Endoplasmic Reticulum Stress Signal Mediators Are Targets of Selenium Action

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Abstract

A monomethylated selenium metabolite, called methylseleninic acid (MSA), has recently been shown to cause global thiol redox modification of proteins. These changes represent a form of cellular stress due to protein misfolding or unfolding. An accumulation of aberrantly folded proteins in the endoplasmic reticulum (ER) triggers a defined set of transducers to correct the defects or commit the cells to apoptosis if the rescue effort is exhausted. Treatment of PC-3 human prostate cancer cells with MSA was found to induce a number of signature ER stress markers: (*a*) the survival/rescue molecules such as phosphorylated protein kinase–like ER-resident kinase (phospho-PERK), phosphorylated eukaryotic initiation factor-2α (phospho-eIF2α), glucose-regulated protein (GRP)-78, and GRP94; and (*b*) the apoptotic molecules such as caspase-12, caspase-7, and CAAT/enhancer binding protein homologous protein or growth arrest DNA damage-inducible gene 153 (CHOP/GADD153). Additional evidence suggested that CHOP/GADD153 might be an important transcription factor in apoptosis induction by MSA. In general, a higher concentration of MSA was required to elicit the apoptotic markers compared with the rescue markers. The apoptotic markers increased proportionally with the dose of MSA, whereas the rescue markers failed to keep pace with the increasing challenge from MSA. GRP78 is the rheostat of the ER stress transducers. In GRP78-overexpressing cells, the ability of MSA to up-regulate phospho-PERK, phospho-eIF2α, GRP94, caspase-12, caspase-7, and CHOP/GADD153 was significantly muted. A generous supply of GRP78 would allow cells to cope better with ER stress, thereby improving the odds for survival and negating the commitment to apoptotic death. The present study thus provides strong evidence to support an important role of ER stress response in mediating the anticancer effect of selenium. (Cancer Res 2005; 65(19): 9073-9)

Introduction

Endoplasmic reticulum (ER) stress is defined as the accumulation of misfolded/unfolded proteins in the lumen of the ER (1). Causal factors of ER stress include disturbance of Ca^{2+} homeostasis, change in redox status, and inhibition of protein glycosylation. The unfolded protein response is a complex but well-orchestrated system of stress management (2). An early step of unfolded protein response is to stop new protein synthesis to lessen the burden of unfolded/misfolded proteins. This process is mediated by the autophosphorylation of an ER-resident protein kinase, protein kinase–like ER-resident kinase (PERK), which in turn inactivates the translation initiation factor, eukaryotic initiation factor 2α (eIF2α), by phosphorylation (3, 4). The expression of ER-resident chaperones, such as glucose-regulated protein (GRP)-78 and GRP94, is also increased (5). These chaperones prevent the aggregation of unfolded/misfolded proteins so that they can be properly refolded. Activating transcription factor 6 (ATF6) and inositol requiring 1α (IRE1α) are the ER-associated transducers responsible for the up-regulation of chaperones and other ER stress–related transcription factors (6–8).

In addition to being a sensor of ER stress, GRP78 also serves as a gatekeeper to the activation of the stress transducers (9, 10). Under normal conditions, most of the GRP78 is bound to PERK, ATF6, and IRE1α to keep them inactive, whereas free GRP78 is present at a low level. When ER stress happens, the misfolded/unfolded proteins complex with free GRP78. The depletion of free GRP78 is a cue for the dissociation of GRP78 from its transducer clients, thus leading to their activation (3). A top priority is to contain the damage by decreasing general protein synthesis (through phospho-eIF2α) and increasing the production of GRP78. If the rescue effort is insufficient to mend the injury, the decision to commit to apoptosis may be triggered, although the mechanism behind this is not yet fully understood (11, 12). ER-resident caspase-12 has been reported to play an important role in rodent models (13, 14). In human cells, caspase-7 (15), caspase-4 (16), and a caspase-8–like protein (17) are known to be recruited to the ER membrane and become activated. Apart from caspases, CAAT/enhancer binding protein homologous protein or growth arrest DNA damage-inducible gene 153 (CHOP/GADD153) is apparently a key proapoptotic transcription factor that is closely identified with ER stress (18, 19). It is commonly accepted that CHOP/GADD153 expression is under the control of ATF6 and IRE1α signalings (20, 21). The downstream targets of CHOP/GADD153 have not been well elucidated; some suggestive evidence has pointed to the Bcl family proteins as well as reactive oxygen species production (19, 22).

