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An ATF4-Signal-Modulating Machine Other Than GADD34 Acts in ATF4-to-CHOP Signaling to Block CHOP Expression in ER-Stress-Related Autophagy

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ABSTRACT

Cells respond to ER-stress via ER-stress sensors, leading to the UPR and subsequent apoptosis; however, occasionally, they activate autophagy without subsequent apoptosis in response to ER-stress. We previously showed that the induction of apoptosis by ER-stress was related to the presence or absence of CHOP expression; nevertheless, how ATF4 expression is elicited without downstream CHOP expression is unknown. We studied the role of GADD34 on the induction of autophagy and/or apoptosis by NaF- or tunicamycin-induced ER-stress in HepG2 cells transfected with GADD34 siRNA. Although NaF and tunicamycin both induced PERK activation followed by eIF2α phosphorylation and ATF4 expression, CHOP expression was only induced by tunicamycin. Concomitant with the signaling change, autophagy was activated both by NaF and tunicamycin, and apoptosis was induced only by tunicamycin. After 4 h, GADD34 mRNA expression was also increased by NaF and tunicamycin. Suppression of GADD34 by GADD34 siRNA increased ATF4 expression in both NaF- and tunicamycin-treated cells. The GADD34 siRNA increased CHOP expression, which corresponded to increased ATF4 in tunicamycin-treated cells; however, the increased ATF4 did not induce CHOP expression in NaF-treated cells. In concert with signal changes, siRNA treatment additively increased the autophagic activity of both NaF- and tunicamycin-treated cells; however, apoptosis was produced and accelerated only for tunicamycin-treated cells. These findings indicate that GADD34 expression induced by ER-stress delays CHOP expression and retards apoptotic cell death, and that an ATF4-signal-modulating machine other than GADD34 acts on ATF4-to-CHOP signaling to block ATF4-induced CHOP expression in ER-stress related autophagy. J. Cell. Biochem. 116: 1300–1309, 2015.

KEY WORDS: ER-STRESS; AUTOPHAGY; PERK PATHWAY; GADD34; CHOP EXPRESSION; APOPTOSIS

Cells have evolved elaborate mechanisms to ensure that proteins are folded and assembled accurately before transport to other organelles. Only correctly folded proteins are allowed to leave the ER. Abnormalities such as the accumulation of unfolded proteins in the ER are collectively termed ER-stress [Schröder and Kaufman, 2005; Faitova et al., 2006]. Many compounds that modify proteins induce ER-stress, and the capacity of cells to respond to toxic stress is critical for survival [Tabas and Ron, 2011]. ER-stress causes the cell to activate a self-protective mechanism termed the unfolded protein response (UPR). The UPR is mediated through three ER-stress sensor proteins, Ire1, AtF6, and PERK, that are located in the ER membrane and signal adaptive responses to the cytosol and nucleus [Mori, 2000; Hetz, 2012]. The UPR initially induces protective events by reducing the ER protein synthesis load and controlling protein quality and folding [Ron and Walter, 2007; Hetz, 2012; Malhi and Kaufman, 2011]; however, if the UPR is overwhelmed, apoptosis can be initiated [Hetz et al., 2011; Malhi and Kaufman, 2011]. A key aspect of ER-stress-induced apoptosis is the induction of CHOP [Zinszner et al., 1998; Oyadomari and Mori, 2004; Tabas and Ron, 2011]. CHOP induces the expression of the pro-apoptotic protein Bim [Puthalakath et al., 2007] and represses expression of the anti-apoptotic protein Bcl2 [McCullough et al., 2001], leading to apoptosis. In contrast, ER-stress can also induce autophagy [Ogata et al., 2006; Yorimitsu et al., 2006]. Multiple connections must exist between ER-stress, autoph-

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agy, and apoptosis; nevertheless, the regulatory association of autophagy with the UPR is not well understood, including the switch between autophagy and apoptosis in the UPR.

Our previous work indicated that in rats, administration of NaF induced extensive autophagosome formation in exocrine pancreatic cells as a result of intracisternal granule accumulation in the ER, and this activation of autophagy was associated with activation of the PERK-eIF2α pathway [Ito et al., 2009]. The PERK-eIF2α pathway has been identified as being required for autophagy [Kouroku et al., 2007; Salazar et al., 2009; Kim et al., 2010; Avivar-Valderas et al., 2011; Yu et al., 2011]. During cellular adaptation to tumor hypoxia, hypoxia activates autophagy through PERK-dependent expression of ATF4 and CHOP [Rouschop et al., 2010; Rzymski et al., 2010]. The accumulated data strongly suggest that ATF4 is important in the activation of autophagy. Recently, we showed that ATF4 was a key signal for ER-stress-induced autophagy and that increasing ATF4 expression via NaF-induced ER-stress did not cause downstream CHOP expression [Matsumoto et al., 2013]; nevertheless, the signal machine working at the ATF4-to-CHOP signaling step to selectively suppress CHOP expression during NaF-induced ER-stress remained unclear.

eIF2α phosphorylation contributes to the reduced formation of translational pre-initiation complexes and activates the translation of ATF4, which accumulates under stress and activates CHOP expression [Fawccett et al., 1999; Harding et al., 2000]. Concomitantly, ATF4 expression following eIF2α phosphorylation increases the translation of growth arrest and DNA damage-inducible protein 34 (GADD34), resulting in the recovery of protein synthesis and the restoration of homeostasis through eIF2α-dephosphorylation [Novoa et al., 2001]. It seems likely that although the GADD34 is a candidate for the ATF4-to-CHOP signal regulator, its transcriptional induction is part of a negative feedback loop that attenuates signaling in the integrated stress response.

In our recent study using HepG2 cells [Matsumoto et al., 2013], NaF, which induces ER-stress by disrupting ER-to-Golgi vesicle transport, only activated autophagy with increased ATF4 expression; in contrast, tunicamycin (Tu), which induces ER-stress by interfering glycosylation, induced autophagy followed by apoptosis with ATF4 and CHOP expression. It was thought that NaF and Tu activated distinct features of the ER-stress pathway to induce autophagy and/or apoptosis. Solidifying the difference between NaF- and Tu-mediated ER-stress induced cell response is important for gaining an understanding of the mechanistic differences between the induction of autophagy and apoptosis for the development of therapeutics against cancer and neurodegenerative diseases. To define the role of GADD34 and the signaling differences between NaF- and Tu-mediated ER-stress on the induction of autophagy, we used GADD34 siRNA to examine the effects of GADD34 on the activation of autophagy and/or apoptosis by NaF, which activates only autophagy, or tunicamycin, which initially activates autophagy and then activates apoptosis.

