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# Endoplasmic reticulum stress preconditioning attenuates methylmercury-induced cellular damage by inducing favorable stress responses

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**We demonstrate that methylmercury (MeHg)-susceptible cells preconditioned with an inhibitor of endoplasmic reticulum (ER) $Ca^{2+}$-ATPase, thapsigargin, showed resistance to MeHg cytotoxicity through favorable stress responses, which included phosphorylation of eukaryotic initiation factor 2 alpha (Eif2α), accumulation of activating transcription factor 4 (Atf4), upregulation of stress-related proteins, and activation of extracellular signal regulated kinase pathway. In addition, ER stress preconditioning induced suppression of nonsense-mediated mRNA decay (NMD) mainly through the phospho-Eif2α-mediated general suppression of translation initiation and possible combined effects of decreased several NMD components expression. Atf4 accumulation was not mediated by NMD inhibition but translation inhibition of its upstream open reading frame (uORF) and translation facilitation of its protein-coding ORF by the phospho-Eif2α. These results suggested that ER stress plays an important role in MeHg cytotoxicity and that the modulation of ER stress has therapeutic potential to attenuate MeHg cytotoxicity, the underlying mechanism being the induction of integrated stress responses.**

Cells and tissues can be protected against a potentially lethal stress by the pre-exposure to the same or different milder stress. Preconditioning cytoprotection has been described in ischemic preconditioning against myocardial infarction[1,2] or delayed neuronal cell death[3], and endoplasmic reticulum (ER) stress preconditioning against renal epithelial cell oxidative injury[4] or cardiomyocyte oxidative injury[5]. ER stress preconditioning can be promoted by the treatment of thapsigargin (TPG), a specific inhibitor of ER $Ca^{2+}$-ATPase[6]. ER $Ca^{2+}$-ATPase maintains the ER $Ca^{2+}$ pool by pumping $Ca^{2+}$ into the ER lumen from the cytoplasm under physiological conditions. Therefore, TPG induces a dose-dependent release of the ER-stored $Ca^{2+}$ pool and promotes ER stress.

Methylmercury (MeHg) is a major environmental toxicant which affects various cellular functions depending on cellular context and developmental phase. MeHg triggers the activation or suppression of several cellular signalling pathways that determine the subsequent cellular fate. Accidental MeHg poisonings involving humans have been recorded, including in Japan[7], Iraq[8], and the USA[9]. MeHg toxicity currently continues to be an environmental risk to human health, especially in susceptible populations who frequently eat substantial amounts of fish or fish predators. The critical role of oxidative stress in the pathogenesis of MeHg toxicity has been demonstrated both *in vitro*[10–16] and *in vivo*[17–19]. Failure to protect cells against MeHg-induced early oxidative stress triggers subsequent ER stress and apoptosis, suggesting that protective factors against ER stress may be important in combating MeHg cytotoxicity, especially in its later stages[14]. Furthermore it has been reported that the overexpression of the ubiquitin-conjugating enzyme cdc34 protects cells against MeHg cytotoxicity[20,21], suggesting that ER stress may play an important role.

Environmental stresses cause various cellular stress responses including initiation of synthesis of stress-related proteins and termination of synthesis of non-stress-related proteins. Some stress-related proteins are predicted to depend on the execution of nonsense-mediated mRNA decay (NMD) due to the presence of upstream open reading frame (uORF) in its 5′-untranslated region (5′ UTR)[22]. NMD is an mRNA surveillance mechanism that eliminates mRNAs containing premature termination codons (PTCs) and PTC-harbored mRNAs are produced

in genetic diseases, cancer, and also normal subjects through alternative splicing[22]. Environmental stresses such as hypoxia[23] and amino acid starvation[22] are known to suppress NMD activity. However, the mechanism of NMD suppression and its role on environmental stress-induced expression of stress-related proteins remain to be elucidated.

In the present study, we adopted ER stress preconditioning approach to investigate cellular stress responses in the context of the mechanism and of protective factors for MeHg toxicity. Our results demonstrate that ER stress preconditioning by the pretreatment with TPG alleviates MeHg-induced cytoxicity through the induction of favorable stress responses. We also showed that the ER stress preconditioning induced suppression of NMD mainly through the phospho-eukaryotic initiation factor 2 alpha (Eif2α)-mediated general suppression of translation initiation.

## Results

**Effect of pretreatment with TPG on MeHg cytotoxicity.** The MeHg-susceptible C2C12 myogenic cell line (C2C12-DMPK160) showed signs of apoptosis within 24 h after exposure to low levels of MeHg[13,14]. Since the cytotoxic effect of MeHg was mediated by the early oxidative stress and subsequent ER stress response[14,20,21], we hypothesized that ER stress preconditioning could provide protection against MeHg cytotoxicity. To address this, we first examined the effect of prior ER stress on MeHg-induced cell damage. Based on the previous cell viability data[14], two points of higher dose than $EC_{50}$ value for MeHg were examined. As shown in Fig. 1A, pretreatment with 0.1–0.3 μg/ml TPG protected C2C12-DMPK160 cells against both 0.4 and 0.5 μM MeHg-induced cytotoxicity.

We next analyzed the protective effect of ER stress preconditioning on MeHg-induced apoptosis by flow cytometry (Fig. 1B). C2C12-DMPK160 treated with 0.4 μM MeHg underwent apoptosis to a greater extent than untreated cells. Pretreatment with 0.3 μg/ml TPG attenuated MeHg-induced apoptosis. The results indicate that pretreatment with TPG effectively protects against MeHg-induced apoptotic processes.

**Effect of pretreatment with TPG on the MeHg-induced increase in intracellular reactive oxygen species (ROS).** Because MeHg-induced apoptosis is triggered by an increase in intracellular ROS levels[13,14], we investigated the effect of ER stress preconditioning on MeHg-induced oxidative stress using flow cytometry. Results demonstrated that a MeHg-mediated increase in ROS occurred 7 h after exposure in cells pretreated with TPG, which was later than in non-preconditioned cells (Fig. 2A). The results suggest that ER stress preconditioning alleviates MeHg-induced oxidative stress.

Since the MeHg-mediated increase in ROS induces the activation of mitogen-activated protein kinase (MAPK) pathways, which are involved in MeHg-induced apoptosis in C2C12-DMPK160[13,14], we investigated the effect of prior ER stress on MAPK pathway activation. As shown in Fig. 2B, the apoptosis–related stress–activated protein kinases (SAPK)/c-Jun $NH_2$-terminal kinases (JNK) pathway was activated by MeHg exposure, while pretreatment with TPG suppressed this effect. In contrast, the cell survival–related extracellular signal–regulated kinase (ERK) pathway was upregulated in TPG-pretreated cells compared to non-pretreated cells.