Apoptosis is one mechanism by which selenium expresses its anticancer effect (23). The goal of the present study is to test the hypothesis that selenium induction of apoptosis is linked to ER stress. The metabolism of both inorganic and organic selenium compounds to methylselenol is essential for anticancer activity (24, 25). Methylselenol is highly redox active, and this characteristic enables it to modify thiol/disulfide interchange in proteins. The above concept has been verified previously with individual proteins, such as protein kinase C (26) and p53 (27), but never on a grand scale until recently. By using a display thiol proteomics approach, we provided evidence of global protein redox modification in various subcellular compartments following treatment with selenium (28). Redox modification of thiol/disulfide bonds is likely to result in protein misfolding or unfolding. Newly synthesized proteins are particularly vulnerable before they are properly folded in the ER. Thus, it is highly plausible that selenium would produce ER stress. The experiments reported in this article were designed to

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investigate (*a*) the ability of selenium to induce signature ER stress markers; (*b*) whether the stress response is strong enough to tip the balance towards apoptosis; and (*c*) if increasing the availability of free GRP78 by overexpression would diminish the sensitivity to selenium induction of apoptosis.

Materials and Methods

Cell culture. With the exception of one study, most of the experiments were carried out with the PC-3 cells. The PC-3 human prostate cancer cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). PC-3 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin/streptomycin, and 2 mmol/L glutamine. Cells were maintained at 37°C in an atmosphere of 5% CO_2 and 95% air. There was one study in which other human prostate cancer cell lines were used. The additional cell lines included LNCaP (from ATCC), LNCaP-LN3 (from Dr. Curtis A. Pettaway, University of Texas M.D. Anderson Cancer Center, Houston, TX), DU145 (from ATCC), and LAPC-4 (from Dr. Charles Sawyers, University of California at Los Angeles Jonsson Comprehensive Cancer Center, Los Angeles, CA).

Selenium reagent. Methylseleninic acid (MSA) was used in all the cell culture experiments. It was synthesized as described previously (29). This selenium compound was developed specifically for *in vitro* studies because of certain unique attributes (30). Selenoamino acids are generally not suitable for cell culture experiments because many epithelial cells have a low capacity in converting the selenoamino acid precursors to the active metabolite.

Western blotting. Cell lysate for SDS-PAGE was prepared in 1× cell lysis buffer (Cell Signaling Technology, Beverly, MA) containing 1 mmol/L phenylmethylsulfonyl fluoride (PMSF; Sigma, St. Louis, MO), 50 mmol/L NaF, and 1 tablet/7 mL of Mini Complete Protease Inhibitor (Roche Applied Science, Indianapolis, IN). Protein concentration of the lysate was determined by using the Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce Biotechnology, Rockford, IL). In preparing for SDS-PAGE, cell lysate was mixed with 1/3 volume of 4× SDS sample buffer [200 mmol/L Tris-HCl (pH 6.8), 8% SDS, 0.4% bromophenol blue, 40% glycerol, 60 μL/mL β-mercaptoethanol] and heated at 100°C for 10 minutes. The GRP78 polyclonal antibody and the CHOP/GADD153 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies to phospho-PERK, phospho-eIF2α, eIF2α, caspase-12, caspase-7, cleaved caspase-7, poly(ADP-ribose) polymerase (PARP), and cleaved PARP were purchased from Cell Signaling Technology. An anti-KDEL monoclonal antibody (mAb) against both GRP78 and GRP94 was purchased from Stressgen Bioreagents (Victoria, BC, Canada). An anti–glyceraldehyde-3-phosphate dehydrogenase mAb (Chemicon, Temecula, CA) or an anti-actin mAb (Sigma) was used for loading control. Polyvinylidene difluoride membranes containing the transferred proteins were blocked in 5% nonfat dry milk in TTBS buffer [10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.1% Tween 20] at room temperature for 1 hour before probing with the primary antibodies and the horseradish peroxidase–conjugated secondary antibodies (Amersham Biosciences, Piscataway, NJ). Protein bands were visualized by the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology) or the ECL Plus Western Blotting Detection System (Amersham Biosciences). Immunoreactive bands were quantitated by volume densitometry with the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Construction of CHOP/GADD153 overexpression vector. CHOP/GADD153 full-length cDNA in pcDNA4/TO was kindly provided by Dr. Christopher P.F. Redfern (31). The cDNA was subcloned into expression vector pIRES2-EGFP (BD Biosciences Clontech, Palo Alto, CA) for overexpression. Briefly, the cDNA fragment was amplified by PCR with the Platinum High Fidelity Taq DNA polymerase (Invitrogen, Carlsbad, CA). The restriction enzyme recognition sites *Bgl*II (in bold) and *Eco*RI (underlined) were incorporated in the 5′-end and 3′-end primers, respectively: 5′-GGACTCAGA**TCTAGA**GATGGCAGCTGAGTCATTG-3′; 5′-GGACTCGAATTCTCAAGCTTGGTGCAGATTCAC-3′. The PCR product was constructed into pIRES2-EGFP between the *Bgl*II and *Eco*RI sites. The authenticity of the subcloned sequence was confirmed by DNA sequencing.

