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Research Article

RESEARCH ARTICLE

Cadmium impairs protein folding in the endoplasmic reticulum and induces the unfolded protein response

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One sentence summary: The toxic metal cadmium disturbs folding of client proteins in the endoplasmic reticulum, where secretory and membrane proteins undergo maturation, and induces a stress response.

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ABSTRACT

Cellular exposure to cadmium is known to strongly induce the unfolded protein response (UPR), which suggests that the endoplasmic reticulum (ER) is preferentially damaged by cadmium. According to recent reports, the UPR is induced both dependent on and independently of accumulation of unfolded proteins in the ER. In order to understand the toxic mechanism of cadmium, here we investigated how cadmium exposure leads to Ire1 activation, which triggers the UPR, using yeast *Saccharomyces cerevisiae* as a model organism. Cadmium poorly induced the UPR when Ire1 carried a mutation that impairs its ability to recognize unfolded proteins. Ire1 activation by cadmium was also attenuated by the chemical chaperone 4-phenylbutyrate. Cadmium caused sedimentation of BiP, the molecular chaperone in the ER, which suggests the ER accumulation of unfolded proteins. A green fluorescent protein-based reporter assay also indicated that cadmium damages the oxidative protein folding in the ER. We also found that an excess concentration of extracellular calcium attenuates the Ire1 activation by cadmium. Taken together, we propose that cadmium exposure leads to the UPR induction through impairment of protein folding in the ER.

Keywords: heavy metal; organelle; molecular chaperone; UPR; endoplasmic reticulum; cadmium

INTRODUCTION

Cadmium (Cd^{2+}) has been recognized as a toxic metal causing serious occupational and public health hazards. As reviewed elsewhere (Jarup and Akesson 2009), Cd^{2+} exposure leads to various types of health damage. Although molecular mechanisms by which Cd^{2+} harms cells have not been fully determined, it appears certain that Cd^{2+} exposure causes the endoplasmic reticulum (ER) stress response, which is known to be induced upon dysfunction of the ER (Liu *et al.* 2006; Hiramatsu *et al.* 2007; Yokouchi *et al.* 2007; Biagioli *et al.* 2008; Tamas *et al.* 2014).

The ER is a membrane-enclosed organelle in which secretory or membrane proteins are folded. Moreover, lipid molecules are metabolized on the ER. Mammalian cells ubiquitously bear three types of transmembrane ER stress sensor, namely, inositol requiring enzyme 1α (IRE1α), protein kinase RNA-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6), on their ER membrane to induce the ER stress response upon dysfunction of the ER (Mori 2000). The best noted ER stress response induced by these ER stress sensors is the transcriptional induction of proteins that contribute to alleviation of the stress conditions. Whereas this cellular event, which is also known as the unfolded protein response (UPR), represents a protective aspect of the ER stress response, the activation of IRE1α and PERK can also lead to apoptosis, probably in order to remove heavily damaged cells from mammalian bodies (Hetz, Chevet

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and Oakes 2015). However, as a side effect of the ER stress response, the ER stress-dependent apoptosis is linked to tissue injury and various diseases (Schonthal 2012; Oakes and Papa 2015). Therefore, Cd^{2+} exposure harms health, possibly by inducing the ER stress response.

Saccharomyces cerevisiae (hereafter simply called yeast) cells do not bear PERK or ATF6, whereas Ire1 orthologues are conserved throughout all eukaryotic species. Besides IRE1α in mammalian cells, yeast Ire1 clusters upon ER stress to exhibit potent endonuclease activity (Kimata *et al.* 2007; Korennykh *et al.* 2009; Li *et al.* 2010). The target of Ire1 in yeast cells is the mRNA transcript from the *HAC1* gene, which has an intron to inhibit its own translation (Ruegsegger, Leber and Walter 2001). Ire1 promotes splicing of the *HAC1* mRNA, the product of which is translated into a transcription factor protein that is responsible for the transcriptional induction of the UPR (Cox and Walter 1996). Because the cellular system that induces the UPR is simpler in yeast than in higher eukaryotes and because Cd^{2+} also induces the UPR in yeast cells (Gardarin *et al.* 2010), yeast cells are likely to be an appropriate model to address the molecular mechanism by which Cd^{2+} damages the ER.

The ER stress, which induces the UPR, is believed to be associated with the accumulation of unfolded proteins in the ER (Kohno 2010). Indeed, yeast Ire1 directly recognizes ER-accumulated unfolded proteins for its cluster formation and activation (Kimata *et al.* 2007; Gardner and Walter 2011; Kimata and Kohno 2011; Promlek *et al.* 2011). However, we have also found that in yeast cells, cellular aberrancy that disturbs membrane lipid homeostasis equally activates both wild-type Ire1 and a mutant version of Ire1 that cannot capture unfolded proteins (Promlek *et al.* 2011). Also in mammalian cells, membrane lipid aberrancy has been reported to activate IRE1α and PERK independently of accumulation of unfolded proteins in the ER (Volmer, van der Ploeg and Ron 2013). These observations reflect the complexity of ER stress and factors that induce it. Cd^{2+}, as well as other heavy metal ions, is reported to cause protein misfolding and aggregation (Tamas *et al.* 2014). However, according to Gardarin *et al.* (2010), Cd^{2+} exposure potently activates Ire1 without damaging the integrity of an ER client protein, carboxypeptidase Y (CPY), in yeast cells. This implies that Cd^{2+} induces the UPR without ER accumulation of unfolded proteins. Therefore, it is still obscure how Cd^{2+} damages the ER to induce the UPR.

In the present study, we addressed the molecular mechanism by which Cd^{2+} exposure damages the ER and induces the UPR using yeast cells as a model organism. Our findings shown here strongly suggest that Cd^{2+} impairs protein folding in the ER and then induces the ER stress response.

