also significantly increased in a dose- and time-dependent manner. In addition, small interfering RNA (siRNA)-mediated knockdown of GRP78 significantly enhanced lead-induced cytotoxicity. Compared with other metal(loid)s, including cadmium chloride, zinc sulfate, copper sulfate, and sodium arsenite, lead nitrate was found to be the most potent metal to induce these chaperones in endothelial cells. In the examined UPR pathways, lead increased the phosphorylation of inositol-requiring enzyme 1 (IRE1) and c-jun N-terminal kinase (JNK). Interestingly, the lead-induced upregulation of GRP78 and GRP94 was almost completely blocked by the JNK inhibitor SP600125 or activator protein-1 (AP-1) inhibitor curcumin. Taken together, these results suggest that lead induces ER stress, but the induction of GRP78 and GRP94 expression via the JNK-AP-1 pathway functions as a defense mechanism against lead-induced cytotoxicity in vascular endothelial cells.

Key Words: lead; endothelial cells; ER stress; GRP78; GRP94; JNK.

Lead is a ubiquitous heavy metal that causes numerous acute and chronic diseases; for example, lead exposure from environmental and occupational sources can result in disorders of the nervous and vascular systems and renal dysfunction (Patrick, 2006b). In particular, lead has been shown to be a risk factor for vascular disorders such as hypertension and atherosclerosis in epidemiological and experimental studies (Prozialeck et al., 2008; Vaziri, 2008). Blood lead concentration has been reported to increase to up to 5µM in workers exposed to lead (Tomokuni et al., 1993). Although we previously demonstrated that lead causes functional damage to cultured endothelial cells (Kaji, 2004), the molecular mechanisms underlying cellular adaptive responses to lead toxicity are not completely understood.

The endoplasmic reticulum (ER) is a multifunctional signaling organelle that controls a wide range of cellular processes (Berridge, 2002). The ER plays a critical role in protein folding, handling of misfolded proteins, posttranslational modification, delivery of proteins to their final destination, and calcium homeostasis. The accumulation of unfolded proteins in the ER induces ER stress and triggers a coordinated adaptive program called unfolded protein response (UPR). Reactive oxygen species are induced by misfolded proteins in the ER, leading to the activation of UPR (Malhotra et al., 2008). The UPR alleviates ER stress by decreasing protein synthesis and upregulating both the protein folding and the degradation pathways, leading to cellular recovery (Mori, 2000; Shen et al., 2004). Some of the major downstream targets of UPRs are glucose-regulated protein 78 (GRP78, also known as BiP) and glucose-regulated protein 94 (GRP94), which are ER-resident molecular chaperones that play important roles in protein folding/assembly and ER calcium binding (Ni and Lee, 2007). GRP78 and GRP94 are members of the family of cytosolic chaperones, heat-shock protein 70 and 90, respectively. Disruption of ER function by glucose starvation, disorders of calcium homeostasis, inhibition of protein glycosylation, and inhibition of protein disulfide bond formation leads to a marked induction of GRP78 and GRP94 expression (Lee, 2001). Therefore, the induction of these chaperones, especially GRP78, is not only critical for cell survival under such stress conditions but also widely used as a marker of ER stress.

Heavy metals such as lead, cadmium, and mercury have been shown to be potent protein folding inhibitors in vitro (Sharma et al., 2008). Several studies have also shown that these heavy metals inhibit Ca2+-ATPase (Hechtenberg and
Beyersmann, 1991; Mas-Oliva, 1989; Sandhir and Gill, 1994). Lead is known to specifically bind to GRP78 (Qian et al., 2000, 2007) and induce GRP78 aggregation within human CCF-STTG1 astrocytoma cells (Qian et al., 2005; White et al., 2007). These results suggest that lead can disrupt ER function via multiple mechanisms. In fact, lead induces the expression of GRP78 in rat glioma cells, primary astroglia cells, proximal tubular cells, and HepG2 cells but not in porcine kidney epithelial LLC-PK1 cells (Liu et al., 2006; Qian et al., 2000, 2001; Qian and Tiffany-Castiglioni, 2003; Stacchiotti et al., 2009; Tully et al., 2000), indicating that lead can induce ER stress but the susceptibility may be dependent on the cell type.