MATERIALS AND METHODS

ANTIBODIES AND REAGENTS

Primary antibodies for Western blot analysis were mouse monoclonal antibody specific for β-actin (Sigma-Aldrich Co., St. Louis, MO), and rabbit antibodies recognizing ATF4 and CHOP (Santa Cruz Biotechnology, Santa Cruz, CA); GADD34 (Abcam, Cambridge, UK); eIF2α, Phospho-eIF2α, Ire1, and PERK (Cell Signaling Technology, Danvers, MA); and Phospho-Ire1 (Novus Biologicals, Littleton, CO). Secondary antibodies were horseradish peroxidase-conjugated goat antibodies recognizing rabbit IgG and mouse IgG (Cappel, Aurora, OH). Small interfering RNAs (siRNAs) for *ATF4* (SI03019345), *CHOP* (SI00059528), *GADD34* (SI02659132) and AllStars negative control siRNA (QIAGEN, Hilden, Germany) were used to suppress mRNA and protein expression. Lipofectamine RNAiMAX Reagent and Opti-MEM I Reduced-Serum Medium (Life Technologies, Inc., Rockville, MD) were used as siRNA transfection reagents. Hoechest33342 (CALBIOCEM, Darmstadt, Germany) and Monodansylcadaverine (Dansylcadaverine, MDC, Sigma-Aldrich Co.) were used for histochemistry. Thapsigargin and tunicamycin were purchased from Sigma-Aldrich Co., and NaF was purchased from Nakarai TECK (Kyoto, Japan). HepG2 cells (Human Hepatocellular Carcinoma Cell Line) were portioned from Health Science Research Resource Bank (Sen-nan City, Japan).

CELL CULTURE, ER-STRESS INDUCTION, AND ACTIVATION OF AUTOPHAGY AND/OR APOPTOSIS

HepG2 cells were maintained in Dulbecco′s modified Eagle′s medium (DMEM, Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) containing 10% fetal bovine serum (FBS, Nichirei Biosciences, Osaka, Japan) at 37°C. After being pre-cultured for 48 h at density of 1.0×10^5 cells/ml, the cells were treated with NaF (1 M in phosphate-buffered saline, 2 mM final concentration), tunicamycin (2 mg/ml in 1 N NaOH, 2 μg/ml final concentration), or thapsigargin (2 mM in DMSO, 250 nM final concentration) for the indicated times to induce ER-stress. To induce autophagy by nutrient deprivation, the cells were cultured in Hank′s Balanced Salt Solution (HBSS) for 4, 6, and 10 h following the pre-culture. Thapsigargin and HBSS treatments were used as positive controls for ER-stress-induced apoptosis and starvation-induced autophagy, respectively.

To assess autophagic activity, the treated cells were stained with MDC (100 mM in a solution of acetic acid/DMEM = 1:5, 100 μM final concentration) at 37°C for 45 min [Biederbick et al., 1995]. HepG2 cells were observed by fluorescent microscopy (IMT-2) using 335 nm excitation and 512 nm emission. For morphometric analysis of autophagic activity, five areas were selected at random in a 6-cm culture dish and images were taken at 400-fold magnification under the microscope. Five cells were then randomly selected in each area and their fluorescence intensities were measured using Scion image. The mean values of the fluorescence intensities measured in 25 cells were used to evaluate autophagic activity. Three independent experiments were performed.

To assess apoptosis, the treated cells were stained with Hoechest33342 (1 mg/ml in distilled water, 2 μg/ml final concentration) at 37°C for 30 min [Yao et al., 2011]. The cells were observed by fluorescent microscopy using 346 nm excitation and 460 nm emission. To calculate the ratio of the appearance of apoptosis (apoptotic ratio), five areas in a 6-cm culture dish were randomly selected and images were taken at 200-fold magnification under the microscope. The numbers of living cells containing clear round nuclei and apoptotic cells containing fragmented nuclei were counted in

each image. The apoptosis ratio in each sample was valued as the average of the values calculated from five images. Three individual experiments were repeated.

WESTERN BLOT ANALYSIS

The cells treated with ER-stress inducers and HBSS were lysed with 1% Triton HEPES buffer, pH 7.5 (20 mM HEPES, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA-Na_2, 10 mM sodium pyrophosphate, 100 mM NaF, 17.5 mM glycero-2-phosphate disodium salt hydrate, 1 mM PMSF, 4 mg/ml aprotinin, and 2 μg/ml pepstatin). Whole cell lysates were resolved by SDS/PAGE (7.5%, 10%, or 12% SDS–polyacrylamide gels), electro-blotted onto Immobilon-p Transfer Membrane (Merck Millipore, Darmstadt, Germany), and probed with the indicated antibodies (ATF4 1:200; CHOP 1:200; eIF2α 1:1,000; Phospho-eIF2α 1:1,000; GADD34 1:25,000; Ire1 1:1,000; Phospho-Ire1 1:1,000; PERK 1:1,000, β-actin 1:20,000). Horseradish peroxidase conjugated goat antibodies to rabbit IgG (1:1000) and mouse IgG (1:2000) were used as secondary antibodies. Following incubation of the second antibody for 1 h at room temperature, the corresponding bands were detected using ECL (Millipore Co., Billerica, MA) and LAS-3000 (FUJIFUILM, Tokyo, Japan). Quantification of protein expression was performed by densitometry of the positive band in each Western blot using Multi Gauge software for Windows (FUGIFILM). Each density value was normalized with actin in each Western blot.

HepG2 CELL siRNA TREATMENT

RNA interference-mediated gene knockdown was achieved using pre-validated QIAGEN HP siRNAs for *ATF4* (SI03019345), *CHOP* (SI00059528), and *GADD34* (SI02659132). All siRNA experiments incorporated a validated negative control siRNA (QIAGEN AllStars negative control siRNA). siRNA knockdown experiments were performed by combining 0.6×10^5 cells (dispersed in 500 μl DMEM containing 10% FCS) in 24-well plates with a mixture of 6 pmol siRNA (0.3 μl), 100 μl Opti-MEM I Reduced-Serum Medium, and 1 μl Lipofectamine RNAiMAX Reagent that had been pre-incubated for 20 min according to the manufacturer's instructions. Cells were then cultured for 48 h prior to 6 or 8 h drug treatment. Following treatment, the cultures were processed as described above.

QUANTITATIVE REAL-TIME RT-PCR ANALYSIS

Total RNA was extracted from HepG2 cells using the GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich Co.) according to the manufacturer's instructions. The expression levels of human ATF4, human CHOP, human GADD34, and housekeeping GAPDH (rat) mRNAs were determined using the specific primers indicated in Table I. The Power SYBR Green RNA-ti-CtTM 1-step Kit (Life Technology Co., Carlsbad, CA) was used to detect the quantitative real-time PCR products according to the manufacturer's instructions. The incubation conditions were as follows: cDNA synthesis at 48°C for 30 min, pre-denature at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C, annealing for 60 s at 60°C, and extension for 60 s at 60°C. PCRs for each sample were performed in triplicate for the target genes and GAPDH.