MATERIALS AND METHODS

Yeast strains and plasmids

The haploid W303 strain (*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*) was genetically transformed to carry the *IRE1*-gene deletion (*ire1*Δ*::TRP1*; Mori *et al.* 1996) and the wild-type *ADE2* locus, and the resulting transformant is named W303-ire1Δ. The yeast centromeric vector pRS313 (Sikorski and Hieter 1989) was modified to carry the *IRE1* gene in our previous study and named as pRS313-IRE1 (Kimata *et al.* 2004). Unless otherwise noted, W303-ire1Δ transformed with pRS313-IRE1 was used here as the *IRE1*+ cells. The W303-ire1Δ cells were also transformed with the ΔIII mutant version of pRS313-IRE1 (Kimata *et al.* 2007) and employed in the experiments shown in Fig. 2. A strain producing the green fluorescent protein (GFP)-tagged version of Ire1 (Ire1-GFP; Aragon *et al.* 2009) was generated from W303-ire1Δ, as previously described (Ishiwata-Kimata *et al.* 2013). The plasmid pPM28 was used for cellular expression of eroGFP (Merksamer, Trusina and Papa 2008). The W303-ire1Δ cells transformed with pRS313-IRE1 and pPM28 were used here as *IRE1*+ eroGFP cells.

Chemicals

We added appropriate amounts of aqueous solutions of 0.5 M $CdCl_2$, 1 M dithiothreitol (DTT), 0.25 M $HgCl_2$, 20 mM $PbCl_2$, 0.5 M CrO_3 (chromium (VI)) and 0.5 M $CaCl_2$ into yeast cultures. Tunicamycin was dissolved in dimethyl sulfoxide at a stock concentration of 2 mg ml^{-1}. A sodium salt of 4-phenylbutyric acid (4-PBA, 500 mM aqueous solution) was added to cultures at appropriate time points.

Yeast culture

The standard synthetic dextrose (SD) medium contained 2% glucose, 0.66% Difco yeast nitrogen base without amino acids (http://openwetware.org/wiki/Composition_of_Yeast_Nitrogen_Base_(YNB)) and auxotrophic requirements. The standard SD medium lacking $CaCl_2$ was used here as the low calcium (Ca^{2+}) medium. Except for lacking inositol, the inositol-depleted medium was the same composition as the low Ca^{2+} medium. For all experiments described here, yeast cells were grown in exponential phase and stressed at 30°C under liquid shaking-culture conditions in the low Ca^{2+} medium, unless otherwise noted. In order to stress cells by inositol depletion, we shifted cells from the low Ca^{2+} medium to the inositol-depleted medium.

Culture density (optical density at 600 nm: OD_{600}) was measured using the spectrophotometer SmartSpec Plus (Bio-Rad). Pb^{2+} gradually and non-biologically causes turbidity of medium, the OD_{600} value of which was measured and used for correction of culture density.

RNA and protein analyses

To monitor splicing of the *HAC1* mRNA, total RNA samples were obtained from yeast cells and subjected to reverse transcription (RT)–PCR amplification of the *HAC1* mRNA species, as previously described (Kimata *et al.* 2003; Promlek *et al.* 2011). The RT–PCR products were then separated using 2% agarose gel electrophoresis, and the ethidium bromide-fluorescence images of gels were obtained to calculate the *HAC1* mRNA splicing efficiency using the following formula: {(band intensity of $HAC1^{i}$)/[(band intensity of $HAC1^{i}$) + (band intensity of $HAC1^{u}$)]}, where $HAC1^{u}$ is the unspliced form and $HAC1^{i}$ is the spliced form (Promlek *et al.* 2011). BiP sedimentation assay was performed as previously described (Kimata *et al.* 2003; Promlek *et al.* 2011). To obtain numerical data, experiments were performed using three independent transformants of the *IRE1* plasmids, and the mean values are expressed as mean ± standard deviation.

Fluorescence microscopic analysis

Cells producing Ire1-GFP were observed as previously described (Ishiwata-Kimata *et al.* 2013). Cells expressing eroGFP were observed under the fluorescence microscope DeltaVision Elite (GE Healthcare) with the following conditions: objective, ×63; excitation, 390/18 (exposure time, 1.0 s) or 475/28 nm (exposure time, 0.1 s); and emission, 525/48 nm. To determine the eroGFP ratio (475/390 nm), the fluorescence intensity

of cells was measured using ImageJ (http://imagej.nih.gov/ij/), and [(fluorescence intensity upon excitation at 475 nm)/(fluorescence intensity upon excitation at 390 nm)] was calculated for 30 cells in each sample to obtain mean ± standard deviation.

RESULTS

Ire1 is clustered and activated in yeast cells exposed to Cd^{2+}

To monitor effects of Cd^{2+} on yeast cells, here we cultured them in the low Ca^{2+} medium since, as described later, Ca^{2+} is likely to have an antagonistic effect on Cd^{2+} toxicity. Probably because Ca^{2+} is provided as calcium pantothenate (0.85 μM) even in the low Ca^{2+} medium, growth rate of cells in it did not seem to differ from that in the standard SD medium, which carries 0.9 mM $CaCl_2$ (Supplementary Table S1). In the experiment shown in Fig. 1A and Supplementary Fig. S1, two different cell lines were stressed by various concentrations of Cd^{2+} for 1 h and checked for *HAC1* mRNA splicing, namely, conversion of the unspliced form (*HAC1*^{u}) to the spliced form (*HAC1*^{i}). We thus found that Cd^{2+} exposure at various concentrations caused the *HAC1* mRNA splicing. Because a W303-congenic strain (Fig. 1A) seemed more sensitive to Cd^{2+} than an SEY6210-congenc strain (Supplementary Fig. S1), we employed the W303-congenic strain in the present study. In the experiment shown in Fig. 1B, cellular growth in the presence of Cd^{2+} was monitored. We then set 50 μM Cd^{2+} as its working concentration because under this condition, cellular growth was retarded but not completely abolished, and *HAC1* mRNA splicing was considerably induced.