However, little is known about heavy metal–induced ER stress in the vascular endothelium. The purpose of the present study was to determine (1) whether lead induces ER chaperones GRP78 and GRP94 as an indicator of ER stress in the vascular endothelium, (2) whether GRP78 acts as a defense mechanism against lead cytotoxicity in the endothelium, and (3) which UPR pathway(s) is involved in lead-induced GRP78 and GRP94 expression, using a culture system of bovine aortic endothelial cells (BAECs) as an in vitro model of the vascular endothelium. We choose the BAEC model because it is a commonly used and well-characterized model for studying metal toxicology.

MATERIALS AND METHODS

Materials. Lead nitrate, zinc sulfate, copper sulfate, and lactate dehydrogenase (LDH) were purchased from Wako (Osaka, Japan). Cadmium chloride, thapsigargin, and dimethyl sulfoxide (DMSO) were obtained from Nacalai Tesque (Kyoto, Japan). Sodium arsenite, tunicamycin, SP600125, and rabbit anti-actin polyclonal antibody were purchased from Sigma-Aldrich (St Louis, MO). Mouse anti-GRP78 monoclonal antibody was obtained from BD Biosciences (San Jose, CA). Rabbit anti-GRP94 polyclonal antibody was obtained from Stressgen (Victoria, Canada). Rabbit anti-phospho-insulin-requiring enzyme 1 (IRE1α) polyclonal antibody was purchased from Novus Biologicals (Littleton, CO). Rabbit polyclonal antibody against activating transcription factor 2 (ATF2) (H-280), X-box binding protein 1 (XBP1) (M-186), IRE1α (H-190), and c-jun N-terminal kinase (JNK) (FL) and mouse monoclonal antibody against phospho-JNK (G-7) and C/EBP homologous protein (CHOP) (B-3) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA). All other reagents and chemicals were of the highest grade available.

Cell culture and treatment. BAECs were obtained from Cell Systems (Kirkland, WA). Cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C using Dulbecco’s modified Eagle’s medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Confluent cultures were used for all experiments. Stock solutions were prepared by dissolving each metal compound in sterilized deionized water. Just before treatment, the culture medium was replaced with serum-free medium, and cells were treated with each chemical. Untreated control cells were incubated with serum-free medium alone. Thapsigargin, tunicamycin, SP600125, and curcumin were dissolved in DMSO. For inhibitor treatment, cells were preincubated with a JNK inhibitor SP600125 or activator protein-1 (AP-1) inhibitor curcumin for 30 min in serum-free medium, exposed to lead nitrate, and maintained in serum-free medium throughout the additional 24-h incubation.

Real-time PCR. Total RNA was extracted using the RNeasy Lipid Tissue Mini kit (Qiagen, Valencia, CA), and complementary DNA (cDNA) was synthesized from the messenger RNA (mRNA) using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster, CA). Real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) with 1 μg cDNA and 0.2 μM primers (Table 1) on a 7500 Real-Time PCR system (Applied Biosystems). Thermal cycling parameters were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s, and 60°C for 1 min. Melting curve analysis and agarose gel electrophoresis with ethidium bromide staining was conducted to ensure a single PCR product of correct amplicon length. Levels of GRP78, GRP94, activating transcription factor 4 (ATF4), and glyceroldehyde 3-phosphate dehydrogenase (GAPDH) mRNA in each RNA sample were quantified by the relative standard curve method. Fold change for each gene was assessed after normalization of the intensity value to GAPDH.

Western blotting. After treatment, cells were washed twice with ice-cold PBS (–). Total cell proteins were prepared by lysis in SDS sample buffer (50mM Tris-HCl, pH 6.8; 2% SDS; and 10% glycerol), followed by incubation at 95°C for 10 min. We determined protein concentration using a BCA protein assay reagent kit (Pierce) before 2-mercaptoethanol and bromophenol blue was added to each sample. The cellular proteins were separated by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and electrotransferred onto a polyvinyl difluoride membrane (Bio-Rad, Hercules, CA) at 2 mA/cm² for 1 h according to the method of Kyhse-Andersen (1984). Membranes were blocked with 5% skim milk in (20mM Tris-HCl, pH 7.5; 150mM NaCl; and 0.1% Tween 20) and then incubated with primary antibodies for 1 h at room temperature. The membranes were washed and then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (Chemi-Lumi One L; nacalai) and scanned by LAS 3000 (Fujifilm, Tokyo, Japan); The bands were quantified using ImageJ software, and the density of each band was normalized to that of actin. Representative blots are shown from three independent experiments.

