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Downregulation of Gap Junction Expression and Function by Endoplasmic Reticulum Stress

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ABSTRACT

Gap junctional intercellular communication (GJIC) plays a critical role in the control of multiple cell behavior as well as in the maintenance of tissue and organ homeostasis. However, mechanisms involved in the regulation of gap junctions (GJs) have not been fully understood. Given endoplasmic reticulum (ER) stress and dysfunction of GJs coexist in several pathological situations, we asked whether GJs could be regulated by ER stress. Incubation of mesangial cells with ER stress-inducing agents (thapsigargin, tunicamycin, and AB_5 subtilase cytotoxin) resulted in a decrease in connexin 43 (Cx43) expression at both protein and mRNA levels. This was accompanied by a loss of GJIC, as evidenced by the reduced numbers of dye-coupled cells after single cell microinjection or scrape loading dye transfer. Further studies demonstrated that ER stress significantly inhibited the promoter activity of the Cx43 gene, reduced [^{35}S]-methionine incorporation into Cx43 protein and accelerated degradation of Cx43. ER stress also decreased the Cx43 protein levels in several different cell types, including human umbilical vein endothelial cells, mouse-derived renin-secreting cells and human hepatoma cells. Furthermore, induction of ER stress by hypoxic chemicals thenoyltrifluoroacetone and cobalt chloride was found to be associated with a reduction in Cx43. Our findings thus reveal a close link between ER stress and GJs. ER stress may represent a novel mechanism underlying the altered GJs in a variety of pathological situations. J. Cell. Biochem. 107: 973–983, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: CONNEXIN 43; GAP JUNCTION; UNFOLDED PROTEIN RESPONSE (UPR); ER STRESS; MESANGIAL CELLS

Intercellular communication via gap junctions (GJs) is thought to play an important role in the control of a variety of cellular functions [Christ et al., 1996; Severs et al., 2001; Saez et al., 2003; Yao et al., 2007]. In the cardiovascular system, cells are intensively interlinked by GJ channels. GJs in vascular cells provide a structural basis for coordinated vasoconstriction or vasodilation and for extensive cross-talk between cells of the vascular wall [Christ et al., 1996; Severs et al., 2001]. The altered expression of GJ proteins and its relevance to vascular diseases have also been extensively documented by many investigators [Christ et al., 1996; Lerner et al., 2000; Severs et al., 2001].

Mesangial cells (MCs) are specialized smooth muscle cells that play a pivotal role in the regulation of glomerular hemodynamics. One of the striking features of MCs is that these cells have extremely high density of GJs [Yao et al., 2007]. GJs in MCs are actively involved in the function of the juxtaglomerular apparatus [Yao et al., 2002, 2007]. However, information is still limited on the regulation of GJs by pathophysiologic factors.

The endoplasmic reticulum (ER) is the initial compartment in the secretory pathway. It is responsible for the synthesis, modification and delivery of proteins to their proper target sites within the secretory pathway. The ER is vulnerable to various forms of stress,

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causing the accumulation of unfolded and misfolded proteins. As a consequence, cells exert a coordinated adaptive response, that is, the unfolded protein response (UPR). UPR includes: (1) transcriptional induction of genes encoding ER resident chaperones to increase the ability of ER in processing unfolded proteins, (2) shut-off of protein synthesis to decrease the protein overload in the ER, and (3) increased clearance of unfolded proteins from the ER through upregulation of ER-associated degradation (ERAD). If the adaptation is insufficient, the cells are eliminated by apoptosis [Schroder and Kaufman, 2005; Kitamura, 2008].

Evidence linking UPR to GJ-mediated intercellular signaling is currently lacking. However, the following considerations have prompted us to speculate that UPR may be critically involved in the regulation of GJ formation and function. First, ER stress and dysfunction of GJs coexist in several pathological situations such as ischemic heart diseases and cancers [Peters et al., 1993; Kaprielian et al., 1998; Chipman et al., 2003; Mesnil et al., 2005; Koumenis, 2006; Moenner et al., 2007]. In addition, they exert several similar effects on cell behaviors. For example, both ER stress and GJ dysfunction promote cell growth and increase cell resistance to stress conditions caused by hypoxia, drugs and radiation [Chipman et al., 2003; Bi et al., 2005; Mesnil et al., 2005]. Second, gap junction protein connexins (Cxs) are transported to the cell surface via the conventional secretory pathway [VanSlyke and Musil, 2002; Thomas et al., 2005]. A substantial fraction of newly synthesized, topologically normal Cx molecules is known to be turned over by ERAD [VanSlyke and Musil, 2002]. These processes are critically interfered with by UPR [Schroder and Kaufman, 2005; Kitamura, 2008]. Third, induction of UPR is recently documented to down-regulate genes encoding integral membrane or extracellular proteins, to alleviate the load of client proteins targeted to the ER [Kimata et al., 2006; Rab et al., 2007]. As a dynamic membrane molecule with half-life (1.5–5.5 h) shorter than many other membrane proteins [VanSlyke and Musil, 2002; Saez et al., 2003], Cx molecules are most likely to be affected by ER stress. Fourth, GJ is presently recognized as a determinant factor governing cell fate against stress [Lin et al., 1998; Azzam et al., 2001; Saez et al., 2003]. To maintain homeostasis in tissue and organs, coordinated cell responses against ER stress should include regulation of GJs. Based on these considerations, this study was designed to address whether and how GJIC is regulated by ER stress.

In the present report, we show that ER stress down-regulates GJ expression and functional gap junctional intercellular communication (GJIC). Regulation of GJ may be an important defense strategy for the cells to adapt to ER stress conditions. ER stress may underlie the dysfunction of GJ in a variety of pathophysiological situations.

MATERIALS AND METHODS

MATERIALS

Anti-Cx40 was obtained from Zymed (Temecula, CA). Anti-Cx45, 78 kDa glucose-regulated protein (GRP78) and C/EBP homologous protein (CHOP) antibodies were purchased from Santa Cruz (Santa Cruz, CA). Anti-caspase-3 antibody was from Cell Signaling (Beverly, MA). FITC-conjugated swine anti-rabbit immunoglobulin was purchased from DAKO (Glostoup, Denmark). Salubrinal was from Calbiochem (La Jolla, CA). AB_5 subtilase cytotoxin (SubAB) that specifically degrades 78 kDa glucose-regulated protein (GRP78) was prepared as described previously [Paton et al., 2004]. All other agents including anti-Cx43 antibody were obtained from Sigma–Aldrich Japan (Tokyo, Japan).