We believe that the *HAC1* mRNA splicing shown here actually represents the activation of Ire1 because this reaction was not observed for cells that lacked the *IRE1* gene (Fig. 1C). Moreover, as shown in Fig. 1D, a similar *HAC1* mRNA splicing was observed when cells were treated with a conventional ER stressor tunicamycin, which inhibits N-glycosylation (Takatsuki, Kohno and Tamura 1975). Figure 1E shows the time course of activation of Ire1 in cells exposed to 50 μM Cd^{2+}. The *HAC1* mRNA splicing efficiency peaked 1 h after the onset of Cd^{2+} exposure and then gradually decreased.

We and others previously reported that Ire1 is activated via its cluster formation, which can be visualized as a dot-like distribution of Ire1-GFP through fluorescence-microscopic observation of yeast cells producing Ire1-GFP (Kimata *et al.* 2007; Aragon *et al.* 2009; Ishiwata-Kimata *et al.* 2013). Whereas in cells cultured under non-stress conditions, Ire1-GFP exhibited a double ring-like distribution pattern, indicating its diffusive distribution on the ER membrane, Cd^{2+} exposure led to its dot-like distribution (Fig. 1F).

We next investigated whether Ire1 was activated when cells were exposed to other heavy metal ions. We set the toxic concentrations of chromium (Cr(VI), CrO_4^{2-}), mercury (Hg^{2+}) and lead (Pb^{2+}) to 500 μM, 5 μM and 500 μM, respectively, because under these conditions, cellular growth was considerably retarded (Fig. 1G). As shown in Fig. 1H, none of these heavy metal ions induced the *HAC1* mRNA splicing.

Cd^{2+} disturbs protein folding in the ER to induce the UPR

As described in the Introduction, the UPR is induced both dependently and independently of unfolded proteins accumulated in the ER (Gardner and Walter 2011; Promlek *et al.* 2011; Volmer, van der Ploeg and Ron 2013). In the present study, we thus investigated how Cd^{2+} exposure damages the ER and activates Ire1. According to our previous reports (Kimata *et al.* 2007; Promlek *et al.* 2011), the ΔIII mutant version of Ire1, which carries a partial deletion on its luminal domain, cannot capture unfolded proteins. We also reported previously that ΔIII Ire1 is poorly activated by the accumulation of unfolded proteins in the ER, whereas wild-type Ire1 and ΔIII Ire1 are equally activated by membrane lipid aberrancy (Promlek *et al.* 2011). In the present study, we reproduced this observation through stressing cells by dithiothreitol (DTT) and inositol depletion (Fig. 2A). DTT is a disulfide-bond reducing reagent and impairs protein folding in the ER. On the other hand, inositol is one of the main components of phospholipids, and its depletion is likely to result in membrane lipid aberrancy (Promlek *et al.* 2011). Importantly, here we show that the ΔIII mutation considerably attenuates the *HAC1* mRNA splicing induced by Cd^{2+} (Fig. 2A). As shown in Supplementary Fig. S2A, loss of the *IRE1* gene enhanced Cd^{2+} toxicity to yeast cells, which was not rescued by ΔIII Ire1. Figure 2B shows that Cd^{2+} did not induced the cluster formation of the ΔIII mutant version of Ire1. According to our previous observation (Promlek *et al.* 2011), the ΔIII mutation does not change the cellular abundance of Ire1.

We next employed 4-PBA, which is known as a chemical chaperone that is supposed to interact with hydrophobic domains of misfolded proteins and thus prevents their aggregation (Kolb *et al.* 2015). When 4-PBA was added into culture simultaneously with the DTT- or Cd^{2+}-stress onset, these stressors failed to induce the *HAC1* mRNA splicing (Fig. 2C). However, according to our data shown in Fig. 2D, 4-PBA did not attenuate the *HAC1* mRNA splicing induced by inositol depletion. Probably because of intracellular residual inositol stock, a certain time is required for the UPR induction by inositol depletion, and in the experiment shown in Fig. 2D, we added 4-PBA into cultures 3 h after the inositol-depletion onset, namely at a time point when the *HAC1* mRNA splicing was not yet induced. We did not add 4-PBA simultaneously with the inositol-depletion onset, because cells are highly damaged and killed by a long-term exposure (>5 h) to 4-PBA (data not shown).

We then investigated whether Cd^{2+} exposure actually perturbs the ER protein folding using other experimental approaches. Unfolded proteins tend to form aggregates with which molecular chaperones can be associated in cells. Indeed, according to our previous studies (Kimata *et al.* 2003; Promlek *et al.* 2011), sedimentation of the ER-located molecular chaperone BiP is caused by the treatment of yeast cells with DTT or by the exogenous expression of a model mutant protein. Here we employed the same strategy to examine whether Cd^{2+} exposure forms ER protein aggregates associated with BiP. In the experiment shown in Fig. 3A, yeast cells were lysed in the presence of a mild detergent, Triton X-100, and subjected to high-speed centrifugation. We then found that the amount of BiP in the pellet fraction sharply increased when cells were exposed to Cd^{2+} before cell lysis. We believe that this result is not due to induction of BiP expression by the UPR alone because the total cellular level of BiP was only slightly increased by Cd^{2+} exposure.

Protein folding in the ER lumen often accompanies disulfide bond formation of cysteine residues. Cellular ability to form disulfide bonds in the ER can be monitored using the eroGFP reporter (Merksamer, Trusina and Papa 2008). This is an ER-located GFP mutant that shifts its excitation spectrum in a manner dependent on the formation of an intramolecular disulfide bond. Here we illuminated yeast cells producing eroGFP with light of two different wavelengths for fluorescence microscopy and