**Pretreatment with TPG upregulates antioxidant protein–coding mRNAs.** Because ER stress preconditioning suppressed early MeHg-induced increases in ROS, basal changes in antioxidant mRNAs were examined to determine the antioxidant capacity of preconditioned cells. As shown in Fig. 3A–C, pretreatment with TPG induced significant upregulation of mRNAs encoding the anti-oxidant enzymes, glutathione peroxidase 1 (Gpx1), thioredoxin reductase 1 (Txnrd1), and manganese superoxide dismutase (Mn-Sod) compared to non-preconditioned cells. Western blot analyses of cell lysates demonstrated that nuclear factor erythroid 2-related factor 2 (Nrf2), Txnrd1, and Mn-Sod antioxidant proteins were induced in

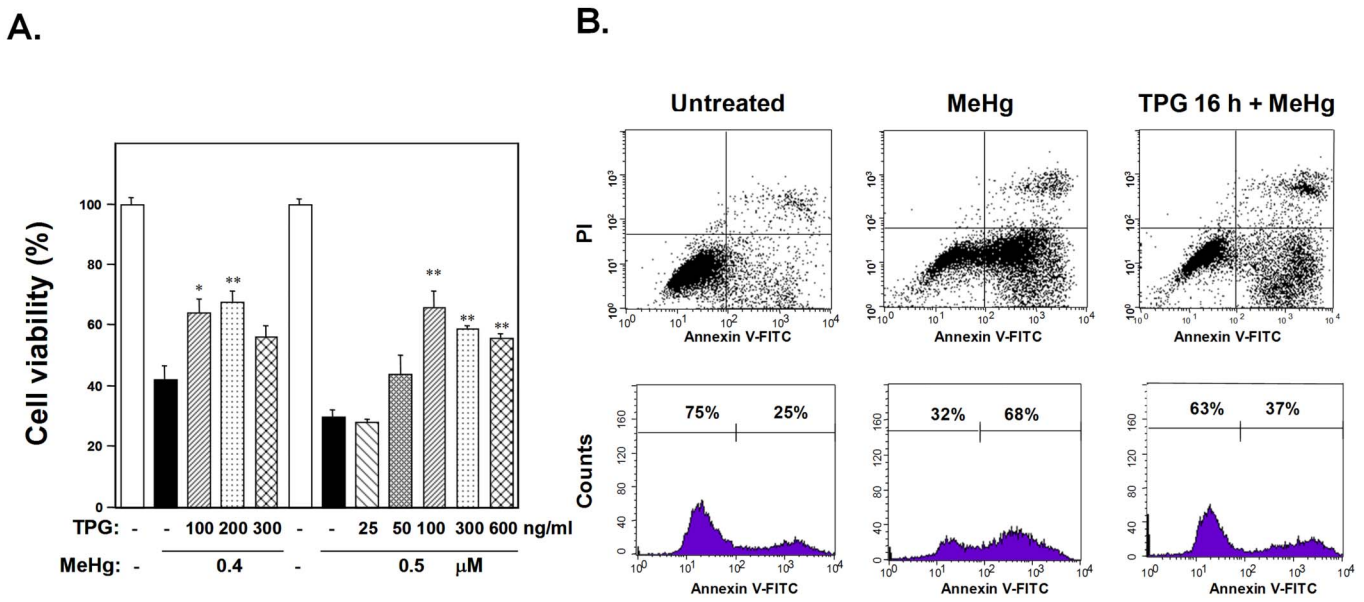


**Figure 1 | Effect of pretreatment with TPG on MeHg cytotoxicity.** (A) Cell viability of C2C12-DMPK 160 cells pretreated with TPG 16 h before exposure to 0.4 or 0.5 μM MeHg was determined. Pretreatment with TPG (100–300 ng/ml) attenuated MeHg cytotoxicity. The viability of untreated cells was regarded as 100%. Values represent means ± SE (n = 6). *, **Significantly different from TPG-untreated and MeHg-treated cells by a one-way ANOVA (*$p < 0.05$, **$p < 0.01$). (B) Apoptosis analysis. The upper panel shows flow cytometry analysis of C2C12-DMPK160 stained with propidium iodide (PI) and FITC-Annexin V. The vertical axis indicates PI fluorescence intensity and horizontal axis Annexin V fluorescence. Exposure to 0.4 μM MeHg for 16 h increased the number of cells undergoing apoptosis (Annexin V-FITC-positive and PI-negative). A minor population of cells was observed to be Annexin V-FITC- and PI-positive, indicating that they were in end-stage apoptosis or already dead. The lower panel shows the profile of frequency of viable cells (Annexin V-FITC- and PI-negative) and cells undergoing apoptosis (Annexin V-FITC-positive and PI-negative). Pretreatment with TPG decreased the frequency of cells undergoing apoptosis.

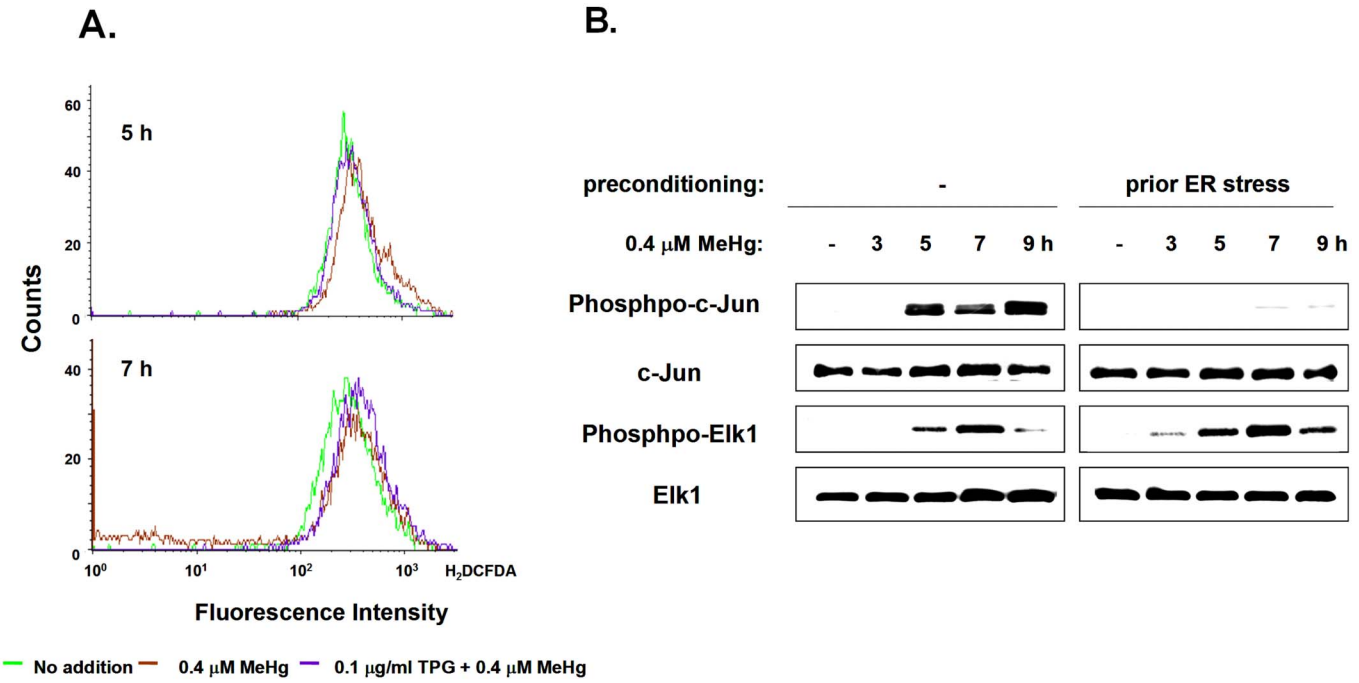


**Figure 2** | (A) Flow cytometry analysis of C2C12-DMPK160 cells labeled with CM-$H_2$DCFDA for investigation of intracellular ROS after 0.4 μM MeHg exposure for 4 or 7 h. Data shown are representative of 3 separate experiments. (B) Effect of pretreatment with TPG on SAPK/JNK, or ERK signaling pathways in C2C12-DMPK160 cells exposed to MeHg. Cells were pretreated with TPG (0.1 μg/ml) for 16 h and exposed to 0.4 μM MeHg. Total cell lysates prepared at the times indicated were analyzed by western blot using the indicated antibody probes. The images for each indicated probe were cropped from the same blot.

preconditioned cells (Fig. 3D). We could not obtain a suitable antibody for the detection of Gpx1 expression.