TABLE I. Quantitative PCR Primers

Gene	Forward	Reverse
ATF4	5′-TGGCCAAGCAC-TTCAAACCT-3′	5′-AACCAGTCAGG-GAGGTTGTTG-3′
CHOP	5′-GGAGCATCAGT-CCCCACTT-3′	5′-CTACACTGGGA-GTTAGGGTGT-3′
GADD34	5′-CCCAGACAGC-CAGGAAAT-3′	5′-TAAAGGACC-GACAGACCC-3′
GAPDH	5′-TGTGAGTGGC-AGTGATGCCA-3′	5′-CCCGATTCGT-CAACCACCAC-3′

STATISTICAL ANALYSIS

Results are presented as the mean ± SD of the indicated number of separate individual experiments, each performed at least in triplicate. After confirming equal variances by Bartlett's test, Turkey's multiple comparison was used to compare morphometric and densitometric measurements.

RESULTS

CHANGES TO ER-STRESS SENSOR SIGNALS CAUSED BY ER-STRESS INDUCERS (NaF, Tu, AND Tg) AND NUTRIENT DEPRIVATION

In control HepG2 cells cultured for 0–10 h, PERK was detected by Western blotting as a broad band consisting of a high-density lower region and a low-density upper region (Fig. 1, upper panel of Western blot). The density patterns of broad PERK-positive bands containing high-density lower band regions did not change among culture times for the control group and 0 h points for each treatment group. In contrast, treatment with NaF, Tu, or Tg altered the density patterns of the broad PERK-positive bands (Fig. 1, upper panel: compare 0 h lane to 4–10 h lanes for each ER-stress inducer-treated group). As measured by densitometry of the high-density portion lower region within the broad PERK-positive bands, NaF, Tu, and Tg treatments led to decreased, showing significant differences at some sampling points compared with the 0 h values for each group (Fig. 1, lower panel graph). NaF and Tg treatments decreased PERK levels at early time points (4, 6, and/or 8 h) following treatment and recovered with time; Tu-treatment decrease PERK level gradually with time. In contrast, there were no differences in the density patterns of the broad PERK bands between 0 h and other times for HBSS treatment, similar to the control (Fig. 1); however, by densitometry, the high-density region of the PERK band substantially decreased with time, especially at the rate stage, but the change was not significant, correlating with decreased cell survival activity.

Another ER-stress signal sensor, Ire1, was also examined. Treatment with Tu and Tg significantly increased Ire1 phosphorylation after 10 and 8 h, respectively. These treatments also increased Ire1 protein expression levels concomitant with the increase in its phosphorylation level. Treatment with NaF did not activate Ire1 at any point (Fig. 2); moreover, HBSS did not activate Ire1 either.

The aforementioned changes in PERK protein levels suggested that NaF, Tu, and Tg activated this ER-stress sensor, but HBSS did not. Our recent study showed that NaF only induced autophagy, Tu prompted autophagy and subsequent apoptosis and Tg elicited apoptosis solely during early stages of treatment, indicating that Tg is unsuitable for

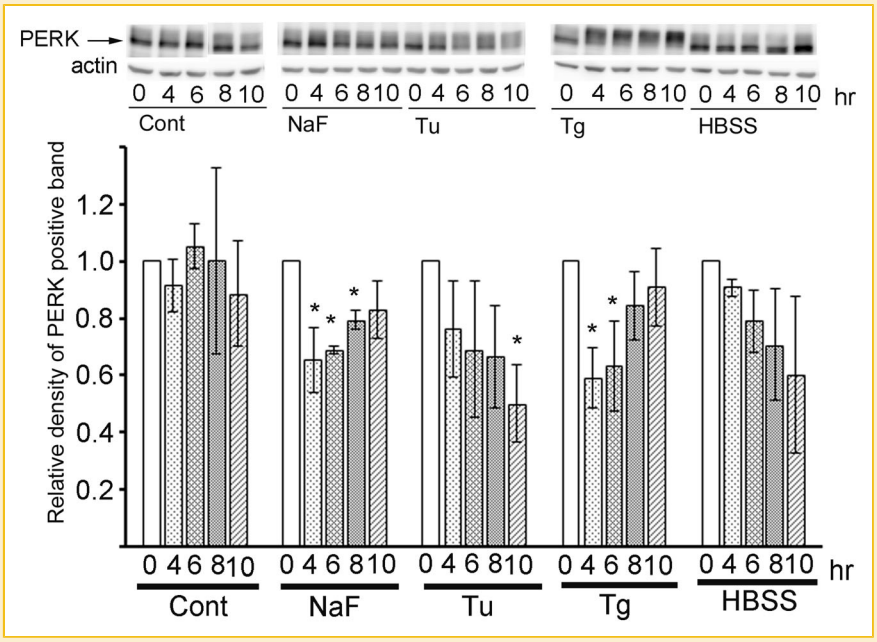

Fig. 1. Change in activated-PERK levels after exposure to ER-stress inducers (NaF, Tu, or Tg) or nutrient deprivation. Whole cell lysates were prepared for PERK Western blotting from HepG2 cells that had been exposed to ER-stress inducers (NaF; Tu, tunicamycin; or Tg, thapsigargin) for 0–10 h, or cultured with Hank's Balanced Salt Solution (HBSS). PERK activation was measured by densitometry of the positive band in each Western blot. Images are representative of three individual experiments. Density changes to the high-density low region of the broad PERK positive band was measured as a marker for activated PERK. Data are presented as the mean ± SD of three individual experiments. The * symbol denotes $P < 0.05$ compared with the 0 h for each treatment group.