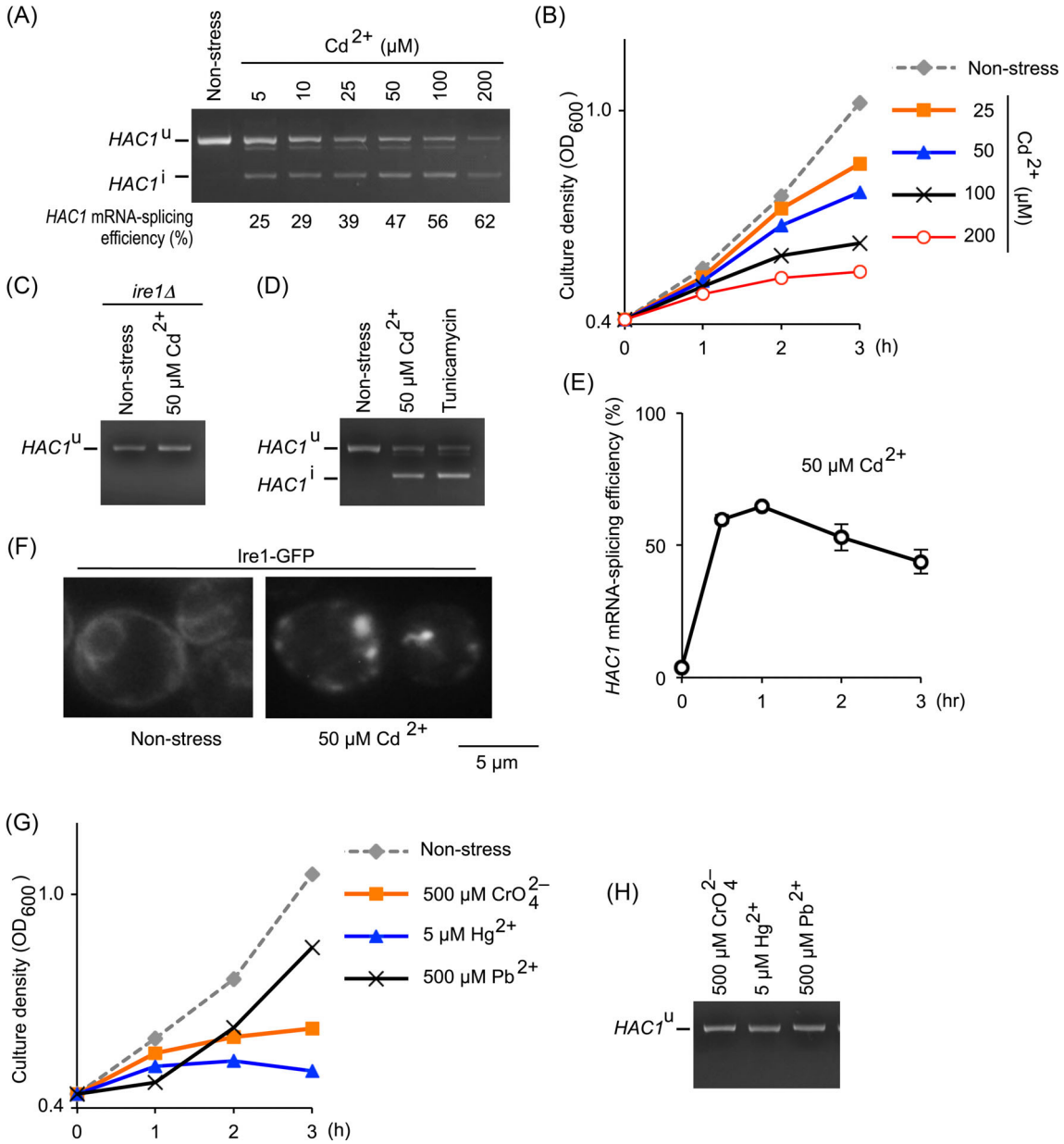

Figure 1. Induction of the *HAC1* mRNA splicing by cadmium exposure. (**A**) After culturing *IRE1*+ cells (W303-ire1Δ transformed with pRS313-IRE1) with the indicated concentrations of $CdCl_2$ for 1 h, total RNA samples were analyzed by RT–PCR amplification of the *HAC1* mRNA species, the products of which were separated using agarose gel electrophoresis and visualized with ethidium bromide staining. (**B**) The *IRE1*+ cells were cultured with the indicated concentrations of $CdCl_2$, and the culture density was monitored. (**C**) A similar experiment to that shown in (A) was performed using the *ire1*Δ strain (W303-ire1Δ transformed with the empty vector pRS313). (**D**) After culturing the *IRE1*+ cells with the indicated chemicals for 1 h, total RNA samples were examined for *HAC1* mRNA splicing. (**E**) The *IRE1*+ cells were cultured in the presence of 50 μM $CdCl_2$ for the indicated periods, and total RNA samples were examined for *HAC1* mRNA splicing. (**F**) After being cultured with 50 μM $CdCl_2$ for 1 h or remaining unstressed, cells producing Ire1-GFP were observed under a fluorescence microscope. (**G**) The *IRE1*+ cells were cultured with the indicated heavy metals, and the culture density was monitored. (**H**) After culturing the *IRE1*+ cells with the indicated heavy metals for 1 h, total RNA samples were examined for *HAC1* mRNA splicing.

merged their images as shown in Fig. 3B, in which cells stressed by Cd^{2+} seemed more reddish than unstressed cells. The quantified data exhibited in Fig. 3B also indicate a change of the excitation spectrum of eroGFP by cadmium exposure, suggesting that Cd^{2+} exposure impairs oxidative protein folding in the ER.

