A 78-kDa glucose-regulated protein is involved in the decrease of interleukin-6 secretion by lead treatment from astrocytes

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Qian Y, Zheng Y, Weber D, Tiffany-Castiglioni E. A 78-kDa glucose-regulated protein is involved in the decrease of interleukin-6 secretion by lead treatment from astrocytes. Am J Physiol Cell Physiol 293: C897–C905, 2007. First published June 13, 2007; doi:10.1152/ajpcell.00059.2007.—Interleukin (IL)-6 is a cytokine produced mainly by microglia and astrocytes and plays a pleiotropic role in the central nervous system. In this study, we cloned rat IL-6 cDNA into an enhanced green fluorescent protein (EGFP) or a red fluorescent protein (DsRed2) vector and rat 78-kDa glucose-regulated protein (GRP78) cDNA into an EGFP vector to construct IL-6-EGFP, IL-6-DsRed2, and GRP78-EGFP chimeras for the investigation of the mechanism of IL-6 secretion from astrocytes. The data showed that constructed IL-6-EGFP and IL-6-DsRed2 chimeras retained the secretory property, and the secretion of IL-6-EGFP from astrocytes could be attenuated by GRP78 depletion with double-stranded RNA interference. Coexpression of IL-6-DsRed2 and dysfunction GRP78-EGFP abolished IL-6-DsRed2 secretion, and two chimeric proteins colocalized inside living astrocytes. Coimmunoprecipitation analysis indicated that IL-6 and GRP78 resided in the same complex. The data further revealed that IL-6-EGFP secretion from astrocytes was blocked by the heavy metal lead (Pb) in a concentration-dependent manner. Analysis of the Pb interaction with protein on a Pb-affinity column demonstrated that Pb bound to GRP78 but failed to bind to IL-6. Therefore, these data suggest that IL-6-EGFP or IL-6-DsRed2 chimeras can be used as imaging probes to study IL-6 secretion from living cells, that GRP78 is involved in IL-6 secretion from astrocytes, and that Pb can block IL-6 secretion from astrocytes via targeting GRP78.

chaperone; cytokine; protein-protein interactions

INTERLEUKIN (IL)-6 is a cytokine that plays pleiotropic roles in normal brain functions and disease progression. Studies in vitro and in vivo have suggested that IL-6 plays a protective role for neurons in the central nervous system (CNS), including the protection of human SY5Y neuroblastoma cells against hydrogen peroxide exposure (3) and neurons in rat organotypic hippocampal slices from degeneration induced by treatment with N-methyl-d-aspartate (28), the stimulation of neuronal differentiation in rat PC12 cells (34, 41), and modulation of synaptic plasticity in rats (2). Furthermore, the intracerebroventricular injection of IL-6 into the ischemic rat brain significantly reduces the damage produced by permanent occlusion of the middle cerebral artery (19). IL-6 knockout (IL-6−/−) mice infected with Theiler’s virus or lesioned with the neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exhibit increased death and neurological deficits compared with wild-type controls (4, 26). In addition, in transgenic mice with astrocyte-targeted IL-6 expression [gliarial fibrillary acidic protein (GFAP) promoter-driven IL-6 gene expression], IL-6 prevents neuroglial degeneration induced by aminonicotinamide (27).

Astrocytes have been recognized as one of the major sources of IL-6 production in the CNS (10). Astroglia-derived IL-6 proteins are secreted into the extracellular space and target other cells, including neurons, via an IL-6 receptor complex composed of the IL-6 receptor and gp130 (10, 36). However, little is currently known about the mechanism by which IL-6 is secreted into the extracellular space. A report (13) has implicated that a 78-kDa glucose-regulated protein (GRP78) is involved in the hypoxia/reoxygenation cycle-induced secretion of IL-6 from rat astrocytes. GRP78 is an endoplasmic reticulum-resident molecular chaperone functioning in protein folding, assembly, and trafficking in the posttranslational quality control of protein synthesis (11, 23). However, the mechanism of GRP78 involvement in IL-6 secretion remains to be identified because the process is manipulated by multiple steps, for instance, by IL-6 transcription, translation, and secretion machinery. A study for the interaction between GRP78 and IL-6 is necessary for understanding the mechanism. In this study, we constructed fluorescent chimeric proteins for GRP78 and IL-6 to investigate the interaction between GRP78 and IL-6 and to verify the function of GRP78 in IL-6 secretion to avoid the effects of IL-6 transcription and translation on IL-6 secretion.