siRNA transfection. Synthetic small interfering RNAs (siRNAs) were purchased from Hayashi Kasei (Osaka, Japan), and transient transfection of siRNAs was performed using RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Briefly, cells were grown to ~50% confluence in a 6-well or 24-well plate and then 3 or 0.6 μl of siRNA duplex (20nM annealed duplex) was mixed with OPTI-MEM (Invitrogen), respectively. In a separate tube, 4 or 0.8 μl of RNAiMAX per reaction mixture was added to OPTI-MEM and incubated for 5 min at room temperature. Both the solutions were mixed and incubated for an additional 20 min at room temperature to allow the formation of complexes. The solutions were then

## TABLE 1
Bovine Gene-Specific Primers for Quantitative Real-Time Reverse Transcription-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’ → 3’)</th>
<th>Reverse primer (5’ → 3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRP78</td>
<td>GCATCGACCTGGGTACACCTTA</td>
<td>CCCTTCAGAGGTAAAGGCAACA</td>
<td>122</td>
</tr>
<tr>
<td>GRP94</td>
<td>AAGAACCCTCAGCTGATGTCACAGA</td>
<td>TGGCCATCTCCTTGCGCTGCA</td>
<td>134</td>
</tr>
<tr>
<td>ATF4</td>
<td>CTGGAGAGAAGATGGTAGCAGCAA</td>
<td>GCCCTCTCCTTGCGGCTGGA</td>
<td>112</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AAACCCCTCAAGAGTGTGAGCA</td>
<td>ACAGTCTTCTGGTGAGGCA</td>
<td>140</td>
</tr>
</tbody>
</table>
addicted to the cells. The sequences of the sense and antisense strands of the siRNAs were as follows: bovine GRP78 siRNA-1, 5′-GGCCACUAAUGGA-GAUACUdTdT-3′ (sense) and 5′-AGUAUCUCCAUAGUGGCCAC-3′ (antisense); bovine GRP78 siRNA-2, 5′-CUCUCUGUGUAUCAC-GAUADdTdT-3′ (sense) and 5′-UAUCUGUACCCAGAGGAGCA-3′ (antisense); and bovine ATF6 siRNA, 5′-CAGCUACCAUAAACAAdTdT-3′ (sense) and 5′-UUGUAAUGGUGGUAGCUGU-3′ (antisense). Nonspecific sequences were used as the siRNA negative control (Qiagen).

Caspase-3/7 activity. Caspase-3/7 activity was measured with Caspase-Glo 3/7 assay kit (Promega, Madison, WI) according to the manufacturer’s protocol. Briefly, cells were seeded in a six-well plate. After incubation, cells were lysed in a cell lysis buffer (20mM Tris-HCl, pH 7.5; 150mM NaCl; 2.5mM sodium pyrophosphate; 1mM EDTA; 1mM sodium orthovanadate; and 1% Triton) and sonicated for eight cycles (30-s pulse and 30-s rest on ice) using a Bioruptor (CosmoBio, Tokyo, Japan). The cells were then centrifuged, and the supernatant was collected. Each sample was normalized to a protein content of 20 μg and then mixed with Caspase-Glo 3/7 reagent for 6 min at room temperature. Luciferase activity was measured with a luminometer (Glomax 20/20; Promega).

Assay of cytotoxicity. Cells were seeded in a 24-well plate. After incubation, the conditioned medium was harvested, and an aliquot of the medium was used for the assay of LDH activity. The LDH leakage assay was performed using the CytoTox Non-radioactive Cytotoxicity Assay kit (Promega) with adding slight modification to the manufacturer’s instructions. Briefly, 40 μl of conditioned medium or LDH standard solutions were mixed with 40 μl of substrate mix solution and incubated at room temperature for 30 min in a fresh 96-well plate. Next, 40 μl of stop solution was added, and the absorbance was measured at 492 nm using a Multiskan plate reader (Thermo Scientific, Waltham, MA).

