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□ This study aims to explore the expression of GRP78, a marker of endoplasmic reticulum (ER) stress, in the cortex of rat brains acutely exposed to methylmercury (MeHg). Thirty Sprague-Dawley (SD) rats were randomly divided into six groups, and decapitated 6 hours (h) after intraperitoneal (i.p.) injection of MeHg (2, 4, 6, 8 or 10 mg/kg body weight) or normal saline. Protein and mRNA expression of *Grp78* were detected by western blotting and real-time PCR, respectively. The results showed that a gradual increase in GRP78 protein expression was observed in the cortex of rats acutely exposed to MeHg (2, 4 or 6 mg/kg). Protein levels peaked in the 6 mg/kg group ($p < 0.05$ vs. controls), decreased in the 8 mg/kg group, and bottomed below the control level in the 10 mg/kg group. Parallel changes were noted for *Grp78* mRNA expression. It may be implied that acute exposure to MeHg induced hormetic dose-dependent changes in *Grp78* mRNA and protein expression, suggesting that activation of ER stress is involved in MeHg-induced neurotoxicity. Low level MeHg exposure may induce GRP78 protein expression to stimulate endogenous cytoprotective mechanisms.

Key words Methylmercury; endoplasmic reticulum stress; GRP78; rat; cortex

INTRODUCTION

Methylmercury (MeHg) is recognized as a global environmental pollutant. Its primary target organ is the central nervous system (CNS). MeHg disrupts multiple biochemical and physiological processes (Aschner *et al.* 2010). In humans, MeHg damages the visual cortex and interferes with somatosensory processing. Signs of intoxication include the constriction of the visual field, hearing loss, sensory impairment of the extremities, muscle weakness, tremors and mental deterioration (Castoldi *et al.* 2001). Several mechanisms have been proposed for MeHg-

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induced neurotoxicity, such as the induction of oxidative stress (Farina *et al.* 2011), disruption of neurotrophic signaling (Andersson *et al.* 1997), disruption of intracellular calcium homeostasis (Hare *et al.* 1993), and alterations in neurotransmitter systems (Minnema *et al.* 1989). Nevertheless, the precise nature of MeHg-induced neurotoxicity remains to be fully elucidated, likely reflecting upon its multifaceted disruptive cellular mechanisms (Atchison and Hare 1994; Ceccatelli *et al.* 2010; Grandjean *et al.* 2010).

The endoplasmic reticulum (ER) is a major organelle serving several specialized functions, such as calcium storage, biosynthesis, folding and assembly of transmembrane and secretory proteins, and the production of phospholipids and sterols. Disturbance in any of these functions may lead to ER stress (Xu *et al.* 2005). One of the most characterized and highly conserved ER stress responses is the unfolded-protein response (UPR) (Kaufman 2002). The UPR diminishes cellular stress by activating protein folding and degradation pathways in the ER and by inhibiting protein synthesis (Mori 2000; Rutkowski and Kaufman 2004). The UPR induces the expression of the 78-kDa glucose-regulated protein (GRP78). GRP78 is an ER-resident molecular chaperone that prevents the aggregation of unfolded or misfolded proteins so that they can be properly refolded, ubiquitinated and presented to the proteasome for degradation (Lee 2001; Brostrom and Brostrom 2003; Schröder and Kaufman 2005).

GRP78 is also known as an immunoglobulin-binding protein, BiP. It is a major functional molecule that is regulated by the ER stress response, and its up-regulation affords protection from cytotoxic injury (Lee 2001). GRP78 also functions as a molecular chaperone under physiological conditions (Pfaffenbach and Lee 2011). Upon stress, a greater abundance of GRP78 proteins is required to promote refolding and repair of denatured proteins, which serves to impede a cellular damage cascade (Pfaffenbach and Lee 2011). A protective role of GRP78 is supported by studies in neurons and glia (Lee *et al.* 1999; Yu *et al.* 1999; Suyama *et al.* 2011). However, when ER stress is prolonged and/or too pronounced, ER resident chaperones are unable to counteract the accumulation of misfolded proteins and an ER-mediated apoptotic program is triggered through the activation of caspase-12 (Nakagawa *et al.* 2000; Lamkanfi *et al.* 2004; Cribb *et al.* 2005).