Transient transfection of PC-3 cells. The expression vector for human GRP78 overexpression was kindly provided by Dr. Richard C. Austin (32). The parental empty vector pcDNA3.1 was used for mock transfection. Briefly, PC-3 cells were seeded in six-well plates at 0.3×10^6/well and cultured for 48 hours before transfection. Transient transfection was done in serum-free medium Opti-MEM I (Invitrogen) by using FuGene6 (Roche Applied Science). After 6 hours of transfection, the medium was replaced with the regular growth medium. Cells were incubated for 18 hours before MSA treatment. For CHOP/GADD153 overexpression, transfected cells were cultured for 24 hours before harvesting for Western blot or apoptosis analysis. The pIRES1-EGFP empty vector was used for mock transfection.

Apoptosis analysis. The Annexin V-Phycoerythrin Apoptosis Detection Kit I (BD Biosciences PharMingen, San Diego, CA) was used for apoptosis analysis by flow cytometry. After MSA treament, cells were trypsinized, combined with the culture medium containing detached cells, and centrifuged at 400 × *g* for 5 minutes at 4°C. Cells were washed twice with cold PBS (pH 7.4) and suspended in 1× binding buffer [10 mmol/L HEPES (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$] at a density of 1×10^6/mL. An aliquot of 100 μL of cell suspension was transferred to a 5-mL tube and mixed with 5 μL of Annexin V-phycoerythrin and 5 μL of 7-amino-actinomycin. The cells were gently vortexed and incubated in the dark at room temperature for 20 minutes. A volume of 400 μL of 1× binding buffer was added to each tube. The stained cells were analyzed by flow cytometry. Cells negative for 7-amino-actinomycin and positive for Annexin V-phycoerythrin were scored as apoptotic cells. In some cases, apoptosis analysis was done with the Cell Death Detection ELISA Kit (Roche Applied Science). This method measures apoptosis by determining the cytoplasmic histone-associated DNA fragments.

Coimmunoprecipitation assays. Cell lysate for coimmunoprecipitation was prepared by scrapping and collecting cells in 1× radioimmunoprecipitation assay (RIPA) buffer [1× PBS (pH 7.4), 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS] supplemented with 1 mmol/L PMSF and 1 tablet/7 mL of Complete Mini Protease Inhibitor. Protein concentration of the cell lysate was determined by the BCA method. For each sample, 500 μg of total protein extract were precleared by incubation with 1 μg of rabbit immunoglobulin G and 20 μL of protein A-agarose beads (Santa Cruz Biotechnology) at 4°C for 30 minutes. The precleared lysate was separated from the protein A-agarose beads by centrifugation at 1,000 × *g* for 5 minutes at 4°C. After that, it was mixed with 10 μL of caspase-7 antibody and 20 μL of protein A-agarose beads. The mixture was incubated at 4°C overnight and then washed five times with the RIPA buffer. The immunoprecipitate was extracted with 80 μL of 1× SDS sample buffer [50 mmol/L Tris-HCl (pH 6.8), 2% SDS, 0.1% bromophenol blue, 10% glycerol, 15 μL/mL β-mercaptoethanol], followed by heating at 100°C for 10 minutes. The supernatant was obtained by centrifugation at 1,000 × *g* for 5 minutes and 30 μL of each sample were resolved by SDS-PAGE to detect the coimmunoprecipitated GRP78.

Statistical analysis. The Student *t* test was used to determine statistical differences between treatment and control values, and $P < 0.05$ was considered significant.