Cell stain. For morphological observation, Giemsa stain was used. Briefly, cells were grown in a 24-well plate and exposed to lead nitrate. The cell layer was washed with PBS(–), fixed with 0.25 ml of May-Grunwald stain solution (Wako) for 3 min, and stained with 0.5 ml of Giemsa stain solution (Wako) for 30 min at room temperature.

Statistical analysis. Statistical analysis was performed using Microsoft Excel (Microsoft, Redmond, WA). Statistical significance was assessed with Student’s t-test, and p < 0.05 was considered significant.

RESULTS

We first examined the effect of lead on the gene expression of GRP78 and GRP94, markers of ER stress, in BAECs. As shown in Figure 1, real-time PCR analysis showed that the expression of GRP78 and GRP94 mRNA increased significantly in response to lead (2–25μM) in a dose-dependent manner. Exposure of the cells to lead also resulted in a marked increase in the expression of both GRP78 and GRP94 proteins in a dose-dependent manner (Fig. 2A). When cells were incubated with 10μM lead for less than or equal to 24 h, GRP78 and GRP94 levels increased in a time-dependent manner (Fig. 2B). Incubation with lead (2–25μM) for 24 h did not increase the release of LDH, a marker of cell damage, in BAECs (data not shown). These results suggest that lead induces ER stress but not non-specific cell damage; induction of GRP78 and GRP94 would function as a defense mechanism against ER stress in BAECs exposed to lead.

To examine whether GRP78, which is markedly induced by lead, plays an important role in protection against lead cytotoxicity in BAECs, we designed bovine-specific siRNA and performed siRNA-mediated knockdown of GRP78 and then tested the susceptibility of the cells to lead cytotoxicity. As shown in Figure 3A, transfection of GRP78 siRNA-1 and siRNA-2 reduced constitutive and inducible GRP78 expression. Control or GRP78 siRNA–transfected cells were treated with lead nitrate and then the activity of caspase-3/7, an earlier marker of apoptosis, and cytotoxicity were assessed. Under the lower exposure conditions (10 or 25μM Pb), knockdown of GRP78 caused an increase in caspase-3/7 activity (Fig. 3B); however, no apparent cell damage was observed as evaluated by LDH release. This could be possibly because of the induction of GRP94 by the knockdown of GRP78 alone (data not shown). To assess the protective role of GRP78 against lead cytotoxicity, the higher exposure conditions (50 or 100μM Pb) were tested. The results of our analysis revealed that lead did not induce non-specific cell damage in the control siRNA–transfected cells, whereas knockdown of GRP78 resulted in a significant increase in the susceptibility to lead cytotoxicity.
when evaluated by the LDH release assay (Fig. 3C). Consistent with this result, the morphological observation revealed that the knockdown of GRP78 resulted in a breakdown of endothelial monolayer in the presence of lead (Fig. 3D).

Of the metal(loid)s examined, lead was found to be the most potent inducer of GRP78 and GRP94 proteins in BAECs (Fig. 4) under our conditions. Although cadmium also significantly induced the expression of GRP78 and GRP94 proteins, no significant increase in the levels of these chaperones was observed in the cells treated with zinc, copper, or arsenite. Inducers of the ER stress response—thapsigargin and tunicamycin—induced the expression of CHOP protein, another indicator of ER stress, which every metal(loid) failed to do in BAECs under our conditions. Furthermore, it was revealed that lead exposure did not induce CHOP in a time- and dose-dependent manner (data not shown). Only slight cell damage was observed upon treatment with cadmium (10 μM), arsenite (10 μM), thapsigargin (1 μM), or tunicamycin (1 μM) for 24 h when evaluated by the LDH release and morphological examination (data not shown).