CELL CULTURE

Rat MCs were established and characterized as described previously [Yao et al., 2002, 2005, 2006]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD) supplemented with 5% fetal bovine serum (FBS). Medium containing 1% FBS was generally used for experiments. Human umbilical vein endothelial cells (HUVEC), mouse-derived renin-secreting cells (As4.1) and human hepatoma cells (Huh-7) were purchased from American Type Culture Collection (Manassas, VA).

NORTHERN BLOT ANALYSIS

MCs were treated with various agents for the indicated time periods. Total RNA was extracted by the single-step method [Chomczynski and Sacchi, 1987], and Northern blot analysis was performed as described before [Yao et al., 2005; Yokouchi et al., 2007]. cDNAs for Cx43 (provided by Drs. G. Olbina and W. Eckhart, Molecular and Cell Biology Laboratory, The Salk Institute for Biologic Studies, San Diego), GRP78 (provided by Dr. Kazunori Imaizumi, Nara Institute of Science and Technology, Nara, Japan) and CHOP (provided by Dr. David Ron, New York University School of Medicine) were used for preparation of radio-labeled probes. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

WESTERN BLOT ANALYSIS

Western blot was performed by the enhanced chemiluminescence system [Yao et al., 2006; Yokouchi et al., 2007]. Briefly, extracted cellular proteins were separated by 10% SDS–polyacrylamide gels and electrotransferred onto polyvinylidine difluoride membranes. After blocking with 3% bovine serum albumin in PBS, the membranes were incubated with anti-Cx43 (1:2,000 dilution), anti-Cx40 (1:1,000 dilution); anti-GRP78 or anti-CHOP antibody (1:1,000 dilution, respectively). After washing with PBS containing 0.1% Tween 20, the filters were probed with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG, and the bands were visualized by the enhanced chemiluminescence system (GE Healthcare, Buckinghamshire, UK). To confirm equal loading of proteins, the filters were soaked in 62.5 mM Tris–HCl (pH 6.8) containing 2% SDS and 100 mM β-mercaptoethanol for 30 min at 60°C and reprobed for β-actin using anti-β-actin antibody (1:30,000 dilution; Sigma–Aldrich Japan).

CYTOTOXICITY ASSAY

Cytotoxicity was evaluated by the release of lactate dehydrogenase (LDH). LDH, a stable cytoplasmic enzyme, is released into culture media upon damage of the cell membrane. Confluent mesangial cells in 96-well culture plates were exposed to ER stress inducers for 24 h, and culture media were collected and assayed for LDH using LDH Cytotoxicity Detection Kit (Takara Bio Inc., Otsu, Shiga, Japan) [Zhu et al., 2006].

IMMUNOCYTOCHEMISTRY

For immunochemistry, cells on glass slides were fixed in 4% formaldehyde for 10 min and permeabilized in 0.2% Triton-X-100 for 5 min. Staining of Cx43 was performed using an anti-Cx43 antibody (Sigma–Aldrich Japan) [Yao et al., 2002, 2006].

EVALUATION OF GJIC BY MICROINJECTION OF LUCIFER YELLOW (LY)

GJIC was assessed by transfer of the membrane-impermeable fluorescent dye, Lucifer Yellow, after single cell microinjection using an automated microinjection system (Zeiss Oberkochen, Jena, Germany), as described previously [Yao et al., 2002, 2005, 2006].

SCRAPE LOADING DYE TRANSFER ASSAY (SLDT)

The scrape-loading dye transfer assay was used to assess GJIC. Briefly, confluent MCs on 35 mm culture dishes were exposed to culture medium containing 0.5% LY. A scrape line on the monolayer was made with a surgical blade. After 2 min, cells were rinsed three times with DMEM/F12 medium to remove background fluorescence. The cells were fixed with 4% paraformaldehyde in PBS and photographed with a digital camera attached to an Olympus fluorescent microscope (magnification: 200×).

TRANSFECTION EXPERIMENT

MCs in subconfluent culture were transfected with pCx43-luciferase vector [Mitchell and Lye, 2005] by using gene juice according to the manufacturer's instructions (Novagen) [Yao et al., 2006]. After 24–36 h, the transfected cells were exposed to various stimuli in fresh media containing 1% FCS for an additional 8 h. After that, the media was removed and the cells were harvested with reporter lysis buffer. Luciferase activity of the lysates was determined using luciferin reagent (Promega Corp., Madison WI), according to the manufacturer's protocol. Luminescence was measured for 10 s in a luminometer (Gene Light 55; Microtech Nition, Chiba, Japan).

[^{35}S]-METHIONINE LABELING AND IMMUNOPRECIPITATION

MCs in confluent culture in 6-well plates were incubated in methionine-, cystine- and L-glutamine-free DMEM (Life Technologies) containing 1% FBS for 1 h and then labeled with [^{35}S]-methionine (100 μCi/ml; Institute of Isotopes, Budapest, Hungary) in the presence or absence of ER stress inducers for 3 h. The cells were washed 3 times with medium and lysed in RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 5 mM EGTA containing 1% Triton, 0.5% deoxycholate, 0.1% SDS) and supplemented with the mixture of protease inhibitors (Nacalai Tesque, Kyoto, Japan). The cellular lysates were homogenated, cleared by centrifuging at 16,000*g* for 10 min, and immunoprecipitated using a rabbit polyclonal anti-Cx43 antibody at 4°C overnight. Immune complex were precipitated with protein-A/G-sephrose (Pharmacia, Piscataway, NJ) for 2 h at room temperature, and then washed four times with RIPA buffer. The resulting pellets were resuspended in 50 μl of 2× Laemmli buffer (125 mM Tris–HCl, 4% SDS, 20% glycerol, 0.02% bromophenol blue, 10% 2-mercaptoethanol; pH 6.8) and boiled for 5 min. The beads were pelleted, and the proteins were resolved by electrophoresis on a 5–20% gradient SDS–polyacrylamide gel and visualized by autoradiography.

STATISTICAL ANALYSIS

Values are expressed as means ± SE. Comparison of two populations was made by Student's *t*-test. For multiple comparisons, one-way analysis of variance (ANOVA) followed by Dunnett's test was employed. Both analyses were done by using the SigmaStat statistical software. $P < 0.05$ was considered to be a statistically significant difference.