**Pretreatment with TPG up-regulates stress-related protein–coding mRNAs.** In order to determine the mechanism by which prior ER stress attenuates MeHg cytotoxicity, the expressions of mRNAs encoding stress-related proteins glucose regulated protein of 78 kDa (Grp78), metallothionein 1 (Mt1), activating transcription factor 4 (Atf4) were investigated. GRP78, the most abundant ER-resident molecular chaperone, functions to protect the ER by binding unfolded proteins and excessive $Ca^{2+}$[24]. By contrast, MT1 is a small sulfhydryl-rich protein and is thought to play roles in heavy metal regulation and scavenging of free radicals. MT1 is known to be induced by ER stress[25] as well as oxidative stress[26,27]. ATF4 is a transcriptional activator that modulates a wide spectrum of downstream genes involved in adaptation to stresses and has been reported to activate the GRP78 promoter[28].

Exposure to MeHg for 9 h upregulated Grp78 mRNA by 4.5 times over the untreated level and an antioxidant Trolox suppressed the MeHg-induced upregulation of Grp78 mRNA, suggesting that MeHg-induced oxidative stress triggers subsequent ER stress. However, increased expression of Grp78 mRNA was induced in preconditioned cells, to levels 7 times above those of non-preconditioned cells (Fig. 4A). The expressions of mRNA encoding another stress-related protein, Mt1 were also significantly upregulated after MeHg exposure compared to untreated cells and co-treatment with Trolox suppressed the upregulation of Mt1 mRNA under MeHg exposure, suggesting that MeHg-induced oxidative stress triggers its upregulation. Pretreatment with TPG upregulated Mt1 mRNA to 4 times the non-preconditioning level, which is greatly elevated compared to that induced by exposure to 0.4 μM MeHg (Fig. 4B). In addition, Atf4 mRNA was also significantly upregulated by pretreatment with TPG compared to non-pretreated cells (Fig. 4C).

**Pretreatment with TPG up-regulates stress-related proteins.** As prior ER stress induced the expression of mRNAs encoding Grp78, Mt1, and Atf4, protein-level changes in several stress-response factors were examined. As shown in Fig. 5A, pretreatment with TPG upregulated the expression of phospho-Eif2α, Atf4, and Grp78 compared to non-preconditioned cells. Although transcription factors ATF6 and XBP1 are known to be related to GRP78 expression, the induction of ATF6 was observed later after MeHg exposure (Fig. 5A) and XBP1 expression was not observed at least from 17 h after pretreatment with TPG to 9 h after MeHg exposure (data not shown). It is possible that the expression of ATF4 depends on the execution of nonsense-mediated mRNA decay (NMD) due to the presence of upstream open reading frame (uORF) in its 5′-untranslated region (5′ UTR)[22]. Therefore we next investigated NMD activity under conditions of prior ER stress. As shown in Figure 5B, pretreatment with TPG led to NMD suppression, evidenced by the upregulation of non-protein coding small nucleolar RNA host gene 1 (Snhg1) mRNA harboring premature translation termination codon (PTC). In addition, Upf1 phosphorylation was decreased in ER stress-preconditioned cells compared to non-preconditioned cells (Fig. 5C). Upf1 phosphorylation is a marker of NMD activation, since the Upf1 phosphorylation and dephosphorylation cycle is essential for NMD[29,30]. Furthermore, ER stress preconditioning led to a decrease in the expression of Upf1, Eif4a3 and Smg-6, but not of Smg-1 and Smg-7 (Fig. 5C). In addition, reduction of Smg-7 expression was observed after MeHg treatment which is enhanced in ER stress-preconditioned cells (Fig. 5C). These results indicate that NMD was suppressed in ER stress-preconditioned cells most likely through phospho-Eif2α–mediated translation suppression for non-stress-related mRNAs[31]. A reduction in the expression of several NMD components might contribute the suppression of NMD.

In order to ascertain whether suppression of NMD upregulated Atf4 protein levels, we examined Atf4 expression in NMD-suppressed cells transfected with synthetic siRNA targeting mouse Smg-1 or Smg-7[15]. As shown in Fig. 5D, NMD suppression did not induce an increase in Atf4 protein expression. By contrast, levels of