Ca^{2+} attenuates Ire1 activation by Cd^{2+}

Both Ca^{2+} and Cd^{2+} exist as divalent ions, and some chemical properties of Cd^{2+} resemble those of Ca^{2+} (Choong, Liu and Templeton 2014). Therefore, one of the possible mechanisms behind the toxicity of Cd^{2+} is that it occupies Ca^{2+}-binding sites of biomolecules and disturbs Ca^{2+} homeostasis (Kozlowski *et al.* 2014; Zhou *et al.* 2015). In the experiment shown in Fig. 4A, 5 mM Ca^{2+} was added into cultures simultaneously with the ER-stress onset. Importantly, 5 mM Ca^{2+} considerably attenuated the *HAC1* mRNA splicing induced by Cd^{2+}. However, the *HAC1* mRNA splicing induced by 2 μg ml^{-1} tunicamycin or 3 mM DTT was not affected by 5 mM Ca^{2+}. Figure 4B shows that in the presence of 5 mM Ca^{2+}, Cd^{2+} did not induce the cluster formation of

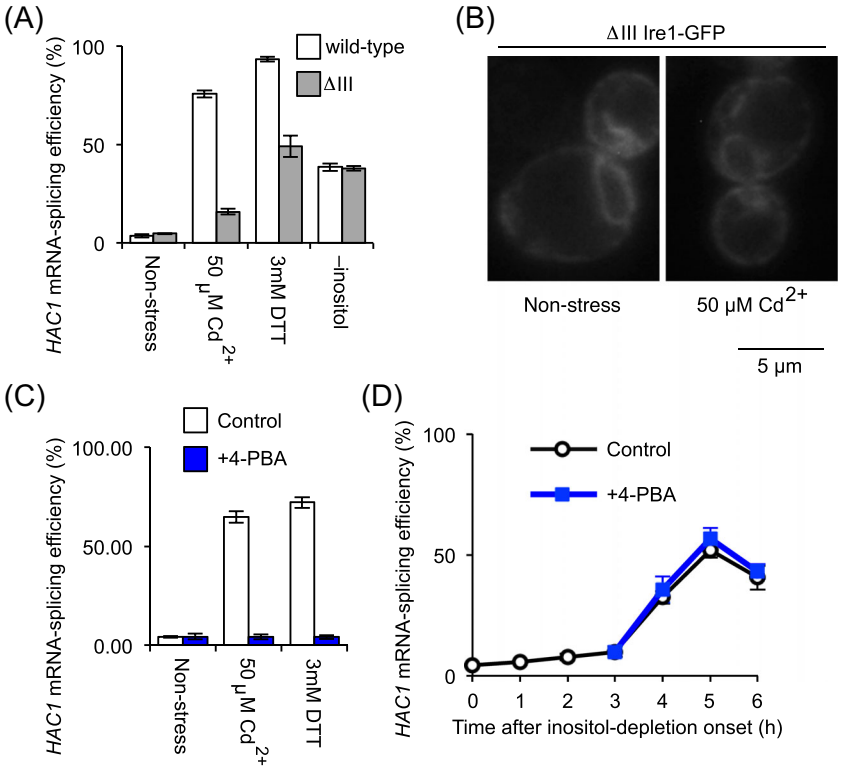

Figure 2. The ΔIII mutation and 4-PBA attenuate Ire1 activation by cadmium exposure. (**A**) The *IRE1*+ cells (wild-type; WT) and congenic cells carrying the ΔIII mutation on the *IRE1* gene were stressed by the indicated stimuli (50 μM $CdCl_2$ for 1 h, 3 mM DTT for 1 h and inositol depletion (–inositol) for 5 h), and total RNA samples were examined for *HAC1* mRNA splicing. (**B**) After being cultured with 50 μM $CdCl_2$ for 1 h or remaining unstressed, cells producing the ΔIII mutant version of Ire1-GFP were observed under a fluorescence microscope. (**C**) The *IRE1*+ cells were stressed by the indicated chemicals for 1 h and checked for *HAC1* mRNA splicing. For the '+4-PBA' samples, 4-PBA (final concentration, 5 mM) was added to cultures simultaneously with the stress onsets. (**D**) The *IRE1*+ cells were stressed by inositol depletion at time 0, and 3 h later 4-PBA was added to the cultures.

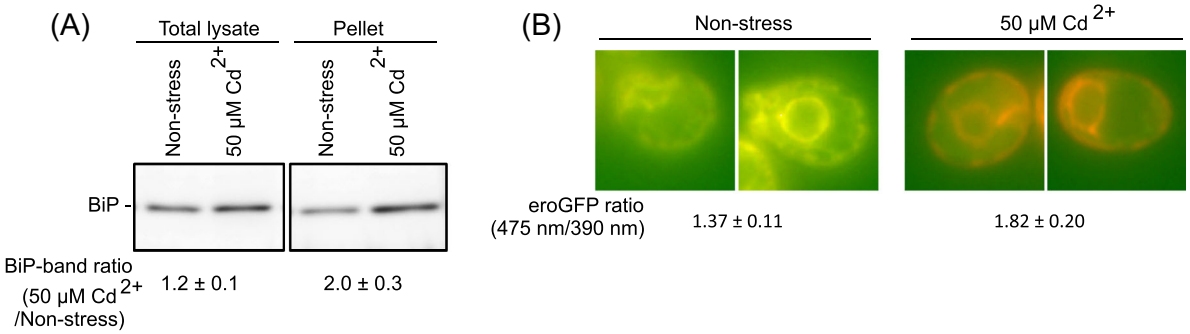

Figure 3. Cadmium exposure impairs protein folding in the ER. (**A**) After being cultured with 50 μM $CdCl_2$ for 1 h or remaining unstressed, the *IRE1*+ cells were lysed and fractionated by centrifugation at 8000×*g* for 15 min. Then, total lysates (equivalent to 0.04 OD_{600} cells) and pellet fractions (equivalent to 1.3 OD_{600} cells) were analyzed by anti-BiP western blotting. (**B**) The *IRE1*+ eroGFP cells (W303-ire1Δ transformed with pRS313-IRE1 and pPM28) were stressed by 50 μM $CdCl_2$ for 1 h or remained unstressed for observation under a fluorescence microscope. The resulting gray-scale images were pseudo-colored green (for excitation with 390 nm light) or red (for excitation with 475 nm light) and merged. The eroGFP ratio (475 nm/390 nm) was obtained as described in Materials and methods.

Ire1. Moreover, 5 mM Ca^{2+} reduced Cd^{2+} toxicity on both *IRE1*+ and *ire1*Δ cells (compare Supplementary Fig. S2B with S2A). Consistent with our observations shown in Fig. 4, Cd^{2+} induced the *HAC1* mRNA splicing only weakly when cells were cultured in the standard SD medium, which contains 0.9 mM $CaCl_2$ (Supplementary Fig. S3).