RESULTS

ER STRESS SUPPRESSES CX43 EXPRESSION AND DISRUPTS GJIC

Incubation of MCs with thapsigargin (TG), which induces ER stress via inhibiting ER Ca^{2+}-ATPase, decreased the protein levels of Cx43 in a time- and concentration-dependent manner, as shown by Western blot analysis (Fig. 1A,B). Time-course analysis demonstrated that a clear inhibition of Cx43 level was observed 3–6 h after stimulation of cells with 100 nM TG, and the effect lasted for at least 24 h (Fig. 1A). Analysis of concentration-effect relationship revealed that TG effectively suppressed Cx43 protein expression at the concentration as low as 10 nM (Fig. 1B).

Tunicamycin (TM), which induces ER stress by inhibition of N-linked glycosylation, similarly inhibited the protein level of Cx43 (Fig. 1C). In addition, SubAB, a subtilase cytotoxin that can rapidly and specifically cleave GRP78 protein [Paton et al., 2006], also downregulated the Cx43 level (Fig. 1D).

In most of the following studies, we have stimulated the cells with 100 nM TG, 5 μg/ml TM or 20 ng/ml SubAB for 12 h (Fig. 1E). Under these experimental conditions, ER stress inducers significantly inhibited Cx43 protein levels (Fig. 1F), without any influence on cell viability, as evaluated by the release of LDH (data not shown).

The mRNA expression of Cx43 was also suppressed by ER stress inducers, TG and TM (Fig. 1G,H). The induction of ER stress by TG and TM was confirmed by the elevation of GRP78 and CHOP, as demonstrated by Northern blot analysis (Fig. 1G,H).

The suppressive effect of ER stress on the Cx43 protein level was confirmed by immunofluorescent staining of MCs with an anti-Cx43 antibody. Normally, Cx43 was mainly localized at perinuclear regions and cell-to-cell contacts (Fig. 2A). Treatment of MCs with TG, TM or SubAB caused an obvious reduction of Cx43 at these locations (Fig. 2A).

The decrease in Cx43 was associated with reduction in GJIC, when it was analyzed using SLDT assay. As shown in Figure 2B. under normal condition, the cells at the scraped border filled with the dye and the dye continued to diffuse into the intact monolayer. Induction of ER stress with TG, TM or SubAB effectively blocked diffusion of the fluorescent dye. Similar results were achieved by analyzing GJIC using microinjection of LY. Microinjection of LY into a single MC led to a rapid diffusion of the dye into the surrounding cells. In the presence of TG, TM or SubAB, the numbers of dye-coupled cells were significantly reduced (Fig. 2C,D).

ER STRESS INHIBITS CX43 SYNTHESIS

To determine the mechanism by which ER stress regulates the Cx43 level, we investigated the effects of ER stress on Cx43 synthesis and degradation, respectively.

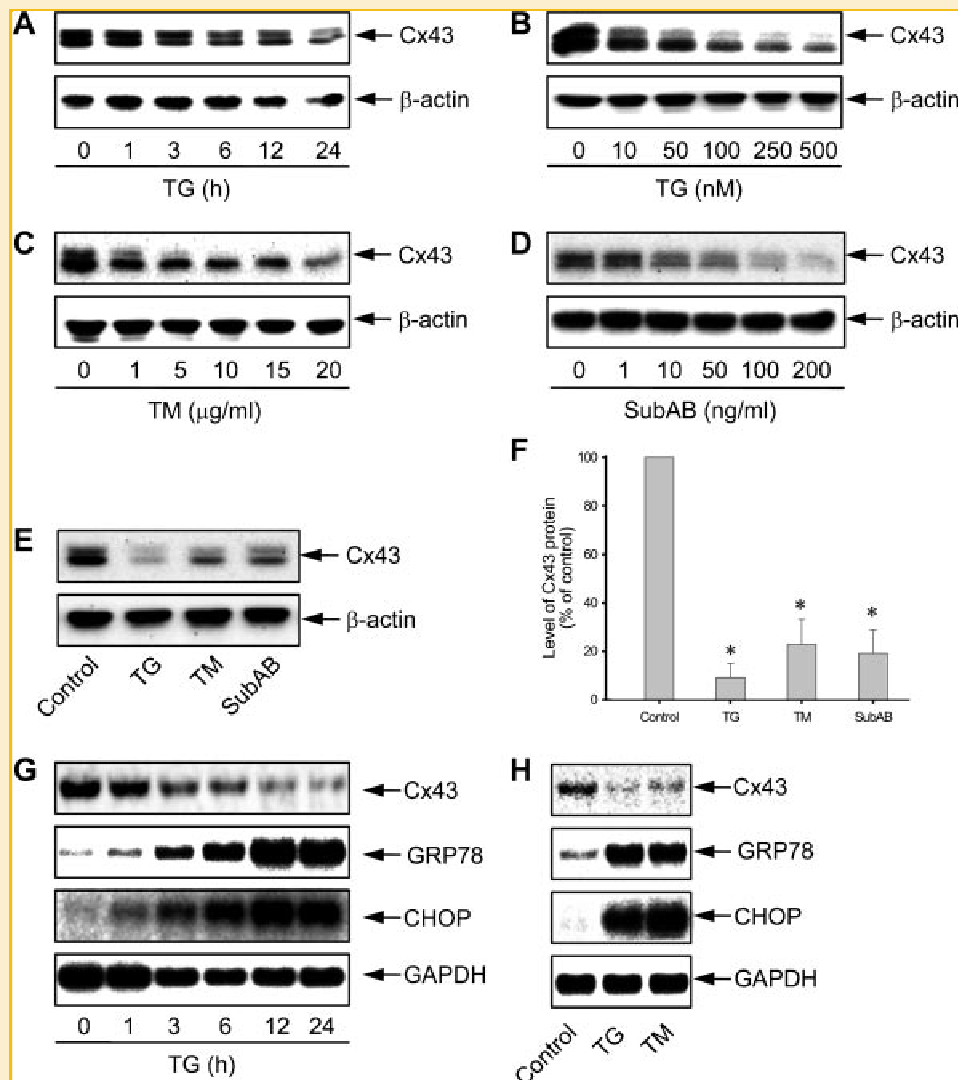

Fig. 1. Inhibition of Cx43 expression by ER stress. A–D: Effects of TG (A and B), TM (C) and SubAB (D) on Cx43 protein levels. MCs were exposed to 100 nM TG (A) for the indicated times (E), or various concentrations of TG (B), TM (C), or SubAB (D) for 12 h. (E–F) MCs were exposed to 100 nM TG (A), 5 μg/ml TM, or 30 ng/ml SubAB for 12 h (E). The cellular protein was extracted and subjected to Western blot analysis of Cx43. Expression of β-actin is shown at the bottom as a loading control. The intensities of Cx43 signal in E was measured using Scion Image, and relative intensity of the each band is shown in (F) (mean ± SE, n = 5), $^{*}P < 0.01$ versus untreated control. (E and F) Effects of ER stress on Cx43 mRNA expression. MCs were treated with 100 nM TG for the indicated time (G), or exposed to 100 nM TG or 5 μg/ml TM for 10 h (H). Cellular RNA was extracted and subjected to Northern blot analysis of Cx43, GRP78, and CHOP. Expression of GAPDH is shown at the bottom as a loading control.