Also, we tested the effect of lead (Pb) on IL-6 secretion from astrocytes. Pb is a developmental neurotoxicant in children (25, 33, 35, 40) and deposits primarily in astrocytes in the brain (12, 38). Occupational exposure to Pb has been suggested as a risk factor in the development of Parkinson’s syndrome (8, 9, 17) and is associated with an increased risk for glioma (1, 6). We (29, 32) have previously reported that Pb selectively binds GRP78 in Pb-affinity column analysis and in image analysis of fluorescent protein chimeras in human living astrocytoma cells. In this study, Pb was used as a probe to assist in understanding the function of GRP78 in IL-6 secretion.

MATERIALS AND METHODS

Cell culture. Primary astroglial cultures were established from the cerebral hemispheres of 1-day-old Sprague-Dawley rat pups as previously described (39), with minor modifications. Animal care procedures used were those documented in an animal use protocol approved by the University Laboratory Animal Care Committee at Texas A&M University. Astrocytes were cultured in Waymouth’s medium (GIBCO-BRL) containing 1 mM glutamate with 10% FBS (GIBCO-BRL) and a 1% penicillin-streptomycin-neomycin antibiotic mixture (Sigma) in T-25 flasks for 4 days. Starting on day 5 (postnatal day 5), astrocytes were maintained in antibiotic-free Waymouth’s medium.
with 10% FBS under 5% CO₂ at 37°C. Starting on postnatal day 11, astrocytes were cultured in 50% DMEM-F-12 medium and 50% human SY5Y neuroblastoma cell-conditioned medium containing 10% FBS under 5% CO₂ at 37°C. Conditioned medium was collected from 70–80% confluent SY5Y cells 2 days after cells had been fed with DMEM-F-12 medium containing 10% FBS. Conditioned medium was filtered through a 0.2-μm pore diameter filter prior to being used. In a preliminary experiment, we found that SY5Y-conditioned medium could maintain a constant level of GFAP expression in astrocytes (Y. Qian et al., unpublished data). The purity of astrocyte cultures was estimated by 4′,6-diamidino-2-phenylindole (Invitrogen) staining for nuclei and immunocytochemical analysis of GFAP with mouse anti-GFAP IgG as a primary antibody (Sigma) and anti-mouse IgG-conjugated TRITC (Sigma) as a secondary antibody for astrocytes. The purity was observed to be ≥95%. Astrocytes between postnatal days 25 and 32 were used in the present study.