Results

Methylseleninic acid induces endoplasmic reticulum stress. Three signature ER stress markers, phospho-PERK, phospho-eIF2α, and GRP78, were used to study the effect of MSA on unfolded protein response. Figure 1*A* shows the Western blot results in cells treated with 10 μmol/L MSA for different lengths of time. Increases in phosphorylation of PERK and its substrate, eIF2α, were evident at 3 hours; these changes were sustained up to 24 hours. The densitometric quantitation of the blots is shown below the bands. The antibody to total PERK is not available commercially. Because PERK is activated by autophosphorylation, the phosphorylation status is indicative of PERK activity. Total eIF2α was not changed by MSA; therefore, the increase of eIF2α phosphorylation was likely due to activation of PERK. The induction of GRP78 by MSA

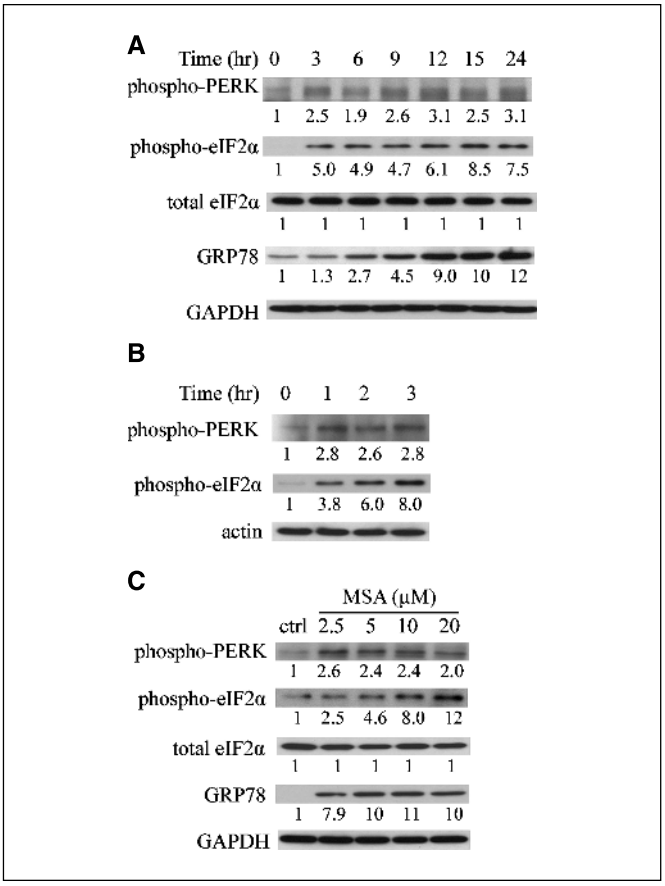

Figure 1. Induction of ER stress markers by MSA in PC-3 cells. *A,* time-dependent response to treatment with 10 μmol/L MSA. *B,* acute response of phospho-PERK and phospho-eIF2α to treatment with 10 μmol/L MSA. *C,* dose-dependent response to MSA treatment at 15 hours. The numbers beneath the bands denote the fold of change relative to the zero hours or control value.

occurred just a little later than the phosphorylation of PERK and eIF2α. GRP78 level started to increase after 6 hours and rose steadily with time. In a separate experiment, we also looked at the unfolded protein response markers during the first 3 hours. As shown in Fig. 1*B*, the phosphorylation of PERK and eIF2α was detectable as early as 1 hour after MSA. The rapid change suggests that ER stress is an immediate occurrence attributable to the reactivity of MSA. Figure 1*C* shows the dose-response data. Cells were treated with 2.5, 5, 10, or 20 μmol/L MSA for 15 hours before harvesting for Western blot analysis. All three unfolded protein response markers were significantly induced by MSA at a concentration as low as 2.5 μmol/L. Interestingly, more MSA caused only very modest augmentation, if any. The results suggest that the effectors charged with helping cells to cope with and recover from ER stress are very sensitive to selenium challenge, but there is a limited capacity of this rescue effort. Depending on the intensity of the challenge, the rescue effort could be easily exhausted. From our previous experience with the PC-3 cells, 2.5 μmol/L MSA produces only minimal growth inhibition. The effective dose range is between 5 and 10 μmol/L.