First, we examined the effect of ER stress on Cx43 gene expression. For this purpose, MCs were transfected with the luciferase gene fused to the Cx43 promoter [Mitchell and Lye, 2005], and the promoter activity was evaluated by luciferase assay [Yao et al., 2006]. As shown in Figure 3A, incubation of MCs with TG, TM, SubAB, or A23187 (which induces ER stress by increasing intracellular calcium pools) for 8 h resulted in a significant suppression of luciferase activity. A similar effect was observed in cells costimulated with platelet-derived growth factor (PDGF) plus 3-isobutyl-1-methylxanthine (IBMX), which activate Cx43 promoter activity in MCs [Yao et al., 2006].

To test for a possible involvement of mRNA stability in the effect of ER stress, MCs were treated with actinomycin D, and the decay of Cx43 message was analyzed. Figure 3B,C shows that Cx43 mRNA was gradually decreased after incubation of cells with actinomycin D. The half-life of Cx43 mRNA was 4.61 ± 0.23 h in control cells (Fig. 3C). Treatment of cells with TG did not significantly affect degradation of Cx43 mRNA (half-life: 4.00 ± 0.53 h; mean ± SE; n = 5; $P > 0.05$).

To examine whether ER stress affected levels of newly synthesized Cx43, MCs were pulse-labeled with [^{35}S]-methionine in the presence or absence of ER stress inducers. The amount of [^{35}S]-labeled Cx43 was analyzed by immunoprecipitation and autoradiography. As shown in Figure 3D (top), TG inhibited [^{35}S]-methionine incorporation into Cx43 in a concentration-dependent manner. A similar effect was observed using TM and SubAB (Fig. 3D, bottom).

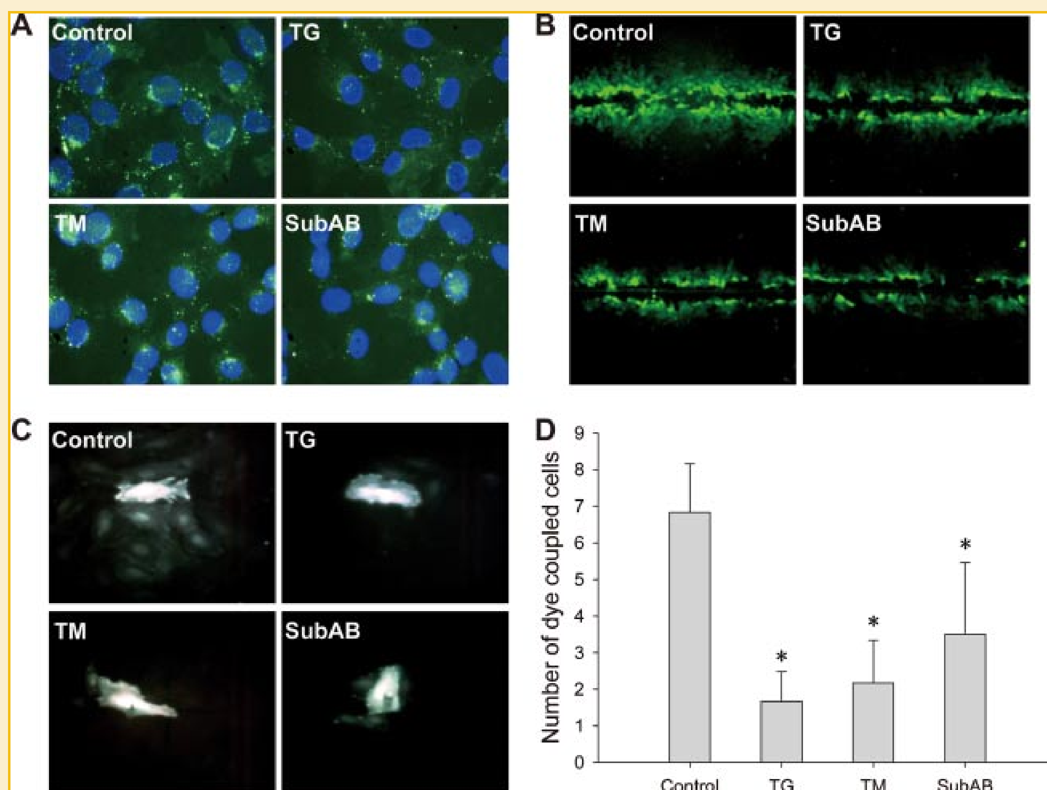

Fig. 2. Effect of ER stress on Cx43 distribution and GJIC. A: Immunofluorescent staining of Cx43. MCs were either left untreated or incubated with 100 nM TG, 5 μg/ml TM, or 20 ng/ml SubAB for 12 h, and then subjected to immunofluorescent staining of Cx43 (green) and nuclei (DAPI stain, blue). Note the obvious disappearance of Cx43 (green) at the perinuclear area in the treated cells. Magnification, 600×. B–D: Disruption of GJIC by ER stress. B: Effects of ER stress on GJIC measured by SLDT assay. Fluorescent micrographs of MCs after SLDT. MCs were either left untreated or exposed to 100 nM TG, 5 μg/ml TM or 20 ng/ml SubAB for 12 h. C: Microphotographic analysis of dye-coupled cells after microinjection of LY. Confluent cultures of MCs were either untreated or treated with 100 nM TG, 5 μg/ml TM, or 20 ng/ml SubAB for 12 h, and LY was injected into a single cell. LY diffusion into adjacent cells was photographed. Magnification, 320×. D: Quantitative analysis of dye-coupled cells. The number of cells into which LY was transferred from the injected cells within 3 min is shown. Data are presented as means ± SE (n = 6–10). $^{*}P < 0.01$ versus untreated control.