Previous reports suggested that several *in vitro* heavy metals, including lead, manganese, cadmium and mercury, can induce ER stress and contribute to damage or adaptation in exposed cells (Castiglioni *et al.* 2001; Chun *et al.* 2001; Qian *et al.* 2000, 2001; Liu *et al.* 2006; Hiramatsu *et al.* 2007; Yokouchi *et al.* 2007; Shinkai *et al.* 2010; Aremu *et al.* 2011; Ji *et al.* 2011). Liu *et al.* (2006) reported that in LLC-PK1 cells inorganic mercury (Hg) failed to significantly induce GRP78 expression, yet it significantly increased the protein levels of two other markers of ER stress, namely

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phospho-eukaryotic initiation factor 2 (eIF2_α) and ATF4. Exposure of Atlantic cod to Hg-enriched sediments for five weeks was associated with enhanced mRNA expression of calreticulin (Olsvik *et al.* 2011b), a Ca²⁺-buffering chaperone in the ER lumen (Qiu and Michalak, 2009). Calreticulin protein expression was also increased in MeHg-treated Atlantic salmon (Olsvik *et al.* 2011a). Increased expression of calreticulin increases Ca²⁺ transients and has been linked to ER stress (Zhang *et al.* 2007). In MeHg-susceptible C2C12-DMPK160 cells, the organometal significantly up-regulated *Grp78* mRNA and ER stress-related proteins, XBP1 and GRP78 (Usuki *et al.* 2008). In agreement with the previous studies, Cambier and colleagues noted that *Hsp5a(Grp78)* and *Hsp90b1(grp94)* were overexpressed in the skeletal muscles of zebrafish fed a MeHg-contaminated diet (Cambier *et al.* 2010). Our previous study showed that acute MeHg (4 mg/kg body weight) exposure increased GRP78 protein expression in the cerebral cortex, hippocampus, brain stem, striatum and cerebellum (Zhang *et al.* 2010a). Thus, a link exists between MeHg exposure and ER stress. However, little is known about the dose-response relationship between ER stress and *in vivo* acute systemic exposure to MeHg.

The objective of the present study was to determine the dose-effect relationship between MeHg exposure and GRP78 expression at the protein and mRNA levels over an expanded dosimetry range. Our results establish that acute exposure to MeHg induced Grp78 protein expression in a non-linear dose-dependent manner. These results suggest that ER stress activation plays a role in MeHg-induced neurotoxicity. Furthermore, low level MeHg exposure may induce a hormetic effect to stimulate endogenous cytoprotective mechanisms.

MATERIALS AND METHODS

Drugs and Reagents

Methylmercury (MeHg, 99.9% purity) was purchased from Sigma (St. Louis, MO, USA). GRP78 polyclonal antibody and β-actin antibody were obtained from the Santa Cruz Biotechnology Company (Santa Cruz, CA, USA). Trizol was bought from Invitrogen (Carlsbad, CA, USA) and the protein extraction kit was obtained from the Kang Cheng Bioengineering Company (Shanghai, China). We bought the BCA protein assay kit from Thermo Scientific (Waltham, MA, USA). Amersham ECL Plus Western Blotting Detection Reagents were obtained from the GE Healthcare Company (Waukesha, WI, USA). The reverse transcription and PCR kits were obtained from Fermentas (Glen Burnie, MD, USA). SYBR Green I Mix was purchased from Stratagene (La Jolla, CA, USA) and the PCR primers were synthesized at the Health Bioengineering Company (Shanghai, China).

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Animals

Adult (4-5 weeks) male Sprague-Dawley (SD) rats (n=30) weighing 200-300 g were provided by the Laboratory Animal Center of Jiangsu University.