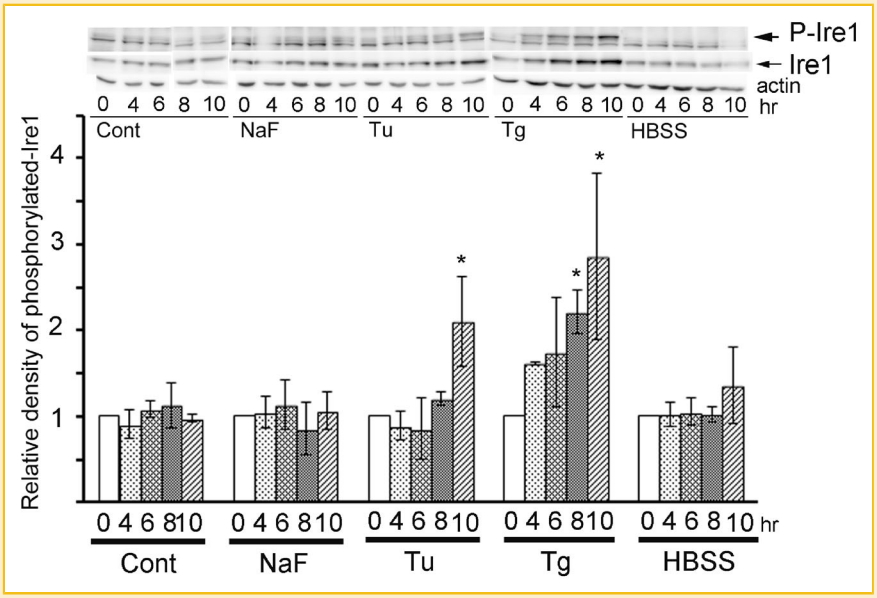

Fig. 2. Changes in Ire1 phosphorylation and expression overtime following exposure to inducers of ER-stress (NaF, Tu, or Tg) or nutrient deprivation. Whole cell lysates were prepared from HepG2 cells that had been exposed to ER-stress inducers (NaF; Tu, tunicamycin; and Tg, thapsigargin) for 0–10 h or cultured in Hank's Balanced Salt Solution (HBSS) for Western blotting of Ire1 and phosphorylated Ire1. First the membrane was reacted with anti-Pospho-Ire1 antibody and analyzed with densitometry. Following actin staining and analysis, the membrane was stripped for 30 min at 60°C using stripping-solution (62.5 mM Tris–HCl pH 6.8 containing 2% SDS and 100 mM β-mercaptoethanol) and then reacted with Ire1 antibody. Activation of Ire1 (measured by expression levels and phosphorylation changes) was measured by densitometry of the positive band in each Western blot. Images are representative of three individual experiments. Data are presented as the mean ± SD of three individual experiments. The * symbol denotes $P < 0.05$ compared with the value at 0 h for each treatment group.

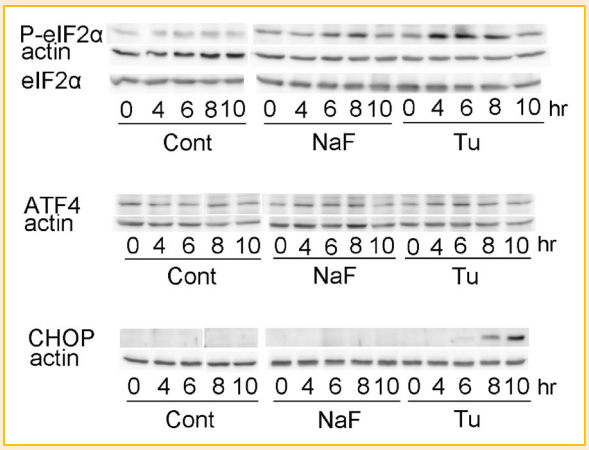

Fig. 3. PERK pathway signal changes after treatment with NaF or Tu. Whole cell lysates from HepG2 cells exposed for 0–10 h to an ER-stress inducer (NaF; or Tu, tunicamycin) were prepared for Western blotting of eIF2α, phosphorylated eIF2α, ATF4, and CHOP. Protein expression and its phosphorylation levels are shown by Western blotting for P-eIF2α, eIF2α, ATF4, and CHOP. Images are representative of three individual experiments.

the study of autophagy-regulatory mechanisms. Consequently, subsequent experiments were performed using the NaF and Tu. Treatment with NaF and Tu moderately increased eIF2α phosphorylation and ATF4 expression (Fig. 3, upper panel for eIF2α phosphorylation and middle panel for ATF4 expression). Treatment with NaF increased eIF2α phosphorylation, peaking at 6 h of treatment with 1.6-fold greater phosphorylation than that measured at 0 h (control), and ATF4 expression at 6 h was 1.2-fold above the control. Treatment with Tu increased eIF2α phosphorylation, peaking at 4 h at 1.8-fold of above the control, and increased ATF4 expression with time to 1.5- (at 4 h), 1.8- (at 8 h), and 2.43-fold (at 10 h) greater than the control. CHOP expression was not detected in the control group cells throughout the culture period or at any point during treatment with NaF, but was dramatically induced by Tu at 8 and 10 h as measured by Western blotting (Fig. 3, lower panels), confirming our earlier data [Matsumoto et al., 2013].

EFFECTS OF NaF AND Tu ON AUTOPHAGY ACTIVATION, AND THE ROLES OF ATF AND CHOP IN AUTOPHAGY INDUCTION

MDC is incorporated into the autophagosome and emits green fluorescence when excited with ultraviolet light. Fluorescence microscopy revealed that ER-stress-induced autophagosomes were detected as green fluorescent granules in the cytoplasm and were collected around the nucleus (Fig. 7A, upper panel). Treatment with both NaF and Tu significantly increased the fluorescence density of the cells at 6 h, which resulted from the accumulation of MDC-containing granules (Fig. 4A).

Transfection of ATF4 siRNA into NaF- and Tu-treated cells completely suppressed the autophagy activated by these ER-stress inducers while transfection of CHOP siRNA did not suppress the NaF- and Tu-induced autophagy (Fig. 4B). The transfection of negative control siRNA (si-Cont, Fig. 4B) had no effect on NaF- or Tu-induced autophagy.

The roles of ATF4 and CHOP in the activation of autophagy were confirmed by transfection of ATF4 siRNA and CHOP siRNA into HepG2 cells that had been exposed to NaF or Tu for 6 h. The effects of ATF4 and CHOP siRNAs on ATF4 and CHOP mRNA levels were confirmed in NaF- and Tu-treated HepG2 cells at 6 h of treatment. The ATF4 and CHOP siRNAs were effective in their suppression of ATF4