ER STRESS ACCELERATES CX43 DEGRADATION

An alternative mechanism by which ER stress could down-regulate Cx43 protein level is to enhance its degradation. To analyze the effects of ER stress on Cx43 degradation, MCs were treated with cycloheximide to inhibit protein synthesis, and the rate of Cx43 protein degradation in the presence or absence of ER stress inducers was evaluated [Leithe and Rivedal, 2004]. As shown in Figure 4A, blockade of protein synthesis using cycloheximide caused a gradual degradation of Cx43. The time required to achieve 50% degradation was 1.77 ± 0.26 h (mean ± SE, n = 3) in control cells. In the presence of TG, Cx43 degradation was significantly accelerated (half-life: 1.07 ± 0.10 h; mean ± SE, n = 3; $P < 0.05$). Like TG-treated cells, Cx43 level in TM-treated cells was also significantly lower than that in control cells (Fig. 4C,D)

To confirm participation of proteasome degradation pathway in ER stress-induced degradation of Cx43, we have investigated the influence of proteasome inhibition on Cx43 levels. As shown in Figure 4E,F, proteasome inhibitor MG132 increased basal level of Cx43 and largely attenuated the reduction of Cx43 triggered by ER stress inducers TG and TM.

ER STRESS DOWNREGULATES CX43 EXPRESSION IN SEVERAL DIFFERENT CELL TYPES

To determine whether the effects of ER stress on Cx43 expression could be observed in other cell types, we evaluated the influence of ER stress on Cx43 expression in HUVEC, As4.1 and Huh-7 cells. The induction of ER stress by various inducers in the tested cells was confirmed by the elevation of GRP78 and/or CHOP. Of note, incubation of cells with SubAB resulted in an expected disappearance of GRP78 in the blots [Paton et al., 2006]. Figure 5A–D shows that the tested cell lines displayed a similar reduction in Cx43 in response to ER stress. We also assessed the selectivity of the effect of ER stress on Cx isoforms in MCs. Similar to Cx43, Cx40 was also downregulated by ER stress in MCs (Fig. 5A).

To answer whether ER stress also down-regulates other membrane proteins, we have examined the level of Thy-1, a glycosylphosphatidylinositol (GPI)-anchored cell surface protein in MCs. As shown in Figure 5E, ER stress did not alter the level of Thy-1, suggesting that the downregulating effects of ER stress on membrane protein were relatively selective.

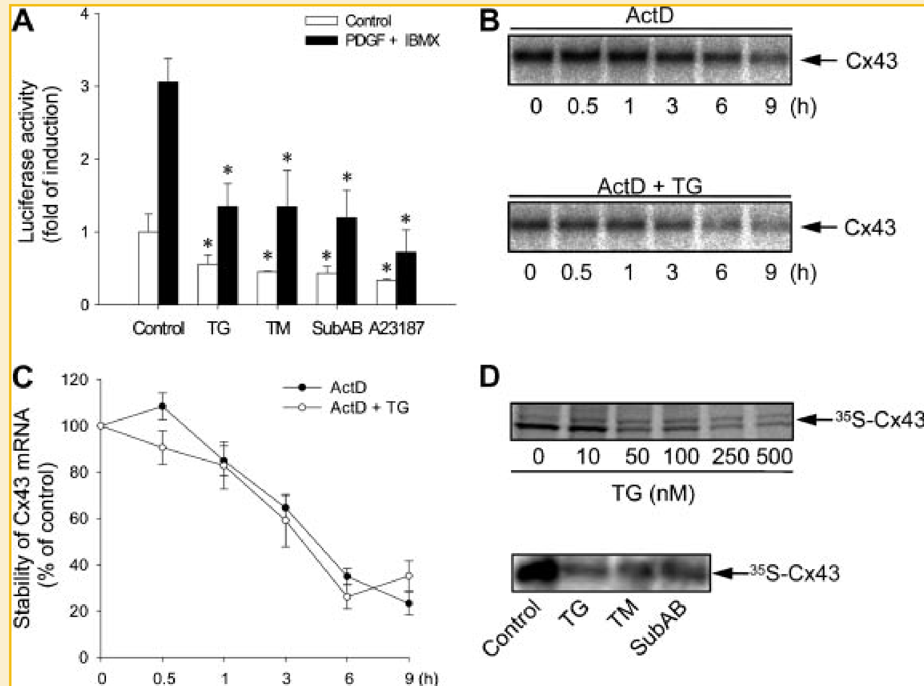

Fig. 3. Effect of ER stress on Cx43 synthesis. A: Suppression of the Cx43 promoter activity by ER stress. MCs were transiently transfected with the Cx43 promoter (pCx1686-Luc) and exposed to TG (100 nM), TM (5 μg/ml), SubAB (20 ng/ml) or A23187 (5 μM) in the presence or absence of IBMX (250 μM) plus PDGF-BB (30 ng/ml) for 8 h. The relative luciferase activity is represented as the induction over untreated control (mean ± SE, n = 4). $^{*}P < 0.01$ versus untreated control. B,C: Effect of ER stress on Cx43 mRNA stability. B: MCs were either left untreated (upper panel) or treated with 100 nM TG (lower panel) for 4 h before exposing to actinomycin D (ActD; 5 μg/ml) for the indicated time periods. Total RNA was extracted and subjected to Northern blotting analysis. C: Results of the densitometric analysis of the northern blots. The blots were subjected to densitometric analysis. Solid circles represent MC treated with ActD alone, and open symbols represent cells treated with ActD and TG (mean ± SE; n = 5). D: ER stress on Cx43 protein synthesis. MCs were labeled with ^{35}S-methionine (100 μCi/ml) in the presence of 100 nM TG (upper panel), or various stimuli (100 nM TG, 5 μg/ml TM or 20 ng SubAB) for 3 h (lower panel). At the end of labeling, cellular lysates were extracted, and subjected to immunoprecipitation followed by SDS-PAGE and autoradiography.

INDUCTION OF ER STRESS BY HYPOXIC CHEMICALS IS ASSOCIATED WITH A REDUCED CX43 LEVEL

Downregulation of Cx43 expression and function by ER stress suggests that ER stress-related signals could be important mechanisms underlying the malfunction of GJs in a variety of pathological situations. Given hypoxia is a well-known trigger of ER stress both in vivo and in vitro [Feldman et al., 2005; Wouters et al., 2005; Xu et al., 2005; Koong et al., 2006; Koumenis, 2006; Lee and Hendershot, 2006; Moenner et al., 2007; Sawada et al., 2008], we asked whether hypoxic ER stress also results in an altered level of Cx43. For this purpose, MCs were exposed to agents that mimic hypoxia in vitro. Thenoyltrifluoroacetone (TTFA) and $CoCl_2$ were used for this purpose. TTFA mimics hypoxia by inhibiting the Q reduction site of mitochondrial respiratory complex II [Sun et al., 2005], and $CoCl_2$ induces hypoxia by stabilizing hypoxia inducible factor-1α [Corley et al., 2005]. As shown in Figure 6, treatment of MCs with TTFA or $CoCl_2$ induced ER stress, as demonstrated by the increased levels of GRP78 and CHOP. The hypoxic ER stress was associated with an obviously reduced level of Cx43. Of note, the increased mRNA expression of GAPDH in $CoCl_2$-treated cells in Figure 6B suggested the presence of hypoxic stress under the experimental condition [Graven et al., 1994].