To investigate the pathway responsible for the induction of GRP78 and GRP94 by lead in BAECs, we examined the effects of lead on the UPR pathways. ER stress–activated gene transcription is mediated by three different, albeit interconnected, pathways—the PKR-like ER kinase (PERK)-ATF4, ATF6, and IRE1-JNK/XBP1 (Bernales et al., 2006; Ron and Walter, 2007). The three major transinducers (PERK, ATF6, and IRE1) of the UPR sense the presence of unfolded proteins in the ER lumen and transduce signals to the nucleus. Therefore, we hypothesized that one or more of these pathways must be activated to induce the ER chaperones in response to lead-induced ER stress. Lead slightly increased mRNA encoding ATF4, a downstream transcription factor of the PERK pathway (Fig. 5A). In addition, lead did not influence the expression of the 90-kDa ATF6α; although siRNA-mediated knockdown of ATF6α significantly decreased the constitutive expression of GRP78 and GRP94, it did not influence the induction of GRP78 and GRP94 proteins by lead (Fig. 5B).

Furthermore, the active form of the 50-kDa ATF6α was also not detected in response to lead (data not shown). In contrast, phosphorylation of IRE1α was significantly induced by lead treatment in a time-dependent manner (Fig. 5C); however, nuclear accumulation of the spliced XBP1 (XBP1s) protein, a downstream transcription factor of the IRE1 pathway, was not detected in response to lead under our conditions (data not shown). On the other hand, JNK phosphorylation, another downstream mediator of IRE1, was increased by lead exposure (Fig. 5D). These results indicate that lead-induced GRP78 and GRP94 expression would be mediated by activation of JNK but not XBP1 in the downstream of the IRE1 pathway.

To confirm that the JNK pathway mediates lead-induced GRP78 and GRP94 protein expression, BAECs were pretreated with SP600125, a specific inhibitor of JNK, and exposed to lead. As expected, pretreatment with SP600125 markedly diminished lead-induced expression of GRP78 and GRP94 proteins (Fig. 6A) in a dose-dependent manner (data not shown). To ascertain the involvement of the transcription factor AP-1 in the induction of GRP78 and GRP94 by lead, BAECs were preincubated with curcumin, an inhibitor of AP-1, and exposed to lead. As expected, pretreatment with curcumin significantly abolished lead-induced expression of GRP78 and GRP94.

**FIG. 2.** Dose- and time-dependent increase of GRP78 and GRP94 proteins by lead in BAECs. (A) Cells were exposed to lead nitrate (2, 5, 10, or 25 μM) for 24 h, and total cell lysates were subjected to Western blot analysis using the indicated antibodies. Each value represents the mean ± SE of three independent experiments. Pb, lead nitrate; *p < 0.05 and **p < 0.01 compared with control.
proteins (Fig. 6B) in a dose-dependent manner (data not shown).

**DISCUSSION**

Previously, we have shown that lead causes functional damage in vascular endothelial cells in a culture system. Specifically, lead inhibits the repair of wounded monolayers (Fujiwara et al., 1997) and decreases fibrinolytic activity (Kaji et al., 1992b). However, lead does not induce nonspecific damage to cells as cadmium does (Kaji et al., 1992a, 1995b), suggesting that there are biological defense mechanisms against lead toxicity in vascular endothelial cells. In the present study, we demonstrated that lead causes ER stress, but a defense mechanism—specifically, induction of the expression of GRP78 and GRP94, ER chaperones—is activated via the JNK-AP-1 pathway, leading to the protection of vascular endothelial cells against lead cytotoxicity. Our new findings are that (1) ER stress is one of the causes of lead toxicity in vascular endothelial cells, (2) induction of GRP78 sufficiently lead. Control siRNA–, GRP78 siRNA–, or GRP78 siRNA–transfected cells (48 h) were exposed to lead nitrate (10 μM) for 24 h and stained with Giemsa; scale bar = 250 μm. Pb, lead nitrate; *p < 0.05 and **p < 0.01 compared with control.
functions as a defense mechanism against ER stress–associated nonspecific cell damage by lead, and (3) the JNK-AP-1 pathway is involved in the induction of GRP78 and GRP94 by lead.

It has been reported that there are several mechanisms underlying lead-induced ER stress. The metal inhibits protein folding and Ca$^{2+}$-ATPase (Hechtenberg and Beyersmann, 1991; Mas-Oliva, 1989; Sandhir and Gill, 1994; Sharma et al., 2008); in addition, lead binds to the GRP78 protein and induces its aggregation (White et al., 2007). Therefore, it is likely that lead causes ER stress by inhibiting protein folding, disrupting calcium homeostasis, inducing GRP78 aggregation, and via other mechanisms in the ER of vascular endothelial cells.