Construction of fluorescent fusion proteins. IL-6 cDNA with an enhanced green fluorescent protein (EGFP) vector (IL-6-EGFP) and a red fluorescent protein (DsRed2) vector (IL-6-DsRed2), as chimeras, respectively, were constructed as follows. A 0.6-kb cDNA of rat IL-6, covering a whole open reading frame, was amplified from the rat cDNA pool with Taq DNA polymerase and a pair of primers designed from the rat IL-6 cDNA sequence (GenBank Accession No. M26744). The sense primer for the IL-6-EGFP and IL-6-DsRed2 chimeras was 5′-ATAGTCGAAGCTTATGAAGTAGCTCCGGAAG-3′ (the underlined portion indicates the HindIII restriction site and bold letters show the start codon). The antisense primer for the IL-6-EGFP chimera was 5′-ATAGTCGATCGTTTGGCCGAGTAGACCC-3′ (the underlined portion indicates the BamHI site). The sense primer for the IL-6-DsRed2 chimera was 5′-ATAGTCGATCGTTTGGCCGAGTAGACCC-3′ (the underlined portion indicates the BamHI site). IL-6 cDNA was cloned into a pEGFP-N3 vector carrying EGFP cDNA at HindIII/BamHI restriction sites to construct chimeric IL-6-EGFP cDNA for the expression of IL-6-EGFP protein or into a pDsRed2-N1 vector (Clontech) carrying DsRed2 red fluorescent protein cDNA to construct chimeric IL-6-DsRed2 cDNA for the expression of IL-6-DsRed2 protein. The expression of both IL-6-EGFP and IL-6-DsRed2 was driven by the promoter of human cytomegalovirus in the vectors. A GRP78-EGFP chimera was constructed as previously described (32). A 1.9-kb cDNA of rat GRP78, covering a whole open reading frame with a KDEL deletion at the COOH terminal, was amplified from the rat cDNA pool with Taq DNA polymerase and a pair of primers designed from the rat GRP78 cDNA sequence (GenBank Accession No. M14050). The sense primer carrying a HindIII restriction enzyme site was 5′-ATAGTCGAAGCTTATGAAGTAGCTCCGGAAG-3′ (the underlined portion indicates an artificially added HindIII site and bold letters show the start codon). The antisense primer carrying a BamHI site was 5′-ATAGTCGATCGTTTGGCCGAGTAGACCC-3′ (the underlined portion indicates the BamHI site). GRP78 cDNA was cloned into a pEGFP-N3 vector (Clontech) carrying EGFP cDNA at HindIII/BamHI restriction enzyme sites to construct chimeric GRP78-EGFP cDNA for the expression of GRP78-EGFP protein.

Transfection and image analysis. Cells were cultured in four-well Lab-Tek Coverglass Chambers (Nalge Nunc) at an initial density of 100,000 cells/well 1 day before transfection for digital imaging capture. Cells were transfected by plasmids containing chimeric fluorescent protein cDNA with Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions. The transfection efficiency was 5–10% for primary rat astrocytes. Images were captured on an Olympus IX70 inverted fluorescence microscope equipped with a xenon lamp, LAMBDA DG-4 excitation filter set, LAMBDA 10-2 shutter driver, differential interference contrast optics, Hamamatsu camera controller, and Compix Imaging System. Ten images were blindly captured from each group, and transected cell numbers on each blindly captured image were counted with AlphaEaseFC software (version 3.1.2, Alpha Innotech).

Fig. 1. Secretion of IL-6 cDNA with an enhanced green fluorescent protein (EGFP) vector (IL-6-EGFP) from rat astrocytes. A: astrocytes were transfected with EGFP or IL-6-EGFP chimera plasmids for 48 h, and images of intracellular EGFP or IL-6-EGFP fluorescence were blindly captured by fluorescence microscopy at the FITC channel. N, NH₂ terminal; C, COOH terminal. Magnification: ×20. B: cells expressing EGFP or IL-6-EGFP 48 h after transfection were counted in blindly captured images with AlphaEaseFC software. Data are expressed as cells per image (means ± SE; n = 10). C and D: extracellular EGFP or IL-6-EGFP protein levels after 48 h of transfection (C) and extracellular IL-6-EGFP protein levels 30 and 48 h after transfection (D) were detected in the medium by dot-blot analysis with an antibody to EGFP and quantified with ImageJ. Data are expressed as arbitrary units (means ± SE; n = 4).
Analysis of GRP78-EGFP and IL-6-DsRed2 colocalization with confocal microscopy. Cells were cultured in two-well Lab-Tek Coverglass Chambers (Nagle Nunc) at an initial density of 200,000 cells/well 1 day before transfection for analysis of confocal microscopy. Cells were cotransfected by GRP78-EGFP and IL-6-DsRed2 plasmids with Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions. Cells were imaged using a Meridian InSIGHT point laser scanning confocal microscope (Meridian Instruments, Okemos, MI) with 100-mW argon ion and 75-mW krypton ion lasers, which was capable of direct ocular viewing in real time and real color. Detection filter sets on a filter wheel were computer controlled and integrated with a cooled intensified charge-coupled device camera. Images for GRP78-EGFP and IL-6-DsRed2 were collected at 488-nm excitation/515-nm emission and 570-nm excitation/600-nm emission parameters, respectively.