DISCUSSION

In this study, we provide the first evidence showing that ER stress is critically involved in the regulation of GJs. Incubation of MCs with several different ER stress inducers, TG, TM, SubAB and A23187, similarly inhibited the expression of Cx43 and disrupted GJIC. Given these agents activate UPR through different mechanisms, we propose that ER stress has the potential to alter the level of GJs and GJIC.

The suppressive effect of ER stress on Cx43 expression was rapid and potent. This may be explained by the fast turnover of Cx43 and participation of several regulatory mechanisms in Cx turnover. The half-life of Cx43 in MCs was about 2–3 h, based on the results of the rate of Cx43 degradation, which is in agreement with previous reports on other cell types (half-lives 1.5–5.5 h) [VanSlyke and Musil, 2002; Saez et al., 2003]. In response to ER stress, multiple mechanisms operated to repress Cx43. ER stress suppressed Cx43 promoter activity and decreased Cx43 mRNA expression, and most likely also accelerated degradation of Cx43 protein.

It should be mentioned that the faster loss of Cx43 protein observed in cells simultaneously treated with cycloheximide and TG did not necessarily reflect accelerated Cx43 degradation. It could

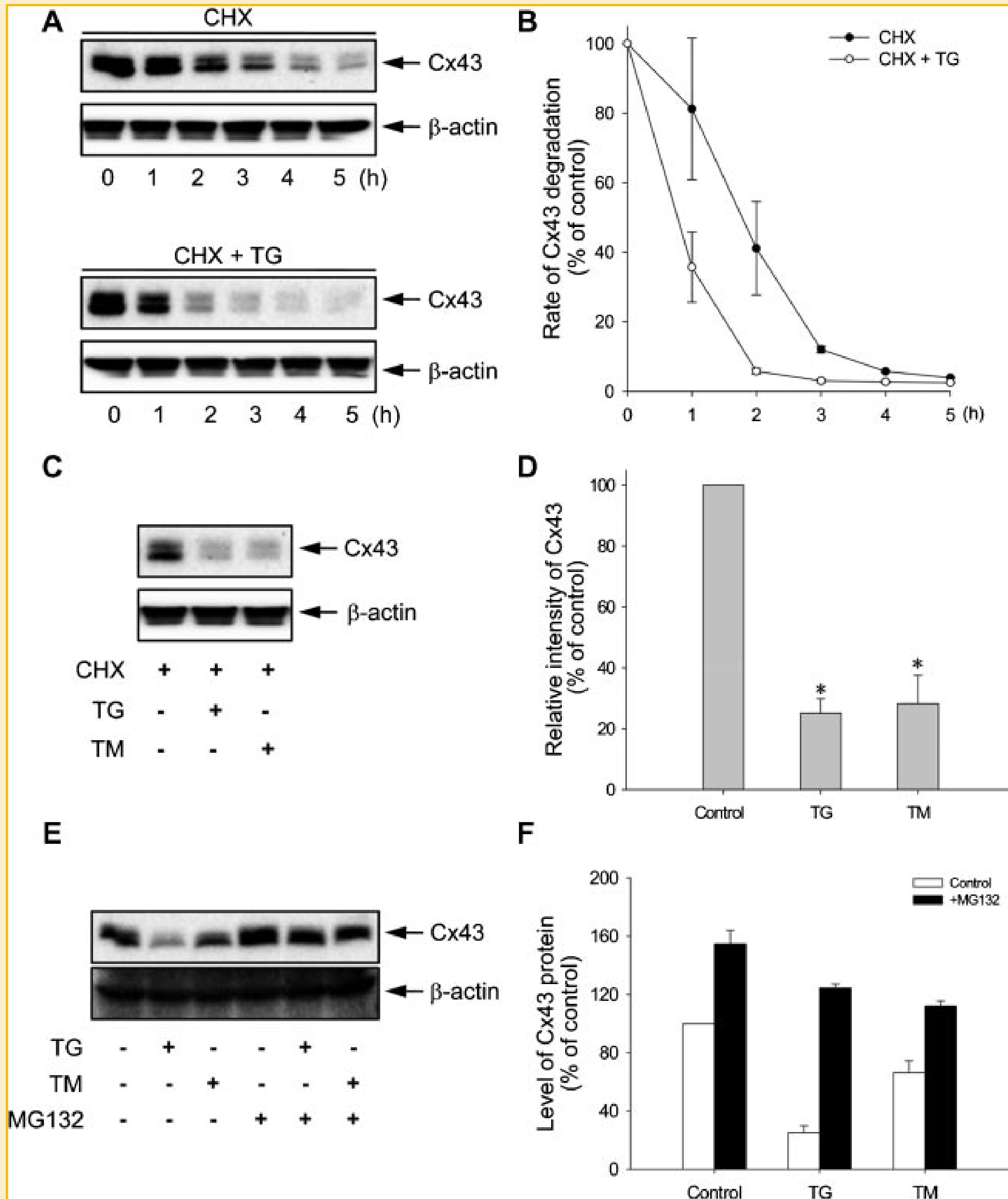

Fig. 4. Effect of ER stress on Cx43 protein degradation. A,B: Time-course effect of TG on Cx43 degradation. MCs were exposed to 50 μg/ml cycloheximide in the presence (lower panel) or absence (upper panel) of 100 nM TG for the indicated length of times. Cellular proteins were analyzed by Western blot with an anti-Cx43 antibody. A representative blot is shown in (A). B: The intensities of Cx43 signal in A was measured using Scion Image, and relative intensity of the band against its intensity at zero point are shown (mean ± SE, n = 3). C,D: Effects of TG and TM on Cx43 degradation. MCs were exposed to 50 μg/ml cycloheximide with or without 100 nM TG or 5 μg/ml TM for 3 h. D: The intensities of Cx43 signal in C. Relative intensity of the band against its intensity at zero points is shown (mean ± SE, n = 4). $^{*}P < 0.01$ versus untreated control. (E and F) Effects of MG132 on Cx43 levels. MCs were exposed to 10 μg/ml MG132 with or without 100 nM TG or 5 μg/ml TM for 6 h. Cellular proteins were analyzed by Western blot with an anti-Cx43 antibody. A representative blot is shown in (E). F: The intensities of Cx43 signal in A was measured using Scion Image, and relative intensity of the band against untreated control are shown (mean ± SE, n = 4).

also be a result of the overlapping effects of these agents on protein translation. However, several considerations made this speculation less likely. First, a saturated concentration of cycloheximide (50 μg/ml) has been used for inhibition of protein synthesis. This left less space for incomplete inhibition of protein synthesis. Second, the faster loss of Cx43 was also induced by TM, which activates ER stress through mechanism different from TG. Third, consistent with the increased Cx43 degradation, inhibition of proteasome degradation pathway with MG132 largely prevented ER stress-induced reduction of Cx43 protein levels. Therefore, the fast loss of Cx43 triggered ER stress inducers was most probably due to increased Cx43 degradation.