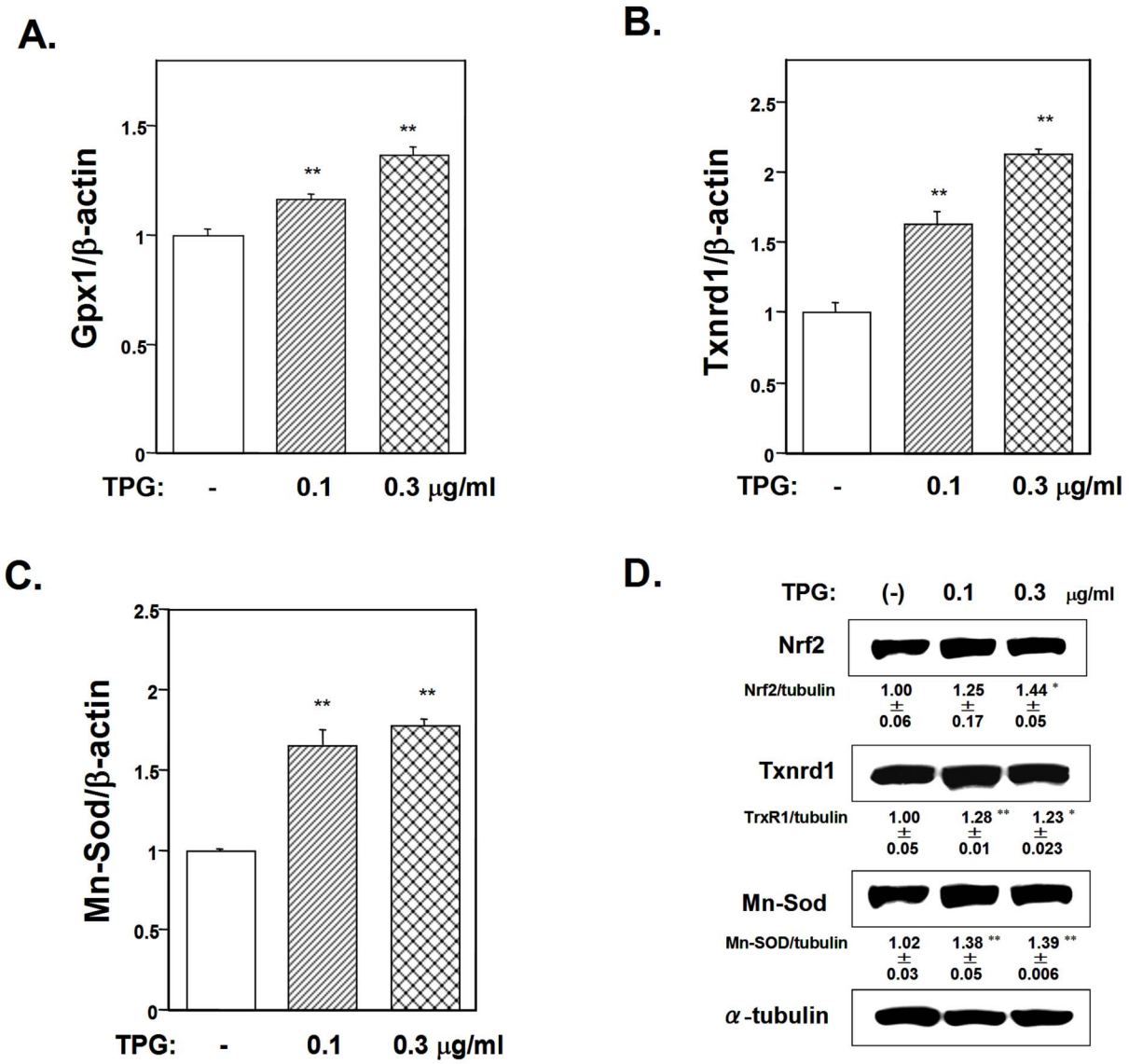


**Figure 3 | Effect of pretreatment with TPG on the expression of antioxidants in C2C12-DMPK160 cells.** Expression of Gpx1 (A), Txnrd1 (B), and Mn-Sod (C) mRNA was analyzed by quantitative real-time PCR. Total RNA was extracted from cells treated with 0.1 or 0.3 μg/ml TPG for 16 h. The histogram depicts the indicated mRNA normalized to β-actin. Values shown are means ± SE of 4 separate experiments.**Significantly different from TPG-untreated cells by a one-way ANOVA (**$p < 0.01$). (D) Western blot analyses of Nrf2, Txnrd1, and Mn-Sod. Western blots of C2C12-DMPK160 cells pretreated with 0.1 or 0.3 μg/ml TPG for 16 h were analyzed with the indicated antibody probes. The densitometric quantification of each protein was normalized to α-tubulin and is represented as fold increase over the non-pretreated cells. Although cropped blots were used, the gels were run under the same experimental conditions. Representative images of 3 samples are shown with quantitative data (means ± SE). *, **Significantly different from TPG-untreated cells by a one-way ANOVA (*$p < 0.05$, **$p < 0.01$).

Atf4 mRNA were increased when NMD was suppressed (Fig. 5E). The results suggest that Atf4 protein expression is not regulated by NMD. In addition, no up-regulation of Grp78 protein was observed in these NMD-suppressed cells.

**Effect of Grp78- or Mt1-knockdown on MeHg cytotoxicity.** Pretreatment with TPG induced the expression of stress-related Grp78 and Mt1 mRNAs to a greater extent than those encoding antioxidative enzymes. In order to determine key factors influencing protection against MeHg cytotoxicity, we investigated the effect of synthetic siRNA-mediated knockdown of Grp78 or Mt1 on cell viability and expression of α-phospho-histone H2ax (γ-H2ax), which is expressed as part of the early response to DNA strand breakage[32]. Transfection of synthetic siRNA targeting Grp78 or Mt1 into C2C12-DMPK160 cells caused the knockdown of each corresponding protein (Fig. 6A). Transfection with synthetic siRNA targeting Grp78 led to a significant decrease in cell viability after 24 h compared to non-silencing siRNA-transfectants or Mt1-knockdown, indicating that Grp78-knockdown alone causes cell injury, even in the absence of MeHg treatment (Fig. 6B). Immunostainning analyses of γ-H2ax demonstrated that Grp78-knockdown augmented MeHg cytotoxicity and blocked protective pathway to cell survival in preconditioned cells exposed to MeHg (Fig. 6C middle and Fig. 6D). On the other hand, pretreatment with TPG protected Mt1-knockdown cells from MeHg-mediated cell injury (Fig. 6C bottom and Fig. 6D).

**Effect of Mt1-knockdown on the mRNA expression of antioxidant enzymes and Grp78.** Since pretreatment with TPG could rescue MeHg cytotoxicity even in Mt1-knockdown cells, the effect of pretreatment with TPG on the mRNA expression of antioxidant enzymes (Gpx1, Txnrd1, and Mn-Sod) and Grp78 was

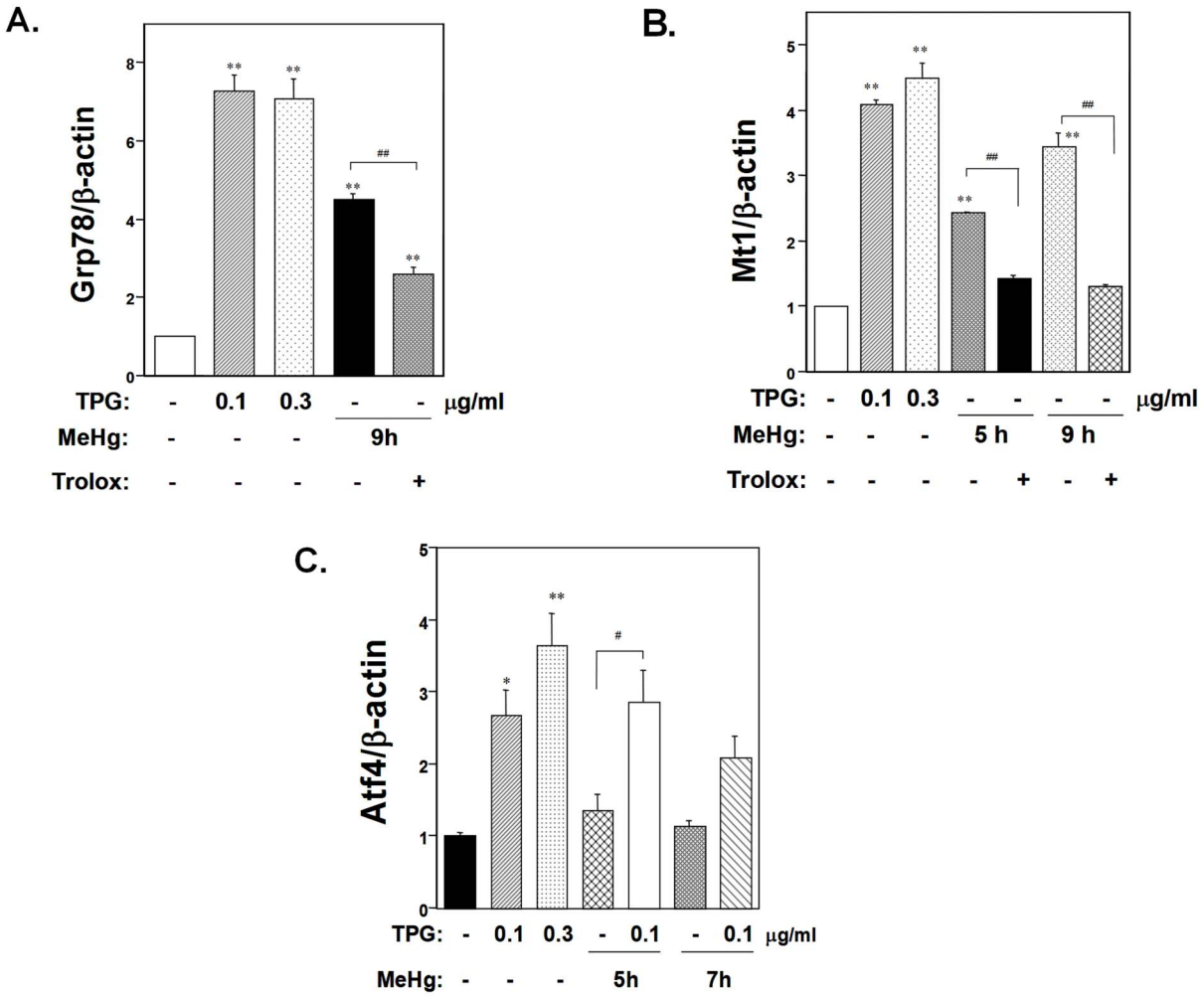


**Figure 4 | Effect of pretreatment with TPG on the expression of Grp78 (A), Mt1 (B), or Atf4 mRNA (C) in C2C12-DMPK160 cells.** Total RNA was extracted from cells treated with 0.1 or 0.3 μg/ml TPG for 16 h, or cells exposed to 0.4 μM MeHg with or without 100 μM Trolox. Expression of Grp78, Mt1, or Atf4 mRNA was analyzed by quantitative real-time PCR. The histogram depicts the indicated mRNA normalized to β-actin. Values shown are means ± SE of 4 separate experiments. *, **Significantly different from TPG- and MeHg-untreated cells by a one-way ANOVA (*$p < 0.05$, **$p < 0.01$). #, ##Significantly different from Trolox-untreated cells by a one-way Welch's *t*-test (#$p < 0.05$, ##$p < 0.01$).

investigated. As shown in Fig. 7A–D, pretreatment with TPG induced significant upregulation of Txnrd1, Mn-Sod, and Grp78 mRNAs in Mt1-knocked down cells compared to non-silencing siRNA-transfectants although expression of Txnrd1 in TPG-untreated cells was downregulated compared to non-silencing siRNA-transfectants. Among them, upregulation of Grp78 mRNA was remarkable. The results suggest the important role of Grp78 in the protection of Mt1-knocked down cells against MeHg-intoxication.