Assay of IL-6-EGFP with dot-blot analysis. Medium was harvested from the wells at 1 ml/well after 48 h from the above-transfected cells for IL-6-EGFP secretion analysis. The medium from each well was loaded onto a nitrocellulose membrane (Bio-Rad) in a Bio-Rad dot-blot apparatus connected to a vacuum pump. Extracellular IL-6-EGFP proteins on the membrane were detected with a monoclonal antibody against EGFP as a primary antibody (1:1,000) and a rabbit anti-mouse IgG conjugated with a peroxidase as a secondary antibody (1:2,500). Signals were visualized on Kodak X-Omat film with the use of an NEN Western Blot chemiluminescent reagent mixture (NEN Life Science). Signal intensities were quantified with ImageJ software (version 1.33d, http://rsb.info.nih.gov/ij/).

Analysis of IL-6 and Pb interactions with a Pb-affinity column. A Pb-affinity column (10 ml) was prepared as previously described (29). A supernatant of rat astroglial homogenates with buffer [20 mM PBS containing 0.5 M NaCl (pH 7.8), 1 mM PMSF, 0.5% Triton X-100, and 5 mM ascorbate] was obtained by centrifugation at 16,000 g for 15 min and loaded onto a Pb-affinity column. Nonbinding proteins were washed away with 20 mM PBS containing 0.5 M NaCl (pH 6.0). Pb-binding proteins were eluted from the column with 50 mM EDTA (pH 8.0). The column by the discharge of Pb with 50 mM EDTA (pH 8.0) was used as a control column. IL-6 in the eluant was detected with Western blot analysis.

Western blot analysis. The procedure was carried out as described in our previous study (29). The antibodies used were a mouse monoclonal antibody against β-actin (Sigma), a rabbit polyclonal antibody against IL-6 (Sigma), a rabbit polyclonal antibody against GRP78 (Stressgen Biotechnologies), a peroxidase-conjugated goat polyclonal antibody against mouse IgG, and a peroxidase-conjugated monoclonal antibody against rabbit IgG (Sigma). Total extracted proteins in PBS containing 1 mM EDTA, 1 mM PMSF, and 0.5% Triton X-100 were separated on a 10% Bio-Rad Ready SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane for Western blot analysis with the antibodies against GRP78 or β-actin as the primary antibody and anti-IgG peroxidase conjugate as the second antibody. Signals for target proteins were visualized on Kodak X-Omat film with the use of the NEN Western Blot chemiluminescent reagent mixture (NEN Life Science). Signal intensities were quantified with ImageJ software (version 1.33d). β-Actin was used as a loading control.

Protein coimmunoprecipitation. Cell lysates were prepared in 1 ml of cold RIPA buffer [50 mM Tris·HCl (pH 7.4), 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, and 1 mM

Fig. 2. Retention of IL-6 in 78-kDa glucose-regulated protein (GRP78)-depleted rat astrocytes. A: GRP78 protein was depleted with a vector-based double-stranded RNA inhibitor (dsRNAi) in rat astrocytes. Cells were transfected with a dsRNAi clone with the use of Lipofectamine 2000 (Invitrogen) for 48 h. An empty vector without dsRNAi was used as a control. Transfected cells were harvested for GRP78 quantification by Western blot (WB) analysis. The procedure was carried out as described in our previous study (29). Each lane was loaded with 25 μg total protein. Band intensities were quantified with ImageJ software. Data are presented as means ± SE; n = 5. **P < 0.01. B: cells in 4-well chamber slides were cotransfected with a dsRNAi clone and IL-6-EGFP or with a control vector (without dsRNAi) and IL-6-EGFP with the use of Lipofectamine 2000 (Invitrogen). After 48 h, images were blindly captured at the green FITC channel on an inverted fluorescent microscope. Magnification: ×20. C: numbers of transfected cells expressing IL-6-EGFP in blindly captured images were counted with AlphaEaseFC software. Data are presented as cells per image (means ± SE; n = 10). D: IL-6-EGFP signals were detected by dot-blot analysis with an antibody to EGFP and quantified with ImageJ. Data are expressed as arbitrary units (means ± SE; n = 5).
After being centrifuged at 10,000 g for 10 min at 4°C, supernatants were transferred to microcentrifuge tubes, and 2 μg of anti-IL-6 or anti-GRP78 rabbit IgG together with 100 μl of suspended protein A beads were added and incubated for 2–3 h at 4°C on a rocker. The immunoprecipitates (beads) were collected by centrifugation at 10,000 g for 10 s, and beads were gently washed with 1 ml of cold PBS for three times. After being washed, beads were resuspended in 100 μl of 2× Laemmli’s buffer (Sigma), boiled for 5–10 min, and centrifuged at 10,000 g for 10 s. Supernatants were reserved for Western blot analysis.