Little is known about mechanisms involved in the regulation of GJ by ER stress. Implication of transcriptional regulation in down-

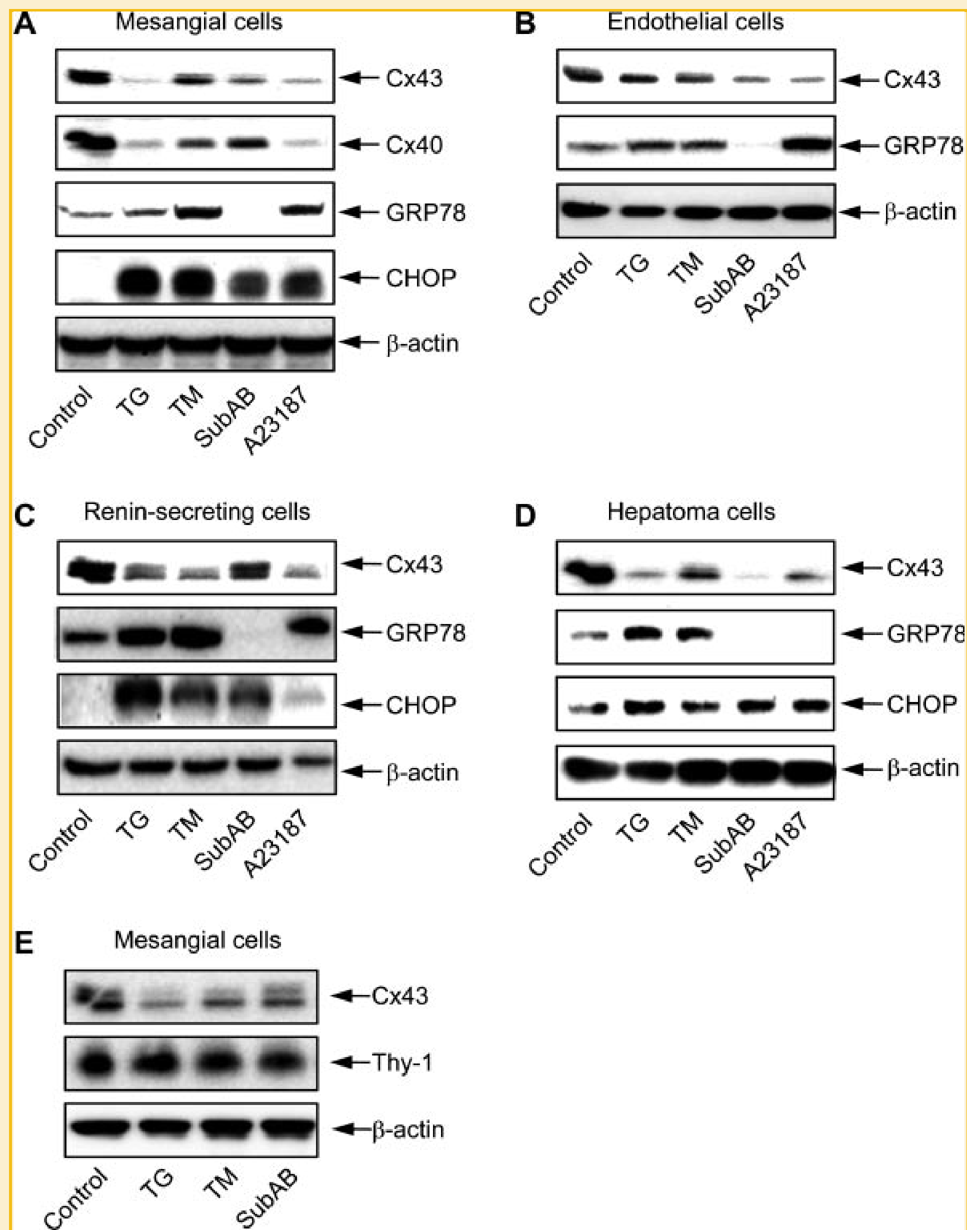

Fig. 5. Effects of ER stress on Cx43 expression in several different cell lines or on Thy-1 expression in MCs. Rat MCs (A), human umbilical vein endothelial cells (HUVEC; B), mouse-derived renin-secreting cells (As4.1; C) and human hepatoma cells (Huh-7; D) were exposed to 100 nM TG, 5 μg/ml TM, 20 ng/ml SubAB or 5 μM A23187 for 12 h. The expression of GJ proteins (Cx40 and Cx43) and markers of ER stress (GRP78 and CHOP) was determined by Western blot. β-Actin levels shown at the bottom of the blots indicate the same amount of loading of the protein. E: Effect of ER stress on Thy-1 protein level. MCs were exposed to 100 nM TG, 5 μg/ml TM and 20 ng/ml SubAB. The expression of Thy-1 and Cx43 was determined by Western blot.

regulation of membrane proteins by ER stress has been described in yeast [Kimata et al., 2006]. However, the mechanisms of this process are presently unknown. Several studies demonstrated that UPR attenuates translation of secretory protein via activation of PKR-like ER kinase (PERK) and subsequent phosphorylation of eIF2α at Ser 51 [Martinez and Chrispeels, 2003; Pakula et al., 2003; Al-Sheikh et al., 2004; Schroder and Kaufman, 2005; Koumenis, 2006]. We have tested the possible role of eIF2α phosphorylation in our experimental setting. Our preliminary results showed that treatment of MCs with salubrinal, an activator of eIF2α [Boyce et al., 2005], did not affect Cx43 expression under both normal and ER stress conditions (data not shown). Recently, ERAD has been reported to mediate the ER stress-induced decrease in several membrane proteins, such as the major histocompatibility complex and the genomic cystic fibrosis transmembrane conductance regulator [Hegde et al., 2006; Rab et al., 2007]. Because the proteasome is one of the major degradation pathways for GJ proteins [VanSlyke and Musil, 2002; Saez et al., 2003], increased Cx43 degradation observed in this study was most likely ascribed to activation of ERAD. In support of the notion, inhibition of proteasome with