## Discussion

In this study, we demonstrated that ER stress preconditioning—by the pretreatment with concentrations (0.1–0.3 μg/ml) of a specific inhibitor of ER $Ca^{2+}$-ATPase, TPG, for 16 h—alleviates MeHg toxicity in MeHg-susceptible C2C12 myogenic cell line. The same effect was demonstrated with another ER stressor tunicamycin (Supplemental Figure 1). The results suggest that the modulation of ER stress has therapeutic potential to attenuate MeHg-induced cell injury.

ER stress preconditioning induced several favorable protective conditions against MeHg cytotoxicity. These included the up-regulation of stress-related proteins Grp78 and Mt1, antioxidant enzymes, phosphorylation of Eif2α, and expression of Atf4. Although the up-regulation of both Grp78 and Mt1 was observed in preconditioned cells, our siRNA-mediated knockdown study revealed that Grp78 is the key factor for protection against MeHg cytotoxicity. Knockdown of Grp78 alone was able to cause cell death, whereas no damage was observed in Mt1-knockdown cells (Fig. 6B). In addition, ER stress preconditioning induced notable up-regulation of Grp78 mRNA in addition to those of antioxidant enzymes in Mt1-knockdown cells (Fig. 7A–D), leading to cytoprotection against MeHg cytotoxicity (Fig. 6C, D). In contrast, Grp78-knockdown cells pretreated with TPG could not attenuate cell death exacerbated by MeHg. The results strongly suggest that Grp78 is a crucial factor in cellular resistance to MeHg cytoxicity induced by ER stress preconditioning.

Under a variety of environmental stresses, gene expression unrelated to stress responses is translationally inhibited, in order to relieve cells of further protein burden. This translational control under stress is triggered by the phosphorylation of the translation initiation factor eIF2α[33]. Here, we demonstrate that ER stress preconditioning induces the phosphorylation of Eif2α and upregulation of Atf4 mRNA and protein (Fig. 4C, 5A). Phospho-Eif2α/Atf4 pathway is referred to as the "integrated stress response" (ISR), of which Atf4 is an important mediator. ATF4 has been reported to regulate the expression of the Heme oxygenase-1 gene in a possible complex with