GRP78 depletion with vector-based double-stranded RNA interference. Vector-based double-stranded (ds)RNA interference (dsRNAi) was constructed as previously described (32) by inserting 70-bp sense and antisense hairpin oligonucleotides digested with BamHII/HindIII [5'-GATCCCGTCAAGGCTCGAAGGTGATTGATACCAGTCACCCTCGTAGACCCGTAGTATTTTTTCAAAAAGCT-3'] (underlined and bolded portions indicate restriction sites; underlined portions indicate sense and antisense sequences)] into a pRNA-U6.1/Neo vector (GenScript) at BamHII/HindIII restriction sites. The constructed dsRNAi vector was delivered into cells with Lipofectamine 2000 transfection reagent (Invitrogen). The empty vector without the insert was used as a transfection control. After transfection for 48 h, cells were harvested for the analysis of GRP78 protein levels with a Western blot assay.

Treatment with Pb. To measure the effect of Pb on IL-6-EGFP secretion and retention, Pb acetate (Sigma) was added to cultures expressing IL-6-EGFP at final concentrations of 0, 1, 10, and 50 μM for 24 h in rat astrocytes. We failed to observe Pb toxicity by counting cell numbers for cell proliferation after 24 h of Pb treatment (data not shown). Pb concentrations at 1–50 μM have extensively been used in rat neural cell lines and primary cell cultures to study Pb neurotoxicity (37). Brain Pb levels are about one to three times the concentration in whole blood Pb (5). Blood Pb levels used to screen Pb poisoning are defined as 10 μg/dl for children by the United States Centers for Disease Control and Prevention in 1991 and 40 μg/dl for occupational safety by the United States Occupational Safety and Health Administration in 1993. Pb contents under similar conditions have been measured in previously published studies of astrocytes. A steady state for intracellular Pb content in astrocytes is reached within 3 days, as we have previously reported (18), but the majority of Pb accumulation occurs before 24 h (30).

Statistical analysis. For experiments containing two groups, means were compared by Welch’s unpaired t-test analysis. For experiments...
RESULTS

To study IL-6 secretion, we constructed an IL-6-EGFP chimera by cloning IL-6 cDNA into an EGFP-containing vector and transfected rat astrocytes to make cells express IL-6-EGFP. IL-6 secretion was monitored by analyzing intracellular IL-6-EGFP retention with fluorescent microscopy and extracellular IL-6-EGFP levels with EGFP antibody. As expected, EGFP fluorescent signals were trapped intracellularly when cells expressed EGFP only (Fig. 1A). However, the intracellular EGFP fluorescent signal intensity and EGFP cell number dramatically decreased by 43% (±43%) when astrocytes expressed IL-6-EGFP chimera (Fig. 1, A and B). In agreement with the change of intracellular EGFP fluorescent signals, a significant increase (+53%) of extracellular EGFP protein levels (Fig. 1C) was observed in IL-6-EGFP-expressing cells compared with EGFP-expressing cells. Extracellular EGFP protein levels increased with time and showed a 63% increase from 30 to 48 h in IL-6-EGFP-expressing cells (Fig. 1D); however, a decrease of intracellular EGFP fluorescent signals was not observed (data not shown). These data indicate that IL-6-EGFP chimeric protein retained the property of IL-6 to secrete from living astrocytes.