Animal treatment protocol

After a 1-week adaption period, thirty SD rats were randomly divided into six experimental groups. In a previous study, we found that, compared with the control group, MeHg-induced alterations of Grp78 protein only in the cerebral cortex and brain stem were statistically significant at the dosage of 4 mg/kg, and magnitude of induction in cerebral cortex was the largest, though the trend was similar in the cerebellum, cerebral cortex, brain stem, hippocampus and striatum (Zhang, *et al.* 2010a). Therefore, we chose cerebral cortex as the optimal structure for the present study and the range of dosages was extended to 2, 4, 6, 8 or 10 mg/kg body weight from 4mg/kg body weight. Regarding to sampling time points, we chose 6 h after administration of MeHg as critical time point because the peak of Grp78 induction was at 6 h after MeHg injection (Zhang *et al.* 2010a). The animals were decapitated 6 h after a single i.p. MeHg injection (2, 4, 6, 8 or 10 mg/kg body weight), and the cerebral cortex was immediately dissected out and placed on ice, following our previous treatment protocol (Zhang *et al.* 2010a). The rats in the control group were i.p. injected with normal saline. The brain cortices were rapidly frozen in liquid nitrogen and transferred to the -80°C freezer. All animal exposure protocols were approved by the Jiangsu University Institutional Animal Care and Use Committee (IACUC) and were adhered to strictly to minimize pain. All exposures and procedures followed the NIH Laboratory Animal Care and Use guidelines.

Primers

Primers were designed and synthesized based on *Grp78* and rat house-keeping gene (*β -actin*) sequence information. The sequences of the primers are shown in Table 1.

Western blot analysis

Western blot analysis was conducted as previously described (Zhang *et al.* 2010a). Briefly, total protein was extracted from the cerebral cortex with a protein extraction kit. The protein concentration was assessed with the BCA protein assay kit (ThermoFisher Scientific, Rockford, IL, USA). Equal amounts (20 μ g protein/lane) of protein and prestained molecular-weight markers were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gels and subsequently transferred to PVDF immunoblotting

*Hormetic effects of methylmercury on GRP78 expression***TABLE 1.** Grp78 and β -actin primers sequences

Gene	Primer's sequence (5'-3')	DNA fragments (bp)	Melting point (°C)
<i>Grp78</i>	For: AACCCAGATGAGGCTGTAGCATA Rev: CACAGTGTTCCTCGGAATCAGTT	162	64
β -actin	For: GAGGGAAATCGTGCGTGAC Rev CTGGAAGGTGGACAGTGAG	444	58

membranes. The membranes were incubated for 60 min at room temperature in blocking buffer containing 5% nonfat dry milk. Next, the membranes were washed in Tris-buffered saline and incubated overnight at 4°C with a rabbit polyclonal antibody to GRP78 (1:1000) followed by incubation with a goat anti-rabbit IgG HRP-linked antibody (1:10000). The membranes were exposed to Amersham ECL Plus Western Blotting Detection Reagents, and a Typhoon 9400 molecular imaging system (excitation wavelength 430 nm, emission wavelength 503 nm) was used for chemical fluorescent scanning. The lane densities were assessed with ImageQuant TL software (GE Healthcare, Waukesha, WI, USA). The ratio of GRP78 gray value and the corresponding β -actin expression level in the various groups was compared to the control group, which was set to 1.

Quantitative real-time PCR

Total mRNA from the cerebral cortex was extracted with Trizol reagent, following the manufacturer's instructions. Then the first-strand cDNA was synthesized using 1 μ g mRNA. Fluorescent real time quantitative PCR (qPCR) was performed to detect the mRNA levels of *Grp78* and β -actin in a Stratagene Mx3000P PCR machine (La Jolla, CA, USA). The qPCR reaction included: 7.5 μ L SYBR Green 1 mix, 0.3 μ L forward primer (10 mM), 0.3 μ L reverse primer (10 mM), 0.3 μ L Reference Dye (ROX) (10 mM), 1 μ L cDNA template and 5.6 μ L ddH₂O. The PCR was performed for 35 cycles (denaturation: 95°C, 30 seconds (s); annealing: *Grp78*, 64°C for 30 s or β -actin 58°C for 30 s; extension: 72°C for 25 s). The relative expression of the target gene was calculated as the ratio between the copy numbers of target gene over that of β -actin. The levels of mRNA in the various groups were compared with the control group, which was set to 1. The specific working conditions of the real-time quantitative PCR are shown in Fig. 1.