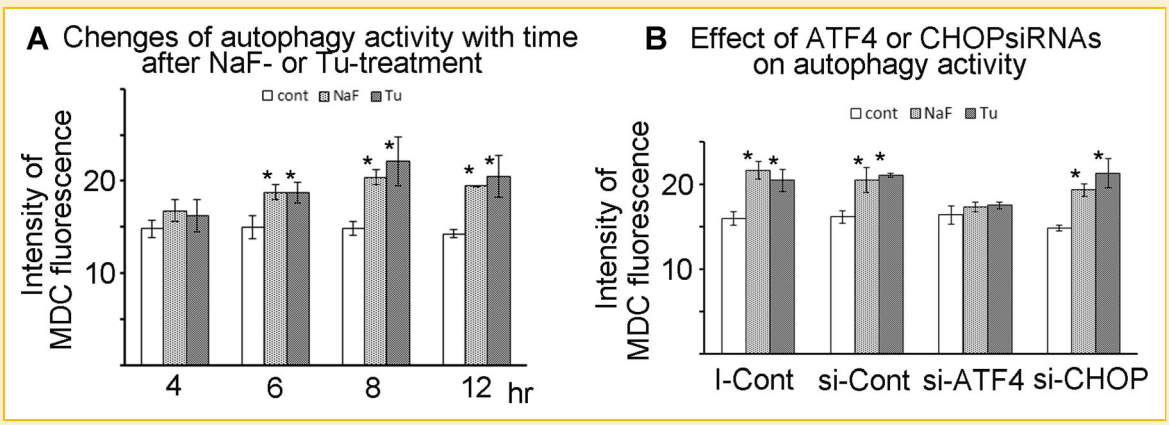

Fig. 4. Changes in autophagic activity after treatment with NaF or Tu and the effects of ATF4 and CHOP siRNAs on drug-induced autophagy. A: Changes in autophagic activity after treatment with NaF or Tu. Densitometric analysis of autophagic activity in HepG2 cells exposed to ER-stress inducers (NaF; or tunicamycin, Tu) for 4–12 h was performed using monodansylcadaverine (MDC) staining. The activity was determined by the fluorescence intensity of MDC incorporated into the treated cells. Data are presented as the mean ± SD of three individual experiments. The * symbol denotes $P < 0.05$ compared with the value at 0 h for each treatment. B: Effects of ATF4 or CHOP siRNAs on autophagic activity. Transfections of HepG2 cells with non-targeting (Negative Cont, si-Cont), ATF4 or CHOP siRNAs were performed according to the manufacturer's instructions. After the 48 h pre-incubation in DMEM containing siRNA, ER-stress inducers were added to the media and the cultures were maintained for 6 h to induce ER stress. Densitometric analysis of autophagic activity in HepG2 cells exposed to ER-stress inducers for 6 h was conducted with MDC with ATF4 or CHOP siRNA added (si ATF4 and siCHOP), negative control siRNA added (si-Cont) or not (I-Cont). The activity was determined using the fluorescence intensity of MDC incorporated into the treated cells. Data are presented as the mean ± SD of three individual experiments. The * symbol denotes $P < 0.05$ compared with the Cont in each treatment group.

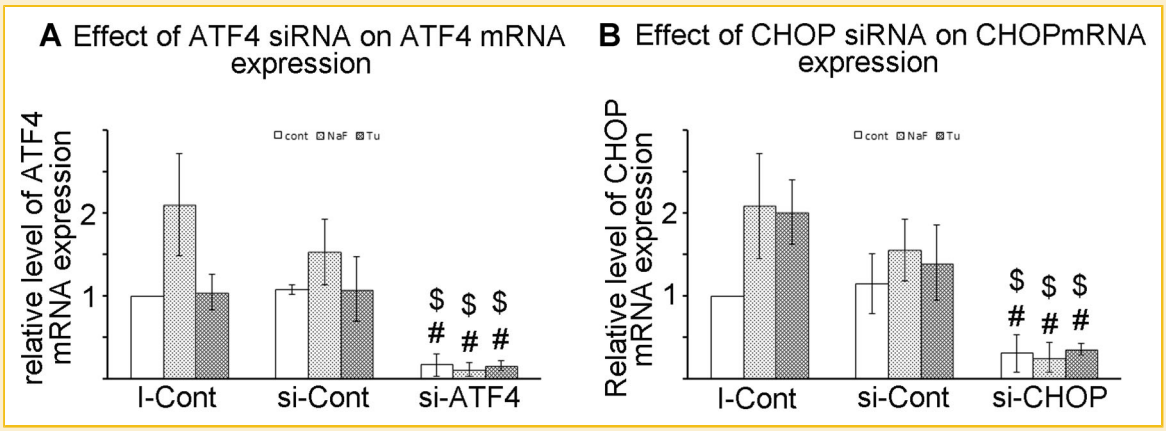

Fig. 5. Effects of ATF4 and CHOP siRNAs on mRNA expression in HepG2 cells exposed to NaF and tunicamycin. siRNA transfections of HepG2 cells with non-targeting (Negative Cont), ATF4 or CHOP siRNAs were performed according to the manufacturer's instructions. Following the first 48 h pre-incubation in DMEM containing siRNAs, each ER-stress inducer was added to the media and the culture was continued for 6 h to induce ER stress. A: Relative of ATF4 mRNA levels were measured by quantitative real-time PCR (qPCR) from ATF4 siRNA knockdown, negative Cont siRNA (si-Cont; non-targeting siRNA) and control (I-Cont; no siRNA) HepG2 cells. The qPCRs were performed in duplicate for each sample. B: Relative CHOP mRNA levels in CHOP siRNA knockdown, negative Cont siRNA (si-Cont; non-targeting siRNA) and control (I-Cont; siRNA) HepG2 cells were measured by quantitative real-time PCR (qPCR). The qPCRs were performed in duplicate for each sample, and samples were collected from three individual experiments. Data are presented as the mean ± SD of three individual experiments. The # and & symbols denote $P < 0.05$ compared with the corresponding treatment (cont, NaF and Tu) in the I- Cont and si-Cont groups, respectively.

and CHOP mRNA expression (Fig. 5A,B), similar to the results of our previous study [Matsumoto et al., 2013].

CHANGES IN GADD34 mRNA EXPRESSION FOLLOWING NaF- OR Tu-TREATMENT AND THE EFFECTS OF GADD34 siRNA ON GADD34 mRNA EXPRESSION

GADD34 mRNA expression was increased 4 h after treatment with NaF and Tu (Fig. 6A). Treatment with NaF increased GADD34 mRNA levels approximately ninefold over the control without ER-stress inducers at 8 h of treatment (Fig. 6B, I-Cont). Treatment with Tu elicited dramatic elevation in the GADD34 mRNA levels between 6- and 30-fold at 4–8 h of treatment (Fig. 6A,B).

The effects of GADD34 siRNA on GADD34 mRNA expression were confirmed in NaF- and Tu-treated HepG2 cells at 6 and 8 h of treatment. As expected, transfection with negative control siRNA (si-Cont, Fig. 6B) did not suppress the increased GADD34 mRNA