IL-6-EGFP secretion was dependent on GRP78. As shown in Fig. 2A, we constructed vector-based GRP78 short interfering (si)RNA (dsRNAi) to deplete GRP78 protein levels and tested intracellular IL-6-EGFP retention in living rat astrocytes. GRP78 protein levels were depleted by ~39% with transient transfection of dsRNAi compared with control vector in rat astrocytes. In parallel with the change of GRP78 levels, the numbers of cells accumulating IL-6-EGFP fluorescent signals significantly increased by ~67% (Fig. 2, B and C). Meanwhile, extracellular IL-6-EGFP protein levels decreased by 40% in GRP78-depleted cells compared with the control (Fig. 2D).

We also constructed another IL-6-DsRed2 chimera, an IL-6 and DsRed2 fusion, to examine the involvement of GRP78 in IL-6 secretion from living cells. The IL-6-DsRed2 chimera also retained the secretory property, as shown in Fig. 3. The DsRed2 group had more DsRed2-positive cells (32 ± 3 cells/image) than the IL-6-DsRed2 group did (9 ± 2 cells/image). Rat astrocytes were cotransfected with IL-6-DsRed2 and KDEL-deleted GRP78-EGFP (see MATERIALS AND METHODS) to study IL-6 secretion from living astrocytes. Cells coexpressing IL-6-DsRed2 and GRP78-EGFP were screened at red and green channels on a confocal microscope. As shown in Fig. 4A, cells retaining IL-6-DsRed2 intracellularly showed expression of KDEL-deleted GRP78-EGFP, suggesting that KDEL-deleted GRP78 caused an amassment of IL-6 intracellularly in living astrocytes. Images captured at red and green channels on a confocal microscope for IL-6-DsRed2 and GRP78-EGFP, respectively, had a complete overlap, suggesting that IL-6 and GRP78 colocalized and that they probably had an interaction with each other in living astrocytes for IL-6 secretion (Fig. 4B). A possible interaction between GRP78 and IL-6 for IL-6 secretion was further supported by protein coimmunoprecipitation analysis. IL-6 was detected in the complex immunoprecipitated with GRP78 antibody (Fig. 5A), and GRP78 was detected in the complex immunoprecipitated with IL-6 antibody (Fig. 5B).

In the next experiment, we studied the effect of Pb on IL-6 secretion from astrocytes by monitoring IL-6-EGFP retention with fluorescent microscopy and extracellular IL-6-EGFP with a dot-blot immunodetection. Astrocytes expressing IL-6-EGFP were treated with 0, 1, 10, and 50 μM Pb for 24 h. As shown in Fig. 6A, the numbers of cells accumulating IL-6-EGFP fluorescent signals increased with Pb in the medium in a concentration-dependent manner. By a computing counting analysis, the numbers of cells accumulating IL-6-EGFP signals per image significantly increased with Pb concentrations (Fig. 6B), suggesting that Pb could increase IL-6-EGFP retention. In contrast, extracellular IL-6-EGFP protein levels, as detected by an immunchemical dot-blot analysis with EGFP antibody, significantly decreased with Pb concentrations (Fig. 6C), implying that Pb could reduce IL-6-EGFP release. However, we failed to observe these effects of Pb in astrocytes expressing EGFP only. As shown in Fig. 6D, Pb treatments with 1, 10, and 50 μM for 24 h did not change EGFP fluorescent signal measurements.
Fig. 6. Retention of IL-6-EGFP induced by lead (Pb) in rat astrocytes. 

A: astrocytes were transfected with IL-6-EGFP plasmids for 24 h and then treated with 0, 1, 10, and 50 μM Pb acetate for another 24 h. Images of intracellular IL-6-EGFP fluorescence were blindly captured by fluorescence microscopy at the FITC channel. Magnification: ×20. 

B: numbers of cells expressing IL-6-EGFP in blindly captured images from A were counted with AlphaEaseFC software. Data are expressed as cells per image (n = 10). 

C: extracellular IL-6-EGFP protein levels were detected by dot-blot analysis with an antibody to EGFP and quantified with ImageJ (n = 5). 