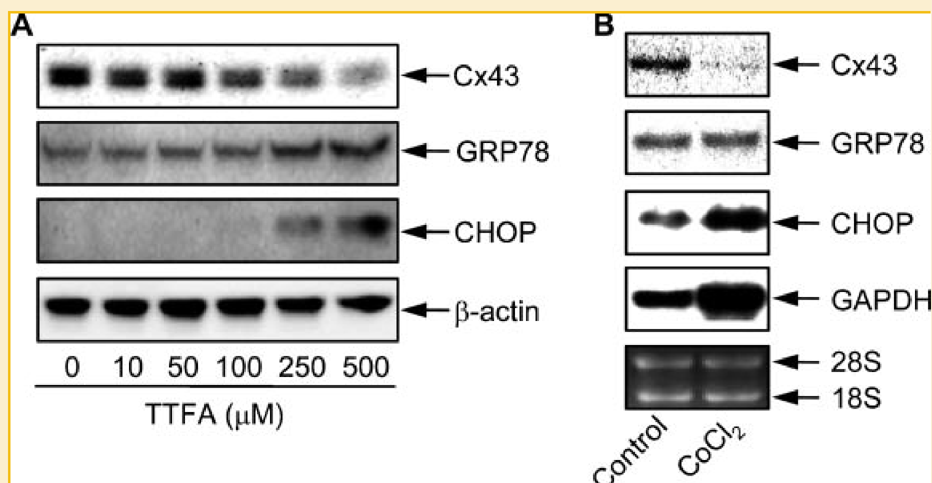

Fig. 6. Induction of ER stress and suppression of Cx43 expression by hypoxia-inducing agents TTFA and $CoCl_2$. MCs were exposed to the indicated concentration of TTFA for 12 h (A) or 100 μM $CoCl_2$ for 8 h (B). Cellular protein or total mRNA was extracted and subjected to Western (A) or Northern blot (B) analysis for expression of Cx43, GRP78 and CHOP. β-actin and 18S ribosomal RNA shown at the bottom of the blots indicate the same amount of loading of protein and RNA, respectively.

MG132 caused accumulation of ubiquitylated Cx43 (Supplementary Fig. 1). In addition, MG132 largely attenuated the suppressive effects of ER stress on Cx43 levels. Further studies are needed to clarify the relevant signaling mechanisms.

Besides rat MCs, ER stress also downregulated Cx43 expression in several different cell types, including human umbilical vein endothelial cells, mouse-derived renin-secreting cells and human hepatoma cells. It is possible that the same regulatory mechanisms may also operate in these cells. Of note, contradictory to our findings, Wang et al. have reported that TM, in a experimental setting similar to ours (4 μg/ml TM, 8 h incubation), promoted GJ channel formation and function in Cx43-overexpressing or cAMP-stimulated Morris hepatoma H5123 cells [Wang and Mehta, 1995; Wang et al., 1995]. The reason for the discrepancy is presently unclear. Because the promoting effects was not observed in control cells that expressed a low level of Cx43, the basal level of Cx43 could be an factor determining cell responses to TM. It is also possible that the effect of ER stress on GJ formation and function could be cell-type specific. Further studies may be needed to clarify why the different cell types displayed different responses to TM in Cx43 expression and function.

Participation of multiple mechanisms in rapid elimination of GJ proteins following ER stress in the current investigation highlights the importance of GJ in UPR. Why should GJ be targeted? What are roles of GJs in UPR? We speculated that elimination of GJs may alleviate the stress and protect cells from the stress-related injury. It is known that UPR inhibits protein synthesis, especially those required to be processed in the ER, such as membrane and secretory proteins, to attenuate the load of client proteins in the ER [Schroder and Kaufman, 2005; Kimata et al., 2006; Rab et al., 2007; Kitamura, 2008]. Given that Cx has a relatively short half-life among plasma membrane proteins [VanSlyke and Musil, 2002; Saez et al., 2003], shutdown of Cx synthesis could lead to rapid attenuation of protein load in ER. In agreement with this notion, we did observe a rapid disappearance of Cx43 at the perinuclear regions following ER stress. In addition, the effects of ER stress on membrane proteins were relatively selective; the level of Thy-1, a surface marker of MCs with half-life over 100 h [Sunyach et al., 2003], was not affected. In addition, the protein levels of IKKγ (a short-lived signal molecule with half-life comparable to Cx43) was also not altered by ER stress [Broemer et al., 2004; Hayakawa et al., 2009]. Another advantage of eliminating GJ proteins over other proteins is that it may prevent the transmission and amplification of 'stress' signals to neighboring cells. GJs has been documented to mediate transfer of molecules like superoxide and calcium ions to propagate a toxic response in several different pathological situations [Lin et al., 1998; Azzam et al., 2001; Chipman et al., 2003]. Given formation of reactive oxygen species (ROS) and leakage of calcium ions from ER during ER stress have been documented [Doutheil et al., 1997; Harding et al., 2003; Haynes et al., 2004]. It is possible that GJs transmit and propagate ER stress-elicited cell damage. In this context, downregulation of GJs may serve as a defensive mechanism for the cells to against ER stress-elicited cell injury.

Another significant finding is that ER stress may be a novel mechanism involved in downregulation of GJs. Presence of ER stress and dysfunction of GJ have been extensively documented in ischemic injuries and cancers [Peters et al., 1993; Kaprielian et al., 1998; Chipman et al., 2003; Mesnil et al., 2005]. Our results demonstrated that the decreased Cx43 levels induced by hypoxia-inducing agents in MCs were closely associated with the occurrence of ER stress. Thus, ER stress may underlie the altered GJ in hypoxic situations.

Collectively, we demonstrated that induction of ER stress results in a rapid elimination of GJ protein and disclosure of GJIC via inhibition of Cx synthesis and promotion of Cx degradation. Targeting GJ could be an important protective mechanism utilized by cells to adapt to ER stress. In addition, our findings provide a presently unrecognized mechanism involved in the regulation of GJs. It may open a new window toward our understanding of the regulatory mechanisms of GJs by pharmacological, carcinogenic, and hypoxic factors.

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