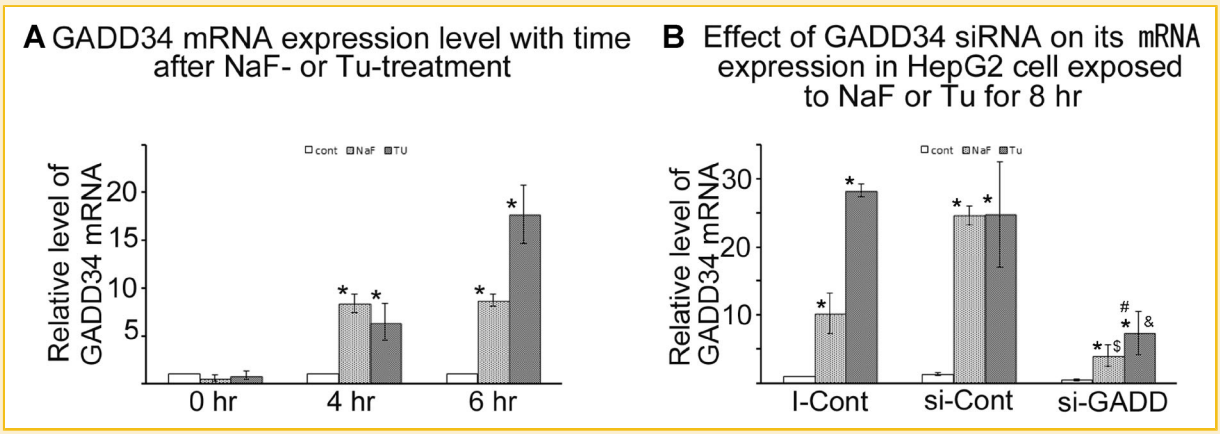

Fig. 6. Changes in GADD34 mRNA expression in HepG2 cells. A: GADD34 mRNA expression levels following treatment with NaF or Tu. Changes in GADD34 mRNA expression following NaF- or Tu- (tunicamycin-) treatment of HepG2 cells were measured by quantitative real-time PCR (qPCR). The qPCRs were performed in duplicate for each sample, and samples were collected from three individual experiments. Data are presented as the mean ± SD of three individual experiments. The * symbol denotes $P < 0.05$ compared with the Cont for each treatment group. B: Effects of GADD34 siRNA on its mRNA expression in HepG2 cells exposed to NaF or Tu for 8 h. HepG2 cells were transfected with a non-targeting siRNA (si-Cont) or GADD34 siRNA according to the manufacturer's instructions. After the first 48 h pre-incubation in DMEM containing siRNA, an ER-stress inducer (NaF or Tu) was added to the media and the culture was maintained for 6 or 8 h to induce ER stress. Relative mRNA levels of GADD34 siRNA (si-GADD), negative Cont siRNA (si-Cont) and non-added (I-Cont) HepG2 cells were measured by quantitative real-time PCR (qPCR). The qPCRs were performed in duplicate for each sample and samples were collected from three individual experiments. Data are presented as the mean ± SD of three individual experiments. The * symbol denotes $P < 0.05$ compared with the values of the respective Cont for each treatment group. The # symbol denotes $P < 0.05$ compared with the respective values of I-Cont. The $ and & symbols denote $P < 0.05$ compared with the respective values of si-Cont.

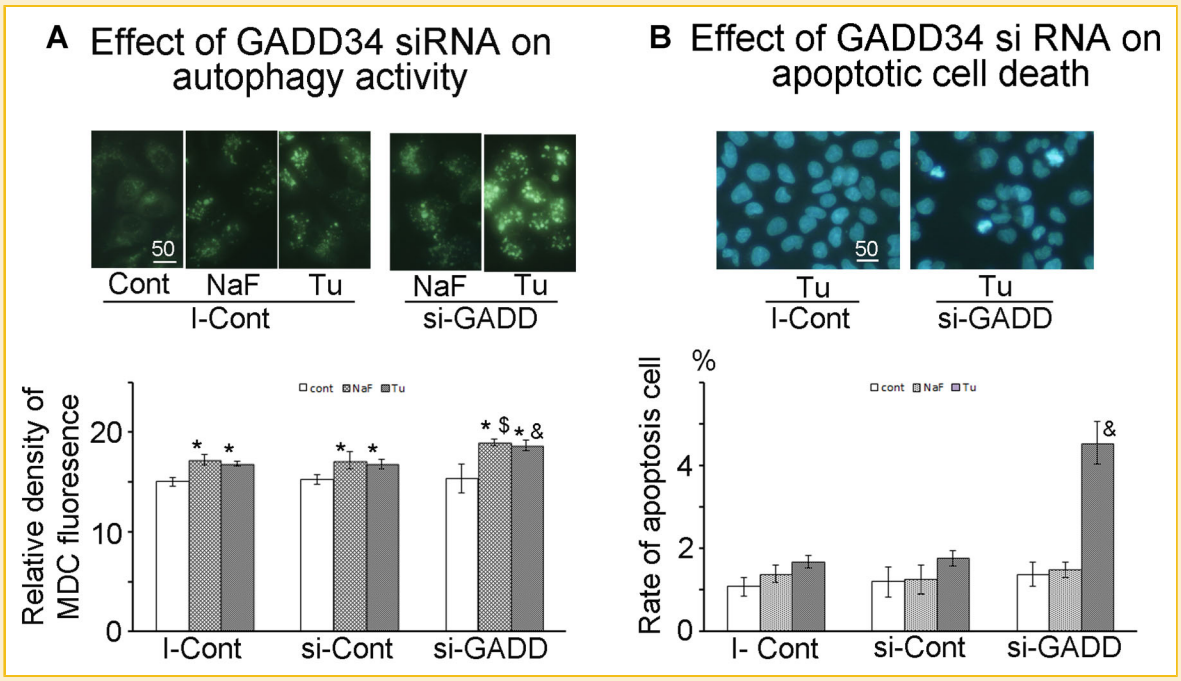

Fig. 7. Effects of GADD34 siRNA on autophagy and apoptosis in HepG2 cells exposed to NaF or Tu for 6 h. A: Effects of GADD34 siRNA on autophagic activity. HepG2 cells were transfected with a Negative Cont siRNA (si-Cont) or GADD34 siRNA as described in the Materials and Methods Section. Autophagic activity was determined using the fluorescence intensity of MDC as described in the Materials and Methods Section. Data are presented as the mean ± SD of three individual experiments. The * symbol denotes $P < 0.05$ compared with the values for the respective Cont for each treatment group. The $ and & symbols denote $P < 0.05$ compared with the values for each ER-stress inducer in si-Cont. Bar = 50 μm. B: Effects of GADD34 siRNA on apoptotic cell death. HepG2 cells were transfected with a Negative Cont siRNA (si-Cont) or GADD34 siRNA as described in the Materials and Methods Section and legend of Figure 5B. Apoptosis was identified using the nuclear staining pattern of Hoechst33342. The incidence of apoptosis is indicated as the percentage of apoptotic cells in a selected area. Data are presented as the mean ± SD of three individual experiments. The & symbol denotes $P < 0.05$ compared with the value of Tu in si-Cont. Bar = 50 μm.

expression in HepG2 cells following NaF- or Tu-treatment. In contrast, transfection of GADD34 siRNA into NaF- or Tu-treated cells strongly suppressed GADD34 expression, which should be increased by treatment with ER-stress inducers (si-GADD34, Fig. 6B). GADD34 mRNA expression levels in NaF-treated cells transfected with GADD34 siRNA were approximately 40% and less than 20% of the corresponding the intact control (I-Cont) and of the negative control (si-Cont), respectively, and the mRNA expression levels in Tu-treated cells were approximately 25% and 30% of the intact and negative controls, respectively. The GADD34 siRNA effectively suppressed the expression of GADD34 mRNA in each treatment condition.

EFFECTS OF GADD34 siRNA ON AUTOPHAGY AND/OR APOPTOSIS ONSET IN HepG2 CELLS EXPOSED TO ER-STRESS INDUCERS

Treatment with NaF or Tu significantly activated autophagy at 6 h (Fig. 7A, I-Cont). Transfection of negative control siRNA (Fig. 7A, si-Cont) did not have an additive effect on the autophagic activity induced by NaF- or Tu-treatment; that is, the negative control siRNA-transfected HepG2 cells treated with NaF or Tu maintained the same levels of autophagic activity as those of NaF- or Tu-treated cells in the intact control group. In contrast, GADD34 siRNA transfection accelerated the autophagic activity induced by treatment with NaF or Tu (si-GADD, Fig. 7A).