D: astrocytes were transfected with EGFP plasmids for 24 h and then treated with 0, 1, 10, and 50 μM Pb acetate for another 24 h. Images of intracellular EGFP fluorescence were blindly captured by fluorescence microscopy at the FITC channel. Magnification: ×20. 

E: EGFP protein levels in the medium of astrocytes treated with 50 μM Pb for 24 h were detected by dot-blot analysis with an antibody to EGFP (n = 5). Data in B, C, and E represent means ± SE. *P < 0.05, significantly different from the control; **P < 0.01, significantly different from the control.
Fig. 7. Analysis of Pb and IL-6 interactions. The supernatant of astroglial homogenates (1 ml) was loaded onto a Pb-affinity column or a Pb-discharged column. After the wash away of non-Pb-binding proteins with 20 mM PBS containing 0.5 M NaCl (pH 6.0), Pb-binding proteins were eluted from the column with 50 mM EDTA (pH 8.0). Total proteins (50 μg) in the supernatant (cell extract), Pb-binding proteins in the eluant (Pb-Column), and a volume-matched fraction from the Pb-discharged column (Non-Pb-Column) were separated on a 10% SDS-PAGE gel. IL-6, GRP78, and β-actin in the supernatant of astroglial homogenates and eluents from the Pb-affinity column and the Pb-discharged column, respectively, were detected by WB analysis using rabbit polyclonal antibodies against IL-6 (1:1,000) and GRP78 (1:1,000) and monoclonal antibody against β-actin (1:5,000) as primary antibodies and anti-rabbit IgG (1:5,000) and anti-mouse IgG (1:2,000) peroxidase conjugates as secondary antibodies.

The intensities inside cells and the numbers of cells accumulating EGFP, suggesting that Pb did not change EGFP retention intracellularly. Consistent with that, 50 μM Pb, the highest dose tested in the study, did not significantly alter EGFP protein levels in the medium (Fig. 6E).

A further experiment indicated that Pb did not directly bind IL-6 to reduce IL-6-EGFP release. We prepared a Pb-affinity column to test IL-6 and Pb interactions. As shown in Fig. 7, IL-6 antibody detected IL-6 in total soluble proteins extracted from rat astrocytes; however, the antibody failed to detect IL-6 in the pool of Pb-binding proteins from the Pb-affinity column. Figure 7 indicates that Pb also did not bind β-actin but specifically bound to GRP78 because the discharge of Pb from the column failed to bind GRP78.

**DISCUSSION**

IL-6 is a secretary protein. The data from this study indicate that IL-6 secretion can be studied using IL-6-EGFP or IL-6-DsRed2 chimeras because both chimeras retained the property to secrete IL-6. The establishment of this method makes possible the study of the mechanism of IL-6 secretion separating from the contamination of medium IL-6 in cultures and the change of IL-6 gene expression. The gene expression of IL-6 is readily induced by stress, including hypoxic injury, physiological compounds, and environmental chemicals (21). The fluorescence-based image analysis provides a method to study the secretory machinery of IL-6. In this study, we used a variety of methods to identify GRP78, a protein chaperone, as a member of IL-6 secretion machinery and the formation of the GRP78-IL-6 complex. The following four lines of evidence support this conclusion.

First, the secretion of IL-6-EGFP was attenuated by the depletion of GRP78 with dsRNAi, consistent with the literature reporting that GRP78 contributes to IL-6 secretion and immunoglobulin secretion (11, 13, 23) and that the unfolded protein response, in which GRP78 functions as a master regulator, contributes to IL-6 secretion from B cells (14). We failed to completely deplete GRP78 protein levels with dsRNAi because GRP78 expression is regulated in a feedback manner and GRP78 depletion probably activated complementary expression (31). This is in agreement with the finding that GRP78 is essential for cell survival and GRP78 knockout (GRP78−/−) is lethal in mice (20).