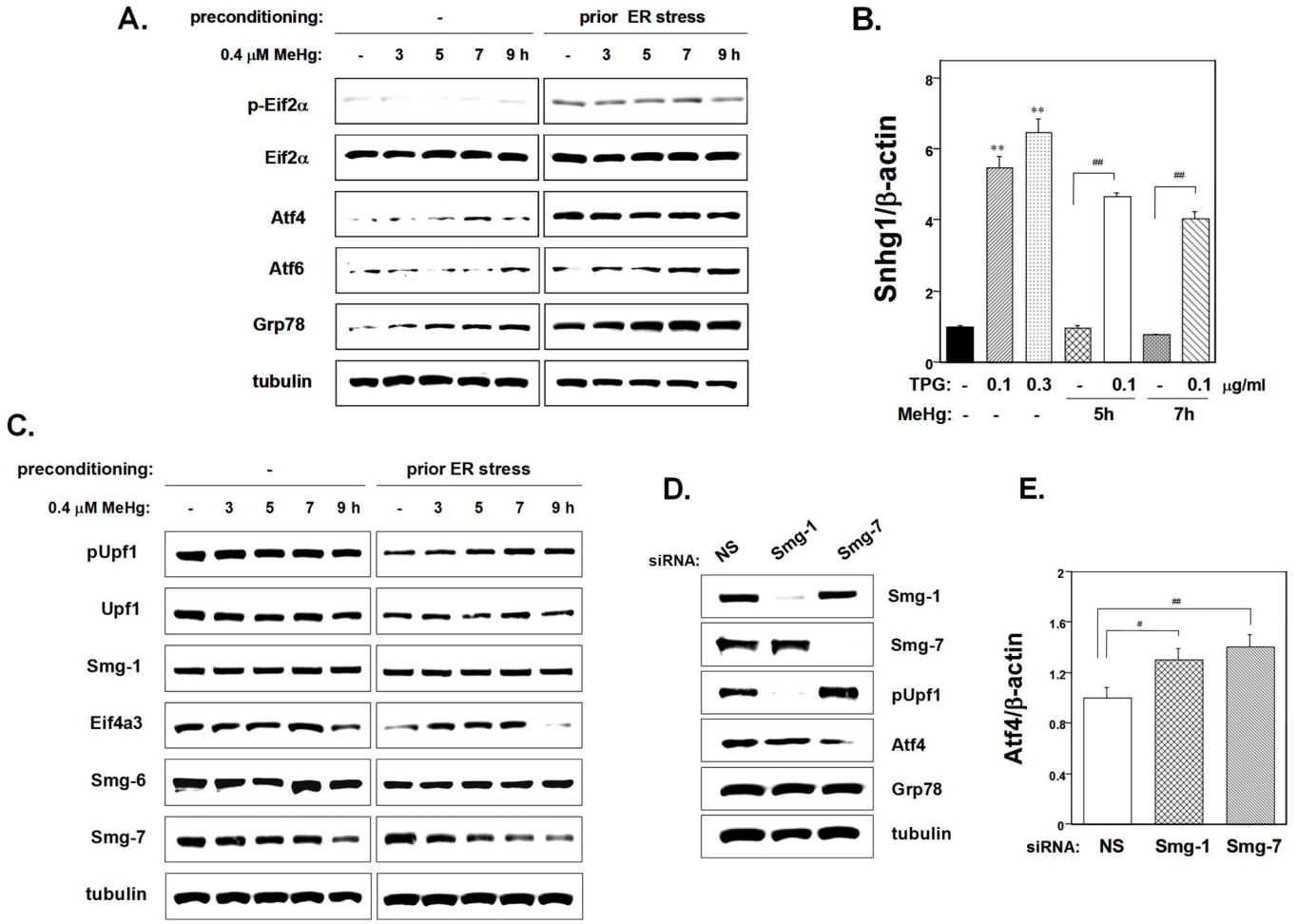


**Figure 5 | Changes in stress-related proteins after pretreatment with TPG.** (A) Effect of pretreatment with TPG on the expression of stress-related proteins after MeHg exposure analyzed by western blotting. Total cell lysates prepared at the times indicated were analyzed with the indicated antibody probes. Although cropped blots were used, the gels were run under the same experimental conditions. (B) Effect of pretreatment with TPG on the expression of Snhg1 mRNA. The histogram depicts Snhg1 mRNA normalized to β-actin analyzed by quantitative real-time PCR. Values are represented as fold increase over that of non-pretreated controls and are means ± SE of 4 separate experiments. **Significantly different from TPG- and MeHg-untreated cells by a one-way ANOVA (**$p < 0.01$). ##Significantly different from TPG-untreated cells by a one-way Welch's *t*-test (##$p < 0.01$). (C) Effect of pretreatment with TPG on the expression of NMD-related factors analyzed by western blotting. Total cell lysates prepared at the times indicated were analyzed with the indicated antibody probes. Although cropped blots were used in this figure, the gels were run under the same experimental conditions. (D) Effect of NMD suppression on the expression of Atf4 and Grp78. Western blots of C2C12-DMPK160 transfected with the indicated synthetic siRNAs were analyzed with the indicated antibody probes. Although cropped blots were used in this figure, the gels were run under the same experimental conditions. NMD inhibition was supported by the decrease (in the case of Smg-1 knockdown) or increase (in the case of Smg-7 knockdown) in Upf1 phosphorylation, a central component of NMD (NS, non-silencing). (E) Effect of NMD suppression on the expression of Atf4 mRNA. The histogram depicts Atf4 mRNA normalized to β-actin analyzed by quantitative real-time PCR. Values are represented as fold increase over that of NS-transfectants and are means ± SE of 4 separate experiments. #, ##Significantly different from NS-transfectants by a one-way Welch's *t*-test (#$p < 0.05$, ##$p < 0.01$).

Nrf2[34], suggesting that the upregulation of antioxidants (Gpx1, Txnrd1, and Mn-Sod) may be caused by the expression of Atf4, induced by ER stress preconditioning.

Although the protein-coding region of ATF4 is poorly translated in unstressed conditions, it is known to be induced following the phosphorylation of eIF2α in stressed cells[33], resulting in the upregulation of ATF4 transcriptional targets[35]. It is possible that ATF4 mRNA is degraded by the NMD mechanism under unstressed conditions, due to the presence of 3 uORFs in its 5′ UTR[22]. In this study, we demonstrated that ER stress preconditioning suppresses NMD activity, most likely through the phospho-Eif2α-mediated general suppression of translation initiation of many factors associated with protein synthesis, evidenced by the upregulation of PTC-harboring non-protein coding Snhg1 mRNA (Fig. 5B). However, NMD suppression by the knockdown of NMD components Smg-1 or Smg-7, could induce the upregulation of Atf4 mRNA (Fig. 5E) but not protein (Fig. 5D). Therefore, the upregulation of Atf4 protein by ER stress preconditioning appears to be mainly mediated by translation facilitation of its protein-coding ORF, enhanced by phospho-Eif2α-induced facilitation of the bypass of inhibitory uORF[36]. ER stress preconditioning downregulated Upf1 and Eif4a3 proteins but not Smg-1, physiological protein kinase of Upf1, or Smg-6. NMD may be suppressed by the combined effects of decreased expression of several NMD components, in addition to suppression of general translation initiation (Fig. 8). Although a recent study reported that SMG-6 mRNA was upregulated in ER stress-induced HeLa cells and suggested that NMD deficiency causes ER stress[37], we could not detect Smg-6 protein upregulation by ER stress preconditioning or Grp78 by NMD suppression in this study.

Finally, ISR induced by ER stress preconditioning caused the delay of oxidative stress and a signaling pathway shift towards cell survival under MeHg exposure. We show that cytoprotection, afforded by

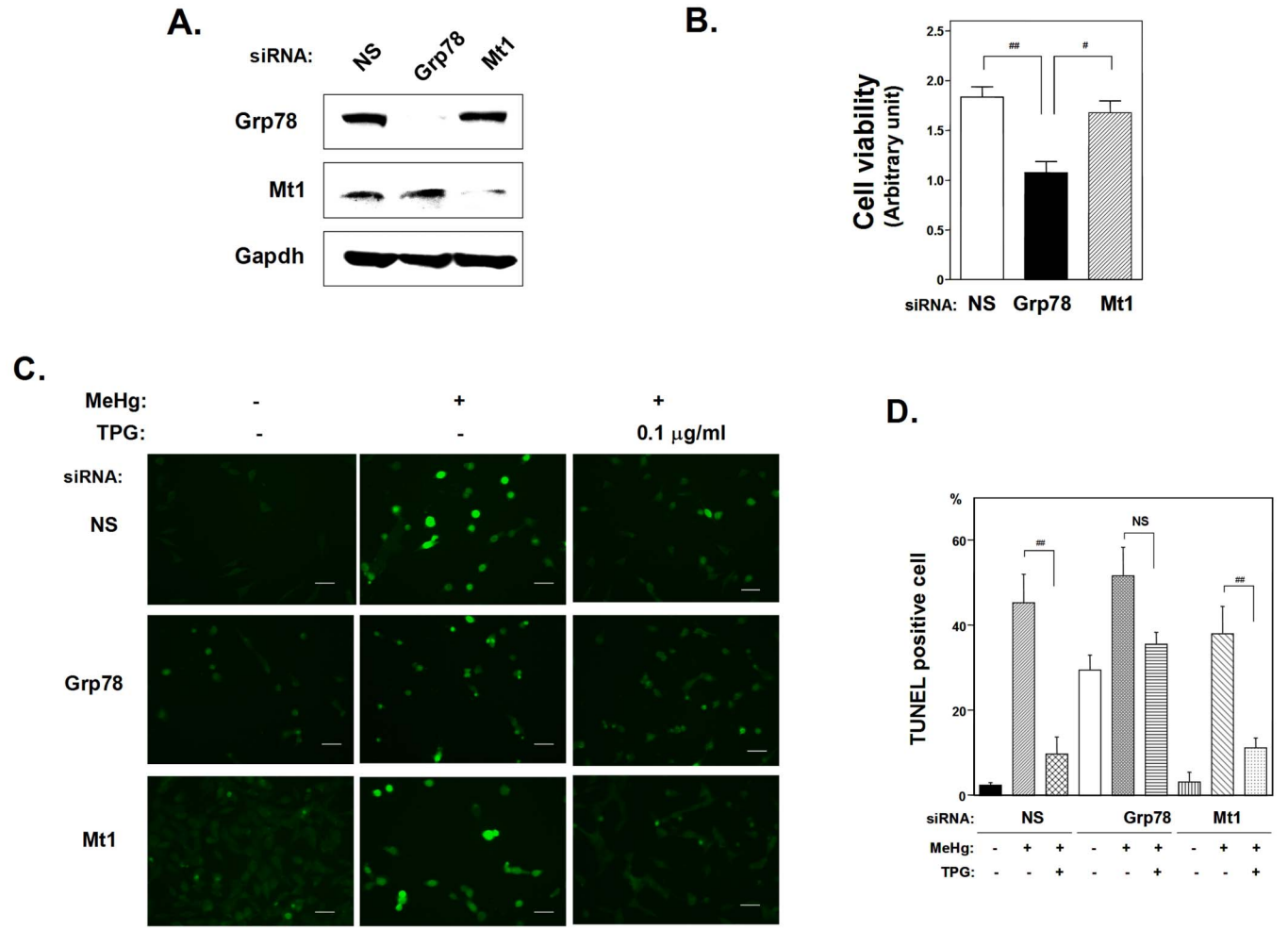


**Figure 6 | Effect of knockdown of Grp78 or Mt1 on MeHg-induced cytotoxicity.** NS, non-silencing. (A) Synthetic siRNA-mediated knockdown of Grp78 or Mt1. Western blots of C2C12-DMPK160 transfected with the indicated synthetic siRNAs were analyzed with the indicated antibody probes. Although cropped blots were used in this figure, the gels were run under the same experimental conditions. (B) Cell viability study. Viability was determined 48 h after transfection of each siRNA. Values represent means ± SD (n = 6). $^{\#,\#\#}$Significantly different from Grp78-knockdown cells by a one-way Welch's *t*-test ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$). (C) γ-H2ax immunostaining. C2C12-DMPK160 cells transfected with the indicated siRNA were pretreated with 0.1 μg/ml TPG 16 h before MeHg exposure. Cells were fixed 11 h after MeHg exposure and stained with anti-γ-H2ax antibody. Representative photographs are shown. Bar = 50 μm. (D) Percentage of γ-H2ax-positive nuclei in Mt1- and Grp78-knockdown, and NS siRNA-transfected cells (means ± SE). Cells were detected by counterstaining of cell nuclei with Hoechst 33342. Three hundred cells in 3–5 fields were analyzed in 3 separate experiments. $^{\#\#}$Significantly different from TPG-untreated and MeHg-treated cells by a one-way Welch's *t*-test ($^{\#\#}p < 0.01$).