The apoptotic ratio of HepG2 cells cultured normally for approximately 24 h after 48 h of pre-culture was in the range of 1–3%. HepG2 cells treated with NaF or Tu had apoptosis rates within the control range by 8 h of treatment. In addition to prolonged treatment culture, NaF slightly increased the incidence of apoptosis (3.3 ± 0.4% and 3.3 ± 0.6% at 12 and 24 h, respectively). In contrast with NaF-treatment, the apoptotic ratio of Tu-treated cells was significantly increased by 1.5-fold between 12 and 24 h of the treatment (3.1 ± 0.7% at 12 h to 5.2 ± 0.2% at 24 h). Only Tu-treatment elicited treatment-related drastic elevation of the apoptotic ratio at 24 h of the treatment culture.

The suppression of GADD34 additively elevated autophagic activity, and accelerated and hastened onset of apoptosis in Tu-treated cell. The apoptotic ratios were 4.5 ± 0.5% and 7.0 ± 0.9% in si-GADD-pretreated HepG2 cells exposed to Tu for 6 and 8 h, respectively (Tu of si-GADD in Fig. 7B). In contrast, although GADD34 siRNA transfection additively elevated autophagic activity in NaF-treated cells similarly to that of the Tu-treatment, the transfection had no effect on apoptosis induction in the cells (NaF of si-GADD, Fig. 7B). Negative control siRNA (si-Cont, Fig. 7B) did not affect apoptotic incidence in HepG2 cells exposed to either NaF or Tu.

EFFECTS OF GADD34 siRNA ON THE EXPRESSION OF ATF4/CHOP PROTEINS AND mRNAs

The effects of GADD34 siRNA on the expression of ATF4 and CHOP proteins and mRNAs were investigated by Western blotting and quantitative real-time RT-PCR. At 8 h of NaF- or Tu-treatment, the

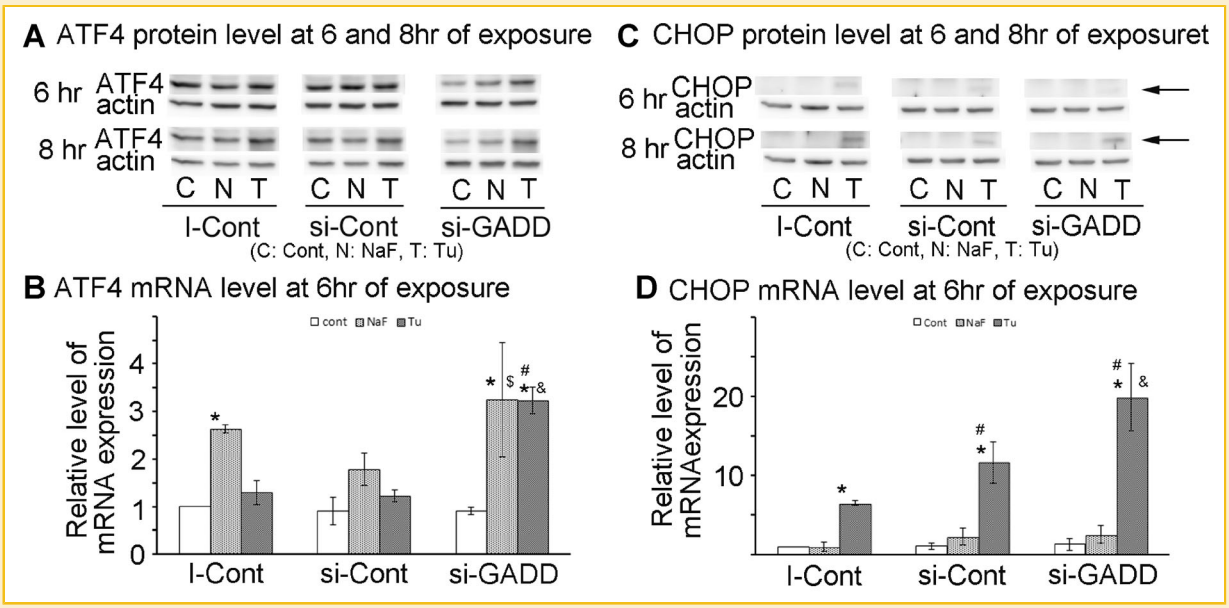

Fig. 8. Effects of GADD34 siRNA on the protein and mRNA expression of ATF4 and CHOP in HepG2 cells exposed to NaF or Tu for 6 or 8 h. A: ATF4 protein levels at 6 and 8 h of exposure, (B) ATF4 mRNA expression levels at 6 h of exposure, (C) CHOP protein levels at 6 and 8 h of exposure, (D) CHOP mRNA expression levels at 6 h of exposure. HepG2 cells were transfected with a Negative Cont siRNA (si-Cont) or GADD34 siRNA as described in the Materials and Methods Section. The effects of GADD34 siRNA on ATF4 and CHOP protein levels (A and C; 6 and 8 h of NaF- or Tu-treatment) and mRNA expression (B and D; 6 h of NaF- or Tu-treatment) were examined in HepG2 cells by Western blotting and real-time PCR, respectively, as described in the Materials and Methods Section. The * symbol denotes $P < 0.05$ compared with the respective values of Cont for each treatment group. The # symbol denotes $P < 0.05$ compared with the respective values of I-Cont. The $ and & symbols denote $P < 0.05$ compared with the respective values of si-Cont.

induced ATF4 protein expression was additionally increased by GADD34 siRNA transfection (Fig. 8A, si-GADD for 8 h, and Table II). This additive effect of GADD34 siRNA on ATF4 protein expression was not as apparent at 6 h (Table II).

ATF4 mRNA expression was elevated by 6 h of NaF- or Tu-treatment to 2.5- and 1.3-fold levels, respectively (Fig. 8B, I-Cont). GADD34 siRNA additively accelerated the ATF4 mRNA expression both in NaF- and Tu-treated cells with significant increases compared with the corresponding treatment cells in the negative control siRNA group (Fig. 8B).

Although definitive expression of CHOP protein was never detected in NaF-treated cells, treatment with Tu initially induced this protein expression at 6 h with measurable levels and increased with time, as shown above (Fig. 3). This Tu-induced CHOP expression was additionally elevated by GADD34 siRNA transfection at 8 h of Tu-treatment, compared with the negative control siRNA (Fig. 8C, si-Cont in lower panels for 8 h and Table II). GADD34 siRNA transfection additively accelerated CHOP mRNA expression at 6 and 8 h of Tu-treatment with significant increases compared with the negative control siRNA (Fig. 8D, showing the data for 6 h of Tu-treatment). For NaF-treated cells, however, GADD34 siRNA did not increase CHOP mRNA expression, but maintained the same expression level as the untransfected NaF-treated cells.