Data analysis

The data are expressed as means \pm S.D. Statistical analysis was conducted with SPSS13.0. Significant differences between groups were evaluated by

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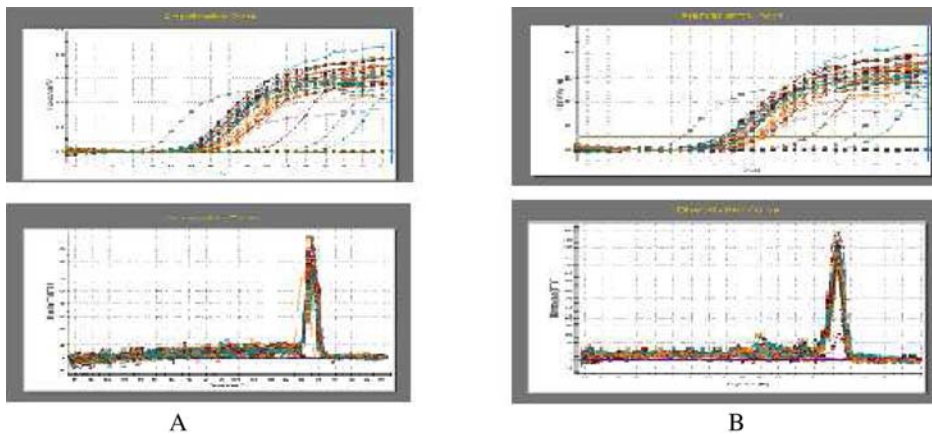


FIG. 1. Fluorescent quantitative amplification and melting curves. A: The amplification and melting curves of β -actin; B: The amplification and melting curves of GRP78.

one-way analysis of variance (ANOVA) with Student-Newman-Keuls test as a *post hoc* test. $p < 0.05$ was considered to be statistically significant.

RESULTS

Effect of MeHg exposure on GRP78 protein expression in rat cerebral cortex

Compared with the control group, GRP78 protein expression showed an initial increasing trend followed by a gradual decrease (Fig. 2). GRP78 protein expression significantly increased ($p < 0.05$) in the 4 mg MeHg/kg group and peaked in the 6 mg MeHg/kg group ($180 \pm 3\%$ of control). Interestingly, higher doses of MeHg (8 and 10 mg/kg) failed to further increase GRP78 protein expression. GRP78 expression in the 8 mg MeHg/kg group was lower than the 6 mg MeHg/kg group, but the expression levels remained higher than in the control group ($p < 0.05$). Notably, GRP78 expression in the highest dosing group (10 mg MeHg/kg) was significantly reduced, dropping to levels below the control group ($p < 0.05$).

Effect of MeHg treatment on *Grp78* mRNA expression in the rat cerebral cortex

To further explore the relationship between GRP78 expression and MeHg exposure in rat cerebral cortex, changes in *Grp78* mRNA were examined with real time PCR (Fig. 3). *Grp78* mRNA levels significantly increased even in the lowest MeHg exposure group (2 mg/kg) *vs.* the controls ($p < 0.05$). Nevertheless, unlike GRP78 protein expression levels, *Grp78* mRNA levels were maintained at approximately the same level in the 2, 4, 6 and 8 mg/kg groups ($p > 0.05$ *vs.* controls) (Fig. 3). Consistent with the protein changes, a significant decrease in *Grp78* mRNA was