DISCUSSION

To explore the mechanisms behind the toxicity of heavy metals, here we investigated the ER stress response triggered by heavy metals in yeast cells. According to our data shown in Fig. 1, Cd^{2+} is likely to induce the ER stress most potently among heavy metals employed in the present study. This observation implies that there is a difference in toxicity mechanisms or target biomolecules of Cd^{2+} compared with the other heavy metals.

As mentioned in the Introduction, a number of previous reports described ER stress induction by exposure of mammals to Cd^{2+} (Liu *et al.* 2006; Hiramatsu *et al.* 2007; Yokouchi *et al.* 2007; Biagioli *et al.* 2008; Tamas *et al.* 2014). Because chronic activation of the ER stress sensors leads to apoptosis and then various diseases (Schonthal 2012; Hetz, Chevet and Oakes 2015; Oakes and

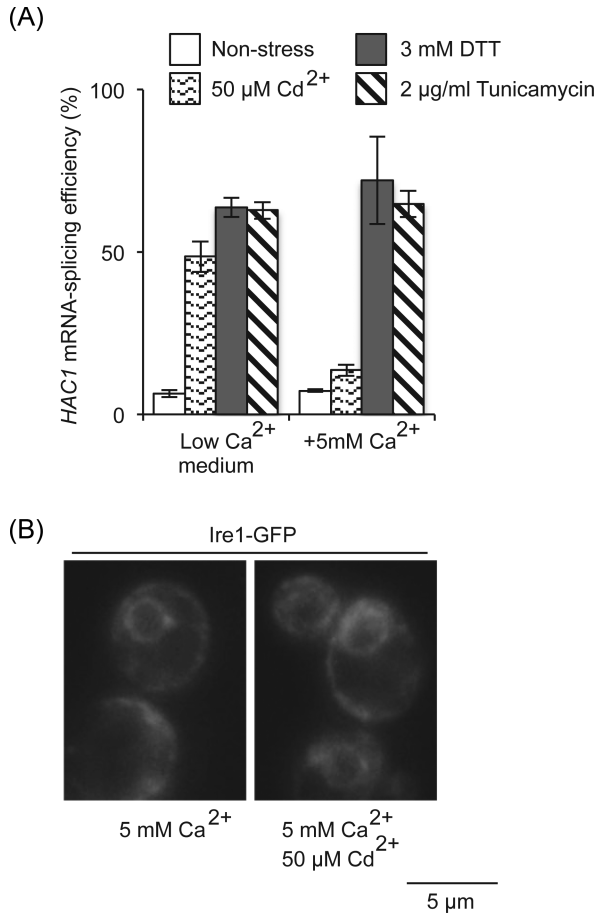

Figure 4. Effect of calcium on Ire1 activation by various ER stressing stimuli. (**A**) After stressing the *IRE1*+ cells with the indicated chemicals for 1 h, total RNA samples were examined for *HAC1* mRNA splicing. For the '+5 mM Ca^{2+}' samples, $CaCl_2$ (final concentration, 5 mM) was added into the cultures simultaneously with the stress onset. (**B**) After being cultured with 5 mM $CaCl_2$ or with 5 mM $CaCl_2$ plus 50 μM $CdCl_2$ for 1 h, cells producing Ire1-GFP were observed under a fluorescence microscope.

Papa 2015), we believe that studies on ER stress by Cd^{2+} would be beneficial for understanding the mechanism behind the toxicity of this metal. Although induction of the UPR by the exposure of yeast cells to Cd^{2+} has already been reported elsewhere (Gardarin *et al.* 2010), the molecular mechanism by which Cd^{2+} leads to the ER stress is still obscure. The UPR contributes to Cd^{2+} tolerance, at least in the case of yeast cells, because yeast cells not carrying the *IRE1* or *HAC1* gene were shown to be hypersensitive to Cd^{2+} (Supplementary Fig. S2A; Gardarin *et al.* 2010).

The primary objective of this study is to understand how Cd^{2+} exposure damages the ER and induces the UPR using yeast cells as a model organism. We focused on this issue because some ER stressing stimuli activate Ire1 via production of unfolded proteins in the ER, whereas other stimuli lead to the Ire1 activation through other obscure mechanisms (Kimata *et al.* 2007; Gardner and Walter 2011; Promlek *et al.* 2011; Volmer, van der Ploeg and Ron 2013). Here we found that the *HAC1* mRNA splicing upon Cd^{2+} exposure was attenuated by the ΔIII mutation of the *IRE1* gene or by addition of the chemical chaperone 4-PBA to the culture medium (Fig. 2). This observation strongly suggests that Cd^{2+} activates Ire1 via the disturbance of protein folding in the ER. The induction of BiP sedimentation by Cd^{2+} exposure (Fig. 3A) supports our theory that Cd^{2+} causes protein aggregation in the ER. In the ER, client proteins often undergo oxidative folding, to which disulfide bond formation of cysteine residues contributes (Depuydt, Messens and Collet 2011). Considering our result from the eroGFP reporter assay (Fig. 3B), we believe that Cd^{2+} exposure impairs disulfide bond formation and oxidative folding of proteins. We thus conclude that Cd^{2+} exposure induces the UPR via production of unfolded proteins in the ER.

However, according to Gardarin *et al.* (2010), Cd^{2+} does not block ER-to-Golgi transport of carboxypeptidase Y (CPY), which requires its intramolecular disulfide-bond formation and folding in the ER (Simons *et al.* 1995). We speculate that the intracellular CPY transport assay is not sensitive enough to find the impairment of ER protein folding caused by Cd^{2+}. It should be noted that even in the case of stressing cells by DTT, the *HAC1* mRNA splicing was induced without a detectable impairment of the intracellular CPY transport, when the concentration of DTT was insufficiently high (data not shown).