FIG. 5. Effects of lead on the activation of the UPR in BAECs. (A) Real-time PCR analysis of ATF4 mRNA levels. Cells were exposed to lead nitrate (2, 5, 10, or 25µM) or thapsigargin (Tg, 1µM) for 12 h. Thapsigargin was used as a positive control for the induction of ATF4. ATF4 mRNA expression levels were normalized to GAPDH mRNA levels and expressed as fold induction relative to control cells. Each value represents the mean ± SE of three independent experiments performed in duplicate. *p < 0.05 and **p < 0.01 compared with control. (B) Effect of knockdown of ATF6α on lead-induced GRP78 and GRP94 expression. Control or ATF6 siRNA–transfected cells (48 h) were exposed to lead nitrate (10 or 25µM) for 24 h, and total cell lysates were subjected to Western blot analysis using the indicated antibodies. Each value represents the mean ± SE of three independent experiments. **p < 0.01 compared with control siRNA–transfected cells. (C) Phosphorylation of IRE1α by lead. Cells were exposed to lead nitrate (10µM) for 6, 12, 18, or 24 h, and total cell lysates were subjected to Western blot analysis using the indicated antibodies. Each band was normalized to that of IRE1α and represents the mean ± SE of three independent experiments. *p < 0.05 and **p < 0.01 compared with control. (D) Phosphorylation of JNK by lead. Cells were exposed to lead nitrate (10µM) for 6, 12, 18, or 24 h, and total cell lysates were subjected to Western blot analysis using the indicated antibodies. Each band was normalized to that of JNK and represents the mean ± SE of three independent experiments. Pb, lead nitrate; *p < 0.05 compared with control.
The induction of GRP78 and GRP94 proteins would be a result of the UPR triggered by lead. We have demonstrated that lead can significantly inhibit DNA synthesis and proliferation (Kaji et al., 1995a). Although the GRP78 and GRP94 proteins could protect the cells against nonspecific cell damage caused by lead-induced ER stress, the functional damage of vascular endothelial cells exposed to lead may be partly attributed to the induction of ER stress. In fact, Outinen et al. (1999) showed that homocysteine induces not only GRP78 upregulation but also DNA synthesis inhibition and growth arrest of human vascular endothelial cells. Moreover, we have reported that lead selectively reduces the secretion of tissue plasminogen activator from vascular endothelial cells (Kaji et al., 1992b) without lowering the mRNA level (Yamamoto and Kaji, 1999). It was suggested that tunicamycin-induced ER stress, which is due to the inhibition of N-linked glycosylation, results in a decrease in the secretion of tissue plasminogen activator (Dorner et al., 1987). The inhibitory effect of lead on the plasminogen activator secretion also may be partly induced by ER stress.

This study shows that the siRNA-mediated knockdown of GRP78 results in a significant increase in caspase-3/7 activity and cytotoxicity induced by lead in BAECs. It was previously reported that knockdown of GRP78 significantly increases the level of reactive oxygen species after exposure to lead in C6 glioma cells (Qian et al., 2005). In addition, lead is known to catalyze oxidative reactions and then generate reactive oxygen species (Patrick, 2006a). Therefore, it is postulated that GRP78 plays a critical role in the protection against lead cytotoxicity through the prevention of not only ER stress but also oxidative stress.

Of the metal(loid)s tested, cadmium as well as lead increased the levels of GRP78 and GRP94 in BAECs; however, other metal(loid)s had no apparent effect, suggesting that lead and cadmium are metals that particularly induce ER stress in endothelial cells. Because only a slight cytotoxicity was observed upon treatment with cadmium and arsenite, it is suggested that the induction of GRP78 and GRP94 expression is a defense mechanism against ER stress induced by these metal(loid)s rather than a response to nonspecific cell damage. Furthermore, interestingly, lead and cadmium induced the expression of GRP78 and GRP94 but not CHOP in BAECs, although thapsigargin and tunicamycin induced CHOP expression. The mechanism underlying the different effects between lead/cadmium and thapsigargin/tunicamycin is unclear; however, it can be assumed that the difference is due to a lesser ability of the heavy metals to induce ATF4, the primary regulator of CHOP induction (Oyadomari and Mori, 2004).