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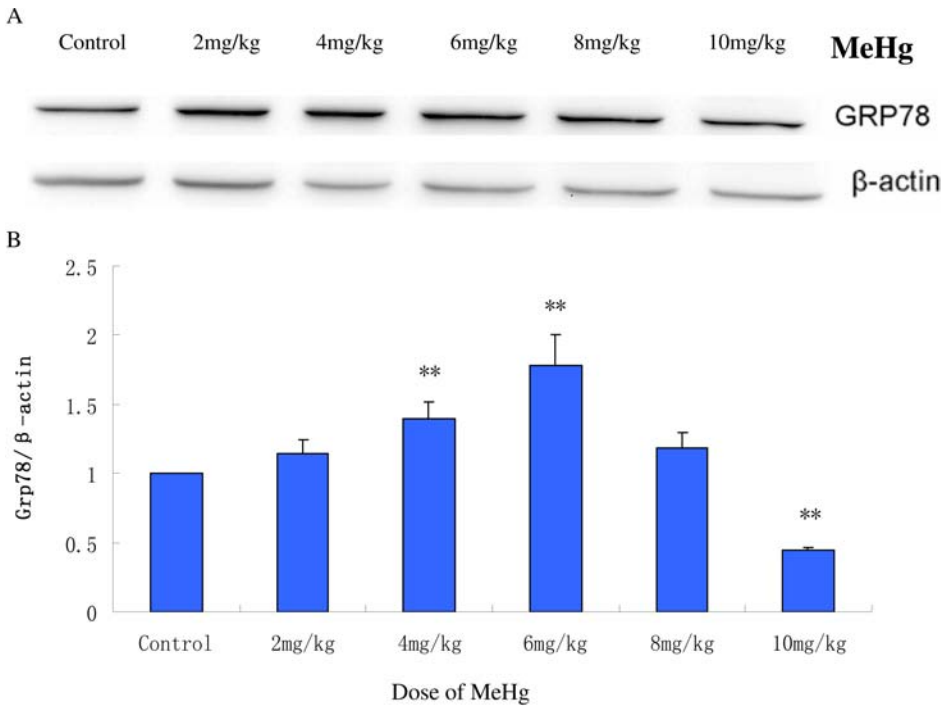


FIG. 2. GRP78 protein expression in the cerebral cortex of rats acutely (6 h) exposed to MeHg. A: Western blot analysis of GRP78 expression. β -actin was used as a loading control. B: Protein bands from the western blots were quantified by scanning densitometry. The relative gray value for the GRP78 protein was expressed relative to β -actin and the control group was set to 1 (n=5). Means \pm SD. ** $P < 0.05$; MeHg treated groups vs. normal saline treated control group (control).

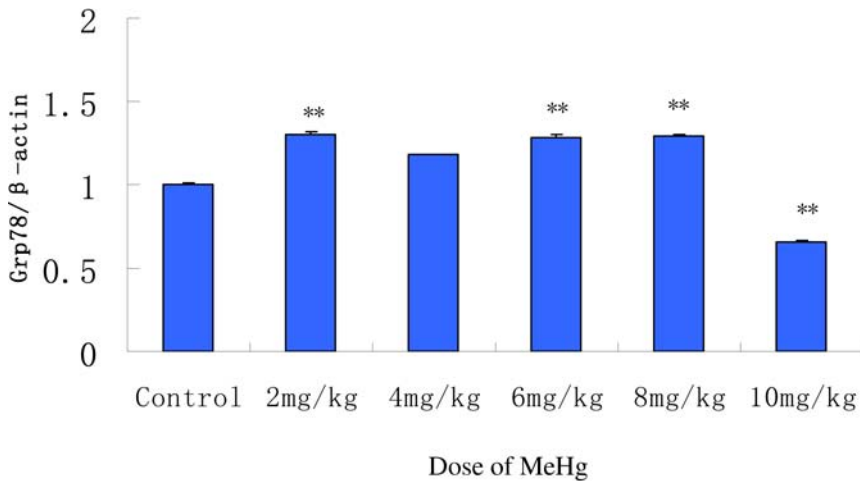


FIG. 3. *Grp78* mRNA expression analyzed by qPCR in the cerebral cortex of rats acutely exposed to MeHg. β -Actin was used as a loading control. The relative value for *Grp78* mRNA was expressed relative to β -actin, and the control group was set to 1 (n=5). Means \pm SD. ** $P < 0.05$; MeHg treated groups vs. normal saline treated control group (control).

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noted in the highest MeHg exposure group (10 mg/kg). Both the *Grp78* mRNA and protein expression were significantly reduced to levels below the controls in this group ($p < 0.05$), suggesting that cellular damage may be inherent to the highest MeHg dosing group.