DISCUSSION

In this study, we examined the role of GADD34 in activation of autophagy and/or apoptosis through the ER-stress sensor in NaF- or

TABLE II. Relative Intensity of ATF4 and CHOP Protein Expressions at 6 and 8 h After NaF- or Tu-Treatment in GAADD34 siRNA Pretreated Cells

	Intact Cont			Negative Cont siRNA			GADD siRNA		
	Cont	NaF	Tu	Cont	NaF	Tu	Cont	NaF	Tu
AT4 (n = 3)									
6 h	1.00	1.23 ± 0.25	2.17 ± 1.09	1.00	0.95 ± 0.42	1.70 ± 1.14	1.00	0.87 ± 0.24	1.82 ± 0.55
8 h	1.00	1.10 ± 0.29	2.20 ± 1.23	1.00	1.30 ± 0.32	2.42 ± 1.35	1.00	1.85 ± 0.45	3.52 ± 0.94
CHOP (n = 3)									
6 h	1.00	2.94 ± 0.95	6.44 ± 4.33[a]	1.00	1.23 ± 0.02	1.56 ± 0.37	1.00	1.17 ± 0.17	3.19 ± 1.65
8 h	1.00	1.43 ± 0.53	3.22 ± 0.63	1.00	2.21 ± 1.31	4.30 ± 1.90	1.00	1.25 ± 0.48	9.88 ± 2.73[b]

[a] $P < 0.05$, versus 6 h Cont value in Intact Cont group.
[b] $P < 0.05$, versus 8 h Tu value in Negative Cont siRNA group.

Tu-treated cells. Suppression of GADD34 by GADD34 siRNA accelerated and elevated the apoptotic ratio of Tu-treated cells with additively elevated CHOP expression, in addition to the activation of autophagy with elevated ATF4 expression. In contrast, GADD34 suppression in cells undergoing NaF-induced ER-stress also accelerated autophagic activity with elevated ATF4 expression, but did not alter the CHOP non-inducible capacity inherent in these cells undergoing ER-stress. We concluded that GADD34 expressed as a result of ER-stress delayed CHOP expression and then retarded apoptotic cell death, and that an ATF4-signal-modulating machine other than GADD34 acts on ATF4-to-CHOP signaling to block CHOP expression induced by ATF4 in ER-stress related autophagy.

Tu, which inhibits glycosylation during protein synthesis resulting in abnormal protein accumulation, activated ER-stress sensors PERK and Ire1, and then induced autophagy after 6 h of treatment and apoptosis after 24 h, as well as increasing ATF4 and CHOP expression. NaF, which interferes with vesicle transport from the ER to the Golgi resulting in the formation of numerous autophagosomes [Matsuo et al., 2000; Ito et al., 2009], activated some downstream signals of the PERK pathway such as eIF2α and ATF4, although it did not increase the expression of CHOP, which act downstream of ATF4. In this study, we could not detect a change in PERK phosphorylation by Western blotting. Accordingly, we evaluated the activation of this ER-stress sensor by examining the change in the pattern of density distribution in the broad PERK-positive band by Western blotting. Western blotting for PERK revealed that NaF decreased the density of the high-density lower region of the broad PERK-positive band, similarly to other ER-stress inducers, Tu and Tg (Fig. 1). Furthermore, HBSS treatment did not alter the density pattern of the broad PERK-positive band. When PERK is activated by ER-stress, it is phosphorylated and there is an associated shift in mobility during SDS–polyacrylamide gel electrophoresis, resulting in the reduced density of the PERK positive band [Harding et al., 1999]. Although GCN2 eIF2α kinase is required for adaptation to amino acid deprivation [Zhang et al., 2002], PERK is not required for eIF2α phosphorylation induced by amino acid starvation [Harding et al., 2000]. Based on these findings, it was considered that NaF increased eIF2α phosphorylation and ATF4 expression by activating PERK, resulting in the activation of autophagy.

Excessive ER-stress caused by functional disruption of the ER such as the accumulation of unfolded protein and disruption of Ca^{++} homeostasis is well known to induce CHOP-mediated apoptosis via overflowed stress outside of UPR-potency. Autophagy is also linked to the ER-stress/UPR pathways [Høyer-Hansen and Jäättelä, 2007; Yorimitsu and Klionsky, 2007]; however, little is known regarding whether autophagy regulates the UPR pathway and how specific UPR targets might control autophagy. The PERK/eIF2α pathway of the UPR has been implicated in autophagy regulation [Tallóczy et al., 2002; Kouroku et al., 2007]. Additionally, MAP1LCB3 and ATG5 are both central in the ubiquitin-like conjugation systems involved in autophagosome formation [Yorimitsu and Klionsky, 2005]. The PERK-dependent transcription factors ATF4 and CHOP are implicated in the translational activation of *MAP1LC3B* and *ATG5* during hypoxia [Rouschop et al., 2010; Rzymski et al., 2010]. Knockdown of ATF4 prevented *MAP1LC3B* induction during hypoxia (ER-stress); however, *MAP1LC3B* induction was not prevented by CHOP knockdown, and CHOP knockdown lead to a reduction in ATG5 [Rouschop et al., 2010]. In our recent study, NaF activated autophagy with increased ATF4 expression without CHOP expression and subsequent apoptosis; in contrast, Tu induced ATF4-activating autophagy with subsequent CHOP-inducing apoptosis [Matsumoto et al., 2013]. These recent findings indicated that ATF4 induced downstream CHOP expression during Tu-induced, but not NaF-induced, ER-stress. The identity of the mechanism modulating CHOP-expression such that it is selectively suppressed in spite of the increased ATF4 expression remains unclear. GADD34 was isolated as a candidate ATF4-to-CHOP signal regulator that attenuates CHOP expression induced by unfolding protein in the ER [Novoa et al., 2001]. ATF4 is known to directly bind and activate an ATF site within the GADD34 promoter [Ma and Hendershot, 2003]. Overexpression of the active form of GADD34 that constitutively dephosphorylates eIF2α can attenuate the eIF2α phosphorylation and severely inhibit ATF4 induction, suggesting that GADD34 is important as a negative feedback regulator of the PERK signal pathway [Blais et al., 2004]. In this study, knockdown of GADD34 using GADD siRNA hastened and accelerated the induction of apoptosis in Tu-treated HepG2 cells with increasing CHOP expression. In NaF-treated HepG2 cells, however, GADD siRNA enhanced NaF-activated autophagy but did not elicit CHOP expression and subsequent apoptosis. These findings indicate that GADD34 transiently suppresses ATF4 and subsequent CHOP expression through its negative feedback potency to dephosphorylate eIF2α and result in retarding the induction of apoptosis, but did not wholly shut down ATF4 signaling to induce CHOP expression. It is considered that when NaF-induced ER-stress induces autophagy only, the suppressive system of ATF4 signaling including the regulation by CHOP promoter sites works to block induction of CHOP expression by ATF4 signaling.

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