Unlike in the case of other ER stressing stimuli, the UPR induced by Cd^{2+} was abolished by excess concentration of Ca^{2+} in the culture medium (Fig. 4). Considering the resemblance of chemical properties between Cd^{2+} and Ca^{2+} (Choong, Liu and Templeton 2014), we believe that Cd^{2+} may mimic Ca^{2+}. Cd^{2+} may impair a cellular system(s) in which Ca^{2+} is involved, leading to the accumulation of unfolded proteins in the ER and to the induction of the UPR. In mammalian cells, the ER acts as a Ca^{2+} reservoir, and thapsigargin, a specific inhibitor of the sarcoplasmic/ER Ca^{2+}-ATPase (SERCA), reduces the Ca^{2+} concentration in the ER and strongly induces ER stress (Schonthal 2012). In yeast cells, however, the Ca^{2+} concentration in the ER is reported to be low even under normal conditions (Strayle, Pozzan and Rudolph 1999), and the UPR is not considerably induced by knockout of the Ca^{2+} pump Pmr1, which is responsible for Ca^{2+} uptake into the ER (Strayle, Pozzan and Rudolph 1999; Jonikas *et al.* 2009). It is thus unclear how important Ca^{2+} is to ER functions in yeast cells. Nevertheless, it should be also noted that severe Ca^{2+} depletion is likely to induce the UPR even in yeast cells. According to Bonilla, Nastase and Cunningham (2002), the UPR is induced when Ca^{2+} chelators are added to yeast cultures. As another explanation for the attenuation of the Cd^{2+}-induced *HAC1* mRNA splicing by Ca^{2+}, Cd^{2+} entry to cells depends on a Ca^{2+} transporter and is repressed by a high concentration of Ca^{2+} in culture medium, albeit not completely (Gardarin *et al.* 2010).

According to previous reports (Gardarin *et al.* 2010; Ruta *et al.* 2014), Cd^{2+} exposure induces Ca^{2+} uptake and increases the Ca^{2+} concentration in the cytosol of yeast cells. Unlike the insight in the preceding paragraph, we believe that, in this case, the relationship between Cd^{2+} and Ca^{2+} is indirect. This is because Ca^{2+} uptake is induced not only by Cd^{2+} but also by DTT or tunicamycin (Bonilla, Nastase and Cunningham 2002). The induction of Ca^{2+} uptake is then likely to be a common outcome of ER stressing stimuli. Because the ER acts as the major source of membrane lipids, we speculate that damage to the ER commonly leads to a shortage of lipidic components in membranous cellular structures including the plasma membrane, which may increase stretching stress on the plasma membrane and open stretch-activated Ca^{2+} channels (Kanzaki *et al.* 1999).

At least in our hands, the standard SD medium gets slightly turbid within 1 month after its preparation, possibly because Ca^{2+} in the medium forms insoluble $CaCO_3$ with atmospheric CO_2. Furthermore, in the case of the standard SD medium, cells cultured in old medium usually show higher Cd^{2+} sensitivity than those cultured in freshly prepared medium (data not shown). Such instability of the standard SD medium may explain

the reason why, in comparison with Gardarin *et al.* (2010), we observed somewhat lower Cd^{2+} sensitivity of cells being cultured in this medium (Supplementary Fig. S3).

Taken together, our insights from the present study and previous reports (Kimata *et al.* 2007; Promlek *et al.* 2011) indicate the multiplicity of ER stressing stimuli. Membrane lipid aberrancy, which is induced, for instance, by inositol depletion, is likely to activate Ire1 without producing unfolded proteins (Promlek *et al.* 2011). On the other hand, DTT, tunicamycin and Cd^{2+} cause accumulation of unfolded proteins in the ER, which is directly recognized by Ire1 (Kimata *et al.* 2007; Gardner and Walter 2011; Promlek *et al.* 2011). We speculate that as a Ca^{2+} mimic, Cd^{2+} may impair a cellular system that requires Ca^{2+} and that contributes to protein folding in the ER. Alternatively, because Cd^{2+} can form stable complexes with thiol groups, this metal ion may preferentially inhibit ER-located enzymes for oxidative protein folding, the active centers of which carry cysteine residues (Depuydt, Messens and Collet 2011). Moreover, it is also possible that Cd^{2+} directly impair proper folding of ER client proteins through forming chelation complexes with them. Finally, we hope that our findings that 4-PBA and Ca^{2+} attenuate the Cd^{2+}-induced ER stress will contribute to finding a way to detoxify Cd^{2+}.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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Conflict of interest. None declared.

REFERENCES

Aragon T, van Anken E, Pincus D *et al.* Messenger RNA targeting to endoplasmic reticulum stress signalling sites. *Nature* 2009;**457**:736–40.

Biagioli M, Pifferi S, Ragghianti M *et al.* Endoplasmic reticulum stress and alteration in calcium homeostasis are involved in cadmium-induced apoptosis. *Cell Calcium* 2008;**43**:184–95.

Bonilla M, Nastase KK, Cunningham KW. Essential role of calcineurin in response to endoplasmic reticulum stress. *EMBO J* 2002;**21**:2343–53.

Choong G, Liu Y, Templeton DM. Interplay of calcium and cadmium in mediating cadmium toxicity. *Chem Biol Interact* 2014;**211**:54–65.

Cox JS, Walter P. A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. *Cell* 1996;**87**:391–404.

Depuydt M, Messens J, Collet JF. How proteins form disulfide bonds. *Antioxid Redox Signal* 2011;**15**:49–66.

Gardarin A, Chedin S, Lagniel G *et al.* Endoplasmic reticulum is a major target of cadmium toxicity in yeast. *Mol Microbiol* 2010;**76**:1034–48.

Gardner BM, Walter P. Unfolded proteins are Ire1-activating ligands that directly induce the unfolded protein response. *Science* 2011;**333**:1891–4.

Hetz C, Chevet E, Oakes SA. Proteostasis control by the unfolded protein response. *Nat Cell Biol* 2015;**17**:829–38.

Hiramatsu N, Kasai A, Du S *et al.* Rapid, transient induction of ER stress in the liver and kidney after acute exposure to heavy metal: evidence from transgenic sensor mice. *FEBS Lett* 2007;**581**:2055–9.