DISCUSSION

This is the first time a dosimetry study on the effects of MeHg exposure in rat brain has been performed to assess the potential for a hormetic effect. Our results showed a typical inverted U-type curve of rat brain (cortical) GRP78 expression at the protein and mRNA levels, which has previously been defined as hormesis (Calabrese and Baldwin 2001; Calabrese and Blain 2004). Hormesis refers to a process whereby a sublethal stressor renders an organism resistant to subsequent stress. This effect has been demonstrated in multiple stress models, both in cell culture to *in vivo* human models, including studies on dietary restriction, exercise, radiation, chemical and heat exposures (Damelin *et al.* 2000; Damelin and Alexander 2001; Cypser *et al.* 2006; Mattson 2008; Le Bourg 2009). The mechanisms that mediate hormesis are ill defined; nevertheless, previous research has implicated members of the heat shock protein 70 (HSP70) family and metallothionein (MT) proteins as contributors to hormesis. Levels of these proteins are known to increase in response to heavy metal exposures (Damelin *et al.* 2000). Additionally, hormesis has been invoked as a potential explanation for the latent period observed in MeHg exposure and the delayed onset of symptoms (Weiss *et al.* 2002). Heinz and colleagues found that when mallard (*Anas platyrhynchos*) eggs were injected with MeHg at doses ranging from 0 to 6.4 ...g/g egg, the eggs' hatching success was significantly increased at the lowest doses and progressively decreased at incremental doses (Heinz *et al.* 2012). The same group also reported that a parental diet containing 0.5 mg MeHg/g body weight enhanced mallard reproduction (Heinz *et al.* 2010). These results are consistent with low-dose MeHg induction of cytoprotective agents, such as glutathione, heat shock proteins (HSP) and metallothioneins (MT1) as recently documented in *Caenorhabditis elegans* (Helmcke and Aschner 2010).

The disruption of protein synthesis is a primary mechanism of MeHg-induced neurotoxicity. Decreased incorporation of [¹⁴C] leucine has been shown in cerebral and cerebellar slices of rats exposed *in vivo* to this organometal (Yoshino *et al.* 1966; Verity *et al.* 1977). However, the mechanisms underlying this inhibition of protein synthesis have not been established. Notably, increased accumulation of unfolded or misfolded proteins in the ER lumen results in compensatory activation of the UPR (Lee 2001). A hallmark of UPR activation is global reduction in the rate of protein synthesis, which is controlled by reversible phosphorylation of the α -subunit of eIF2. The phosphorylation of eIF2 α prevents formation

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of the eIF2-tRNA^{met}-40s complex, thereby impeding the initiation of protein synthesis (Harding *et al.* 1999; Kaufman 1999). Thus, high-dose MeHg-induced UPR activation and eIF2 α phosphorylation may arrest protein synthesis (Verity *et al.* 1977; Syversen 1981; Fair *et al.* 1987). These mechanisms reduce the flux of nascent proteins entering the ER, and then alleviate the increased burdens of protein processing in the ER (Kaufman 1999).

The inverted U-typed curve noted in our study is a common phenomenon (Calabrese and Blain 2004). Upregulation of the expression of stress proteins, such as HSP70 may play a role in the underlying mechanisms of hormesis (Damelin *et al.* 2000). Qian *et al.* (2001) demonstrated the hormetic effects of lead and mercury on *Grp78* mRNA in C6 rat glioma cells. Low-level inorganic mercury also transcriptionally activated *Grp78* in HepG2 cells (Sutton *et al.* 2002). Though obtained from *in vitro* cultured cells, these data corroborate MeHg's ability to induce a hormetic dose-response effect in ER stress responses. Taken together, our studies along with earlier work on GRP78 expression in response to cadmium, lead, inorganic and organic mercury, nickel and iron (Zhang *et al.* 2010b), suggest that GRP78 is a universal target for heavy metal exposure, and that the ER serves as an intracellular sensor for injuries incurred by toxic metals. Furthermore, though the present acute exposure protocol has merits as it provides the necessary means for obtaining dose range for chronic exposure and data on the difference between acute and chronic effects, the effects of chronic (long-term) exposure to MeHg in rats should be conducted to most closely mimic the human exposure via fish and rice consumption (Grotto *et al.* 2011; Li *et al.* 2010).

In summary, acute exposure to MeHg produced a hormetic effect on cortical ER stress-activated UPR in the rat brain. This effect may be attributed to low-level MeHg activated UPR which serves to combat MeHg-induced oxidative stress and calcium imbalance to keep cellular homeostasis. High levels of GRP78 may bind to MeHg thus chelating free MeHg and reducing its ability to bind to cellular targets (Zhang *et al.* 2009). However, the mechanisms and relevance of the MeHg-induced biphasic change in GRP78 expression warrant further study.

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