A second line of evidence is the study of IL-6-DsRed2 secretion and KDEL-depleted GRP78-EGFP colocalization with IL-6-DsRed2. That the secretion of IL-6-DsRed2 was abolished by the expression of dysfunctional GRP78-EGFP further supports that GRP78 is involved in IL-6 secretion. In the GRP78-EGFP chimera, the KDEL motif consisting of the final four residues at the COOH terminal of GRP78 was deleted (see MATERIALS AND METHODS). It has been predicted that KDEL poses at the surface of the GRP78 molecule and functions as a navigator for GRP78 cycling back to the endoplasmic reticulum in protein trafficking (24). Thus, KDEL motif deletion or KDEL spatially blocked by EGFP fusion will cause GRP78 dysfunction. When cells coexpressed KDEL-deleted GRP78-EGFP and IL-6-DsRed2, the overexpressed GRP78-EGFP competed with endogenous and functional GRP78 to reduce IL-6-DsRed2 secretion, and IL-6-DsRed2 accumulation was observed. The dysfunctional GRP78-EGFP also provided an imaging tool to capture its "cargo" molecules and study protein-to-protein interactions in living cells. The colocalization of GRP78-EGFP and IL-6-DsRed2 suggests that GRP78 and IL-6 probably had a physical interaction in IL-6 secretion. However, the colocalization of two fluorescent biomolecules by the analysis of confocal microscopy can be observed in the same complex or two separate complexes because the colocalization suggests a 10- to 100-nm distance between two fluorophores and the biomolecule sizes are 5–20 nm in diameter (7). This remains to be answered by the coimmunoprecipitation analysis.

A third line of evidence is the study of IL-6 and GRP78 coimmunoprecipitation analysis. IL-6 and GRP78 were detected in the same complex, supporting that IL-6 and GRP78 formed in the complex and, furthermore, that IL-6-DsRed2 and GRP78-EGFP colocalized in the same complex. Thus, GRP78 could function in IL-6 secretion via the formation of the GRP78-IL-6 complex. However, it remains to be understood whether GRP78 directly or indirectly interacted with IL-6 in the process of IL-6 secretion from living cells even though they formed in the same complex.

A final line of evidence is the study of the Pb effect on IL-6-EGFP secretion. Our previous studies (29, 32) reported that Pb can specifically bind GRP78. Thus, Pb binding to GRP78 was expected to have a negative effect on IL-6 secretion. This was supported by the observation that IL-6-EGFP retention significantly increased and IL-6-EGFP secretion significantly decreased with Pb concentrations in rat astrocytes. The data further support that GRP78 contributed to IL-6 secretion and mediated environmental toxin-modified secretion of IL-6 from astrocytes. The mechanism by which Pb reduced IL-6 secretion probably involves the change of GRP78 configuration by Pb binding and then the abolishment of the GRP78 and IL-6 interaction, because
IL-6 disappeared in the Pb-GRP78-binding fraction. This is consistent with the finding that GRP78 and IL-6 interactions were necessary for IL-6 secretion and also imply that the dysfunctional GRP78-EGFP and IL-6-DsRed2 colocalization occurred before a failure of IL-6-DsRed2 secretion. GRP78 can also bind misfolded proteins to refold the proteins in the unfolded protein response (31). We failed to directly detect the abolishment of the IL-6 and GRP78 interaction by Pb treatment in living cells because the image analysis in this study is not advanced enough to identify the association and dissociation between IL-6 and GRP78, as mentioned above. A fine assay of fluorescence resonance energy transfer (FRET) will be developed to further identify GRP78 and IL-6 interactions and the machinery of IL-6 secretion. The occurrence of a FRET between two fluorophores implies that they have a distance of 1–10 nm, indicating a direct physical interaction (15).

The observation that Pb could modulate IL-6 secretion from astrocytes is consistent with the finding that Pb alters the kinetic profile of the IL-6 appearance in the mouse brain (16) and that Pb preferably accumulates in astrocytes in the brain (12, 18, 38). Pb continues as a pervasive neurotoxicant in the environment (22) and causes developmental neurotoxicity in children, characterized in part by reduced attention span (25), reduced IQ scores (35, 40), and increased aggression (25). The children, characterized in part by reduced attention span (25), accumulate lead: dependence on cell type and on degree of differentiation. Naunyn Schmiedebergs Arch Pharmacol 357:373–379, 1998.


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GRANTS

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