Induction of GRP78 and GRP94 proteins would be a result of the UPR triggered by lead. We have demonstrated that lead can significantly inhibit DNA synthesis and proliferation (Kaji et al., 1995a). Although the GRP78 and GRP94 proteins could protect
Both YY1 and TFII-I are coactivators of ATF6 (Baumeister et al., 2005). TFII-I is also induced by ER stress and regulated by tyrosine phosphorylation, leading to the activation of protein-protein interactions (Hong et al., 2005; Parker et al., 2001). Additionally, in mammalian cells, upon ER stress, XBP1 mRNA is alternatively spliced by the ER transmembrane kinase IRE1, and XBP1S binds to the ERSE, resulting in target gene activation (Lee et al., 2003). On the other hand, part of the GRP78 induction is attributed to other transcription factors, such as ATF4 (Luo et al., 2003) and AP-1 (He et al., 2000; Song et al., 2001), through an ERSE-independent pathway. In the present study, cleavage of ATF6α was not observed, and siRNA-mediated knockdown of ATF6α failed to block the induction of GRP78 and GRP94 following exposure to lead. We also found that TFII-I mRNA is not induced by lead and that genistein, a specific tyrosine kinase inhibitor, had no effect on lead-induced GRP78 and GRP94 expression (data not shown). Furthermore, nuclear accumulation of XBP1S was not observed and ATF4 mRNA was hardly induced following exposure to lead. These results suggest that the induction of GRP78 and GRP94 by lead does not require the activation of ATF6, TFII-I, XBP1, or ATF4 in endothelial cells. However, we found that lead increased the phosphorylation of IRE1α and JNK and that lead-mediated induction of GRP78 and GRP94 was suppressed by SP600125, suggesting that the activation of the IRE1-JNK pathway is responsible for the induction of GRP78 and GRP94 by lead.

Supporting our findings, previous studies demonstrated that the activation of the IRE1-JNK pathway is mediated knockdown of ATF6α failed to block the induction of GRP78 and GRP94 following exposure to lead. We also found that TFII-I mRNA is not induced by lead and that genistein, a specific tyrosine kinase inhibitor, had no effect on lead-induced GRP78 and GRP94 expression (data not shown). Furthermore, nuclear accumulation of XBP1S was not observed and ATF4 mRNA was hardly induced following exposure to lead. These results suggest that the induction of GRP78 and GRP94 by lead does not require the activation of ATF6, TFII-I, XBP1, or ATF4 in endothelial cells. However, we found that lead increased the phosphorylation of IRE1α and JNK and that lead-mediated induction of GRP78 and GRP94 was suppressed by SP600125, suggesting that the activation of the IRE1-JNK pathway is responsible for the induction of GRP78 and GRP94 by lead. Supporting our findings, previous studies demonstrated that SP600125 also prevented the induction of GRP78 by 7-ketocholesterol in vascular smooth muscle cells (Pedruzzi et al., 2004). IRE1 can activate a stress kinase JNK via its association with TNF receptor-associated factor 2 and apoptosis signal-regulating kinase 1, leading to cell apoptosis (Matsuzawa et al., 2002; Nishitoh et al., 2002; Urano et al., 2000). However, it is known that JNK activation is involved in not only apoptosis but also AP-1–dependent gene activation. Indeed, the AP-1 inhibitor curcumin abolished the lead-induced GRP78 and GRP94 expression. Although the precise mechanism underlying this induction remains to be elucidated, our results suggest that lead induces the expression of GRP78 and GRP94 in endothelial cells via the activation of JNK-AP-1 pathway.

The present study provides the first evidence that endothelial ER is a target of lead toxicity and that the induction of ER chaperones GRP78 and GRP94 is a defensive response in vascular endothelial cells. Furthermore, our study revealed that the JNK-AP-1 pathway, but not other UPR pathways, mediated the induction of ER chaperones. This may be a unique response of vascular endothelial cells to lead.

FUNDING


REFERENCES