prior ER stress against MeHg toxicity, is mediated by enhanced activation of the ERK pathway and inhibition of the SAPK/JNK pathway (Fig. 2B). ERKs play a central role in cell survival, whereas JNK and p38 MAPK pathways are preferentially activated by environmental stresses and are actively involved in various stress responses and apoptosis[38,39]. In this study, a counteracting effect altering the balance between JNK and ERK phosphorylation was observed under MeHg exposure in ER stress–preconditioned cells. Similar results have been described previously, demonstrating that a MeHg-resistant cell line showed activation of the ERK signaling pathway, related to cell survival[40]. Prior ER stress can shift the balance of SAPK/JNK and ERK pathways under MeHg exposure to promote cell survival. An increase in ERK and a decrease in JNK activation are important distal mediators of the ER stress preconditioning in protection against MeHg cytotoxicity.

In conclusion, we demonstrated that ER stress preconditioning can protect cells against MeHg toxicity by the induction of ISR, activation of phospho-Eif2α/Atf4 pathway. The upregulation of Atf4 protein by ER stress preconditioning appears to be mainly mediated by phospho-Eif2α-induced facilitation of the bypass of inhibitory uORF. The induction of ISR led to the upregulation of Grp78, which is critical to protect cells against MeHg cytotoxicity. The results indicate that ER stress plays an important role in MeHg cytotoxicity and that the modulation of ER stress has therapeutic potential to attenuate MeHg-induced cell injury. Atf4 and Grp78 are the major targets in ER stress-induced protection against MeHg toxicity.

## Methods

**Chemicals and reagents.** We purchased TPG, Trolox, and dithiothreitol (DTT) from Sigma-Aldrich, a Cell Counting Kit from DOJINDO, nitrocellulose membranes from Bio-Rad, SAPK/JNK and p44/42 MAPK Assay Kits from Cell Signaling Technology, a SYBR Green Master Mix from Roche, Lipofectamine RNAiMAX and 5-(and 6-)chloromethyl-2′,7′-dichlorodihydrofluorescin diacetate, acetyl ester (CM-$H_2$DCFDA) and Geneticin (G418) from Life Technologies, Dulbecco's modified Eagle's medium (DMEM) and glutamine from Nissui Pharmaceuticals, fetal bovine serum (FBS) from HyClone. Antibodies were purchased from the following suppliers: anti-Mn-Sod (Santa Cruz); anti-Eif2α, anti-phospho-Eif2α, anti-Grp78, and anti-glyceraldehyde 3-phosphate dehydrogenase (Gapdh) (Cell Signaling Technologies); anti-Atf4, anti-Nrf2, anti-Mt1 (Santa Cruz). siRNAs were purchased from Qiagen: mouse Grp78, FlexiTube siRNA SI02674427; mouse Mt1, FlexiTube siRNA SI01319269; and All Star Negative Control siRNA.

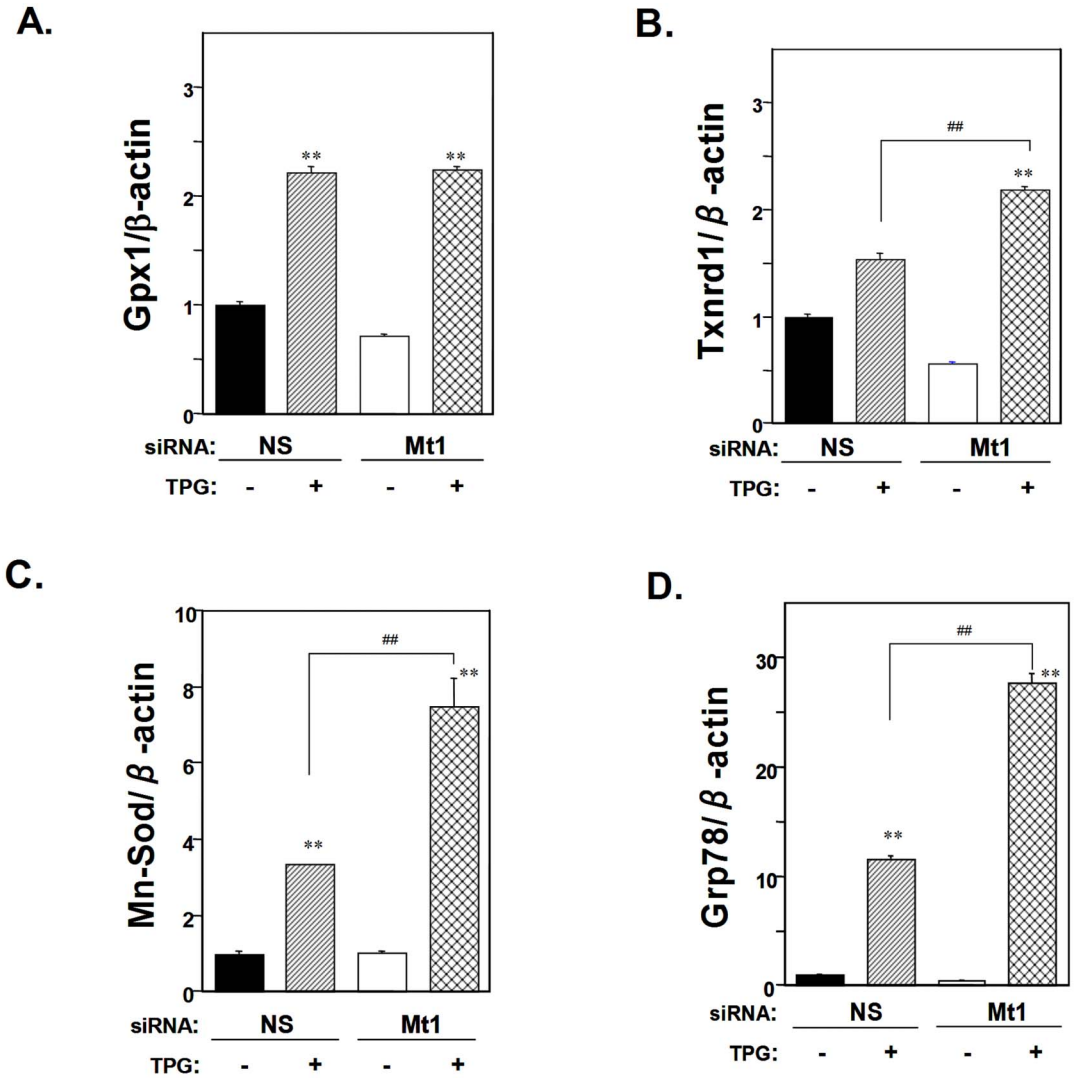


**Figure 7 | Effect of pretreatment with TPG on the expression of antioxidants and Grp78 mRNAs in NS siRNA-transfectants and Mt1-knocked down cells.** The histogram depicts each mRNA normalized to β-actin, represented as fold increase over non-pretreated controls. Values shown are means ± SE of 4 separate experiments. **Significantly different from TPG-untreated cells by a one-way ANOVA (**$p < 0.01$). ##Significantly different from NS-transfectants by a one-way Welch's *t*-test (##$p < 0.01$).