Ishiwata-Kimata Y, Yamamoto YH, Takizawa K *et al.* F-actin and a type-II myosin are required for efficient clustering of the ER stress sensor Ire1. *Cell Struct Funct* 2013;**38**:135–43.

Jarup L, Akesson A. Current status of cadmium as an environmental health problem. *Toxicol Appl Pharmacol* 2009;**238**:201–8.

Jonikas MC, Collins SR, Denic V *et al.* Comprehensive characterization of genes required for protein folding in the endoplasmic reticulum. *Science* 2009;**323**:1693–7.

Kanzaki M, Nagasawa M, Kojima I *et al.* Molecular identification of a eukaryotic, stretch-activated nonselective cation channel. *Science* 1999;**285**:882–6.

Kimata Y, Ishiwata-Kimata Y, Ito T *et al.* Two regulatory steps of ER-stress sensor Ire1 involving its cluster formation and interaction with unfolded proteins. *J Cell Biol* 2007;**179**:75–86.

Kimata Y, Kimata YI, Shimizu Y *et al.* Genetic evidence for a role of BiP/Kar2 that regulates Ire1 in response to accumulation of unfolded proteins. *Mol Biol Cell* 2003;**14**:2559–69.

Kimata Y, Kohno K. Endoplasmic reticulum stress-sensing mechanisms in yeast and mammalian cells. *Curr Opin Cell Biol* 2011;**23**:135–42.

Kimata Y, Oikawa D, Shimizu Y *et al.* A role for BiP as an adjustor for the endoplasmic reticulum stress-sensing protein Ire1. *J Cell Biol* 2004;**167**:445–56.

Kohno K. Stress sensing mechanisms in the unfolded protein response: similarities and differences between yeast and mammals. *J Biochem* 2010;**147**:27–33.

Kolb PS, Ayaub EA, Zhou W *et al.* The therapeutic effects of 4-phenylbutyric acid in maintaining proteostasis. *Int J Biochem Cell Biol* 2015;**61**:45–52.

Korennykh AV, Egea PF, Korostelev AA *et al.* The unfolded protein response signals through high-order assembly of Ire1. *Nature* 2009;**457**:687–93.

Kozlowski H, Kolkowska P, Watly J *et al.* General aspects of metal toxicity. *Curr Med Chem* 2014;**21**:3721–40.

Li H, Korennykh AV, Behrman SL *et al.* Mammalian endoplasmic reticulum stress sensor IRE1 signals by dynamic clustering. *Proc Natl Acad Sci USA* 2010;**107**:16113–8.

Liu F, Inageda K, Nishitai G *et al.* Cadmium induces the expression of Grp78, an endoplasmic reticulum molecular chaperone, in LLC-PK1 renal epithelial cells. *Environ Health Persp* 2006;**114**:859–64.

Merksamer PI, Trusina A, Papa FR. Real-time redox measurements during endoplasmic reticulum stress reveal interlinked protein folding functions. *Cell* 2008;**135**:933–47.

Mori K. Tripartite management of unfolded proteins in the endoplasmic reticulum. *Cell* 2000;**101**:451–4.

Mori K, Kawahara T, Yoshida H *et al.* Signalling from endoplasmic reticulum to nucleus: transcription factor with a basic-leucine zipper motif is required for the unfolded protein-response pathway. *Genes Cells* 1996;**1**:803–17.

Oakes SA, Papa FR. The role of endoplasmic reticulum stress in human pathology. *Ann Rev Pathol* 2015;**10**:173–94.

Promlek T, Ishiwata-Kimata Y, Shido M *et al.* Membrane aberrancy and unfolded proteins activate the endoplasmic reticulum stress sensor Ire1 in different ways. *Mol Biol Cell* 2011;**22**:3520–32.

Ruegsegger U, Leber JH, Walter P. Block of HAC1 mRNA translation by long-range base pairing is released by cytoplasmic splicing upon induction of the unfolded protein response. *Cell* 2001;**107**:103–14.

Ruta LL, Popa VC, Nicolau I *et al*. Calcium signaling mediates the response to cadmium toxicity in Saccharomyces cerevisiae cells. *FEBS Lett* 2014;**588**:3202–12.

Schonthal AH. Endoplasmic reticulum stress: its role in disease and novel prospects for therapy. *Scientifica (Cairo)* 2012;**2012**:857516.

Sikorski RS, Hieter P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics* 1989;**122**: 19–27.

Simons JF, Ferro-Novick S, Rose MD *et al*. BiP/Kar2p serves as a molecular chaperone during carboxypeptidase Y folding in yeast. *J Cell Biol* 1995;**130**:41–9.

Strayle J, Pozzan T, Rudolph HK. Steady-state free Ca^{2+} in the yeast endoplasmic reticulum reaches only 10 microM and is mainly controlled by the secretory pathway pump pmr1. *EMBO J* 1999;**18**:4733–43.

Takatsuki A, Kohno K, Tamura G. Inhibition of biosynthesis of polyisoprenol sugars in chick embryo microsomes by tunicamycin. *Agric Biol Chem* 1975;**39**:2089–91.

Tamas MJ, Sharma SK, Ibstedt S *et al*. Heavy metals and metalloids as a cause for protein misfolding and aggregation. *Biomolecules* 2014;**4**:252–67.

Volmer R, van der Ploeg K, Ron D. Membrane lipid saturation activates endoplasmic reticulum unfolded protein response transducers through their transmembrane domains. *Proc Natl Acad Sci USA* 2013;**110**:4628–33.

Yokouchi M, Hiramatsu N, Hayakawa K *et al*. Atypical, bidirectional regulation of cadmium-induced apoptosis via distinct signaling of unfolded protein response. *Cell Death and Differ* 2007;**14**:1467–74.

Zhou X, Hao W, Shi H *et al*. Calcium homeostasis disruption – a bridge connecting cadmium-induced apoptosis, autophagy and tumorigenesis. *Oncol Res Treat* 2015;**38**:311–5.