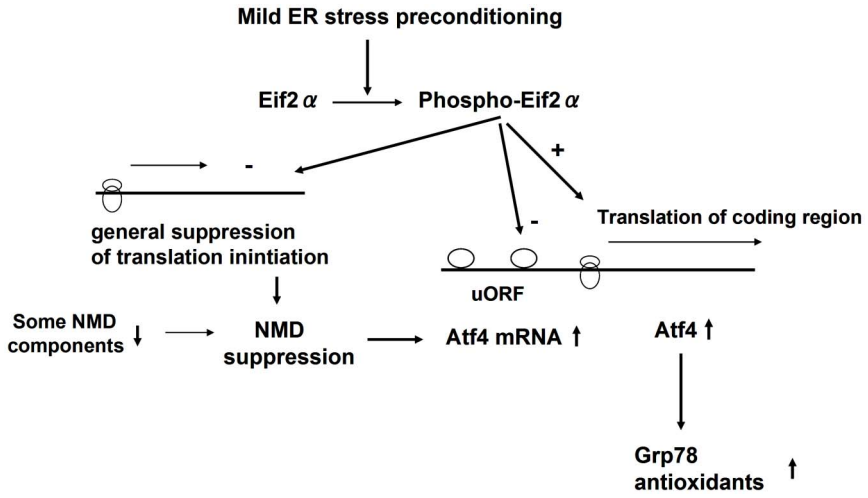


**Figure 8 | Summarized stress responses induced by ER stress preconditioning.** ER stress preconditioning induces Eif2α phosphorylation. Translation of Atf4 coding region is enhanced by phospho-Eif2α-induced facilitation of the bypass of inhibitory uORF. NMD activity is suppressed by the combined effects of decreased expression of several NMD components, in addition to suppression of general translation initiation mediated by phospho-Eif2α, leading to Atf4 mRNA up-regulation.

**Cell culture and drug treatments.** C2C12-DMPK160[13–15] cells were cultured in DMEM supplemented with 10% FBS, 300 μg/ml glutamine and 0.4 mg/ml G418 and exposed to MeHg in serum-free Cosmedium (Cosmo Bio Co., Ltd) as described previously[13,14]. Trolox was prepared as described previously[12,13]. TPG stock was dissolved in dimethylsulfoxide. Tunicamycin was prepared as described previously[14]. For the preconditioning study, TPG or tunicamycin was added to the cells for 16 h before MeHg treatment. After removal of TPG, cells were exposed to MeHg. Viability was determined using a Cell Counting Kit and WST-1 dye 24 h after MeHg exposure.

**Flow cytometry analysis.** Flow cytometry analysis for ROS or apoptosis were previously described[15]. Briefly, for analyses of ROS, cells ($1.3 \times 10^5$ cells/35-mm dish), with or without pretreatment with 0.1 μg/ml TPG, were incubated with 500 nM CM-$H_2$DCFDA for 30 min and then exposed to 0.4 μM MeHg. For analysis of apoptosis, cells ($1.3 \times 10^5$ cells/35-mm dish) with or without pretreatment with 0.3 μg/ml TPG were exposed to 0.4 μM MeHg for 16 h. Harvested cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences).

**Quantitative real-time PCR.** Total RNA was extracted, and first-strand cDNA was prepared as described previously[15]. Quantitative real-time PCR was performed using a LightCycler DX 400 System (Roche). Gpx1, Txnrd1, Mn-Sod, Grp78, Mt1, Snhg1, and Atf4 mRNAs were amplified using a SYBR Green Master Mix and specific primer sets. Specific primer sets for Gpx1, Txnrd1, Mn-Sod, Grp78, Mt1 have been described previously[14,15]. Primer sets for Snhg1 and Atf4 (Gene Bank accession numbers: NR_002896 and NM_009716, respectively) were as follows: Snhg1 5′-AAAAGGATGGGTGTACGCTC-3′ (nucleotides 40–59) and 5′-TGGTACGGCTCCTTTGTTCT-3′ (nucleotides 287–306), and Atf4 5′-TGCACTTTTCTCTCATGGGG-3′ (nucleotides 106–125) and 5′-GGAGCACACAACTTAAACCG-3′ (nucleotides 336–355). Transcript levels were normalized to β-actin mRNA as described previously[15].

**Western blot analysis.** Samples prepared as described previously[24] were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for analysis of Txnrd1, Mn-Sod, Eif2α, phospho-Eif2α, Atf4, Nrf2, Grp78, and Gapdh in the presence of DTT. For the NMD components (phospho-Upf1 (pUpf1), Upf1, Smg-1, Eif4a3, Smg-6 and Smg-7), 5% SDS-PAGE was adopted. The gels were transferred to nitrocellulose membranes, and then incubated with indicated antibody probes. The antibodies of Txnrd1 and α-tubulin[15], and NMD components[29–31] have been described elsewhere. For the assay of SAPK/JNK activity, SAPK/JNK Assay Kit was used according to the manufacturer's directions. Briefly, a phospho-SAPK/JNK antibody linked to agarose beads was used to pull down SAPK enzyme from cell extracts. Upon addition of kinase buffer, c-Jun fusion protein, and ATP, SAPK phosphorylates the c-Jun substrate. Phospho-c-Jun antibody can then be used to measure SAPK activity by immunoblotting. For the assay of ERK signaling, p44/42 MAPK Assay Kit was used. Briefly, immobilized phospho-p44/42 MAPK mAb was used to immunoprecipitate active p44/42 MAP kinase from cell extracts, then an *in vitro assay* was performed using Elk-1 protein as a substrate. Elk-1 phosphorylation was then detected by Western blotting using phospho-Elk-1 antibody. Total c-Jun or Elk1 was estimated as described previously[13]. The proteins were detected as described previously[41]. The densitometric quantification was performed using the NIH Image software and the data normalized to the α-tubulin protein is represented as a fold-increase over the control.

**siRNA preparation and cell transfection.** Mouse Smg-1 and Smg-7 siRNAs have been described previously[15]. The transfections of synthetic siRNAs (Grp78, Mt1, Smg-1, and Smg-7) were carried out with Lipofectamine RNAiMAX.

**Immunocytochemistry.** siRNA-transfected cells were cultured at a density of $10^5$ cells on gelatin-coated glass coverslips in a 35-mm dish. To detect γ-H2ax, after treatment with MeHg for 11 h, the coverslips were fixed and immunostained with a mouse γ-H2ax antibody as described previously[41]. Hoechst 33342 staining was performed as described previously[12]. After washing 5 times in PBS, the coverslips were mounted and analyzed as described previously[14].

**Statistical analysis.** Statistical analysis was conducted by using Graph Pad PRISM 5.0 (GraphPad Software). Data were analyzed by a one-way ANOVA for multiple data analyses or a one-way Welch's *t*-test for two data comparison and were expressed as mean ± SEM. A difference was considered statistically significant when $p < 0.05$.


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## Author contributions

F.U. designed research; F.U. and M.F. performed research; A.Y. contributed reagents/materials/analysis tools; F.U. and A.Y. analyzed data and wrote the paper. All authors reviewed the manuscript.

## Additional information

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