Methylseleninic acid induces endoplasmic reticulum stress–associated apoptotic response. The effects of MSA on two ER stress–associated proapoptotic markers, cleaved caspase-12 and CHOP/GADD153, were examined. Figure 2*A* shows the results of the time course experiment in cells treated with 10 μmol/L MSA for up to 24 hours. The cleavage of caspase-12 rose steadily with the duration of MSA treatment. The densitometric quantitation of the blots is shown below the bands. Full-length caspase-12 was not detectable; this might be due to the possibility that human caspase-12 is a truncated gene incapable of producing the full-length product (33). The basal level of CHOP/GADD153 was extremely low. On exposure to MSA, CHOP/GADD153 expression started to climb after 6 hours, peaked at 12 to 15 hours, and dropped down to a low level at 24 hours. PARP cleavage was also evaluated as an indicator of total caspase activation. Based on our experience, PC-3 cells have a low basal level of apoptosis (or PARP cleavage) due to routine handling alone. Hence, a modest amount of PARP cleavage was detectable even at zero time. The data show that PARP cleavage became noticeable at 6 hours and gained momentum progressively with longer treatment. By coincidence or not, the apoptotic alarm seemed to start ticking at around the same time when CHOP/GADD153 induction was taking hold. Figure 2*B* shows the dose dependence results. Cells were treated with 2.5, 5, 10, or 20 μmol/L MSA for 15 hours. Cleaved caspase-12, CHOP/GADD153, and PARP cleavage responded to 5 μmol/L MSA, but not to 2.5 μmol/L MSA. Furthermore, the magnitude of increase was proportional to the dose of MSA above 5 μmol/L, especially for cleaved caspase-12 and CHOP/GADD153. Two interesting points emerged in contrasting the results in Figs. 1 and 2. First, it took more MSA to elicit the ER stress apoptotic markers compared with the unfolded protein response markers (5 μmol/L for the former versus 2.5 μmol/L for the latter). Second, the apoptotic markers increased proportionally with the dose of MSA, whereas the unfolded protein response markers failed to keep pace with the increasing challenge from MSA.

Sustained expression of CHOP/GADD153 correlates with apoptosis augmentation in methylseleninic acid–treated cells. As shown in the previous figure, the induction of CHOP/GADD153 by MSA was transient. Because MSA is metabolized to inactive dimethylselenide with time, a small amount of MSA could be lost

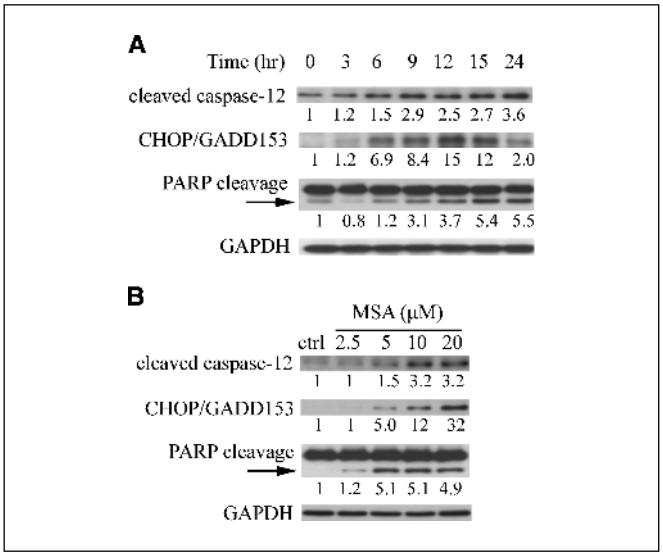

Figure 2. Induction of ER stress proapoptotic response by MSA in PC-3 cells. *A,* time-dependent response to treatment with 10 μmol/L MSA. *B,* dose-dependent response to MSA treatment at 15 hours. The numbers beneath the bands denote the fold of change relative to the zero hours or control value.

from the cultured cells. A second challenge with MSA might be expected to reinvigorate the expression of CHOP/GADD153. For this experiment, the spent MSA medium was replaced with fresh MSA medium after 9 hours. Cells were left in culture for a total of 24 hours from the first MSA dosing before harvesting for apoptosis analysis by flow cytometry. As shown in Fig. 3*A*, in the unreplenished culture, CHOP/GADD153 protein dropped back to basal level at 24 hours, whereas in the replenished culture, it was still elevated. The apoptosis data (determined by Annexin V staining) under these two conditions are shown in Fig. 3*B*. There were considerably more apoptotic cells in the replenished culture than in the unreplenished culture, suggesting a potential contribution of CHOP/GADD153 in mediating the effect of MSA on apoptosis. The role of CHOP/GADD153 in apoptosis induction in this cell model was confirmed by a CHOP/GADD153 overexpression experiment. The results, as determined with the Cell Death Detection ELISA Kit, are shown in Fig. 3*C*. It should be noted that these cells were not treated with MSA. Compared with mock transfection, CHOP/GADD153 transfection produced substantially more apoptotic cells.

Overexpression of GRP78 alleviates methylseleninic acid–induced endoplasmic reticulum stress. As noted in the Introduction, a change in equilibrium between free and bound GRP78 in the ER is the spark that ignites the stress transducers (3, 9, 10). More free GRP78 is conducive to lowering the severity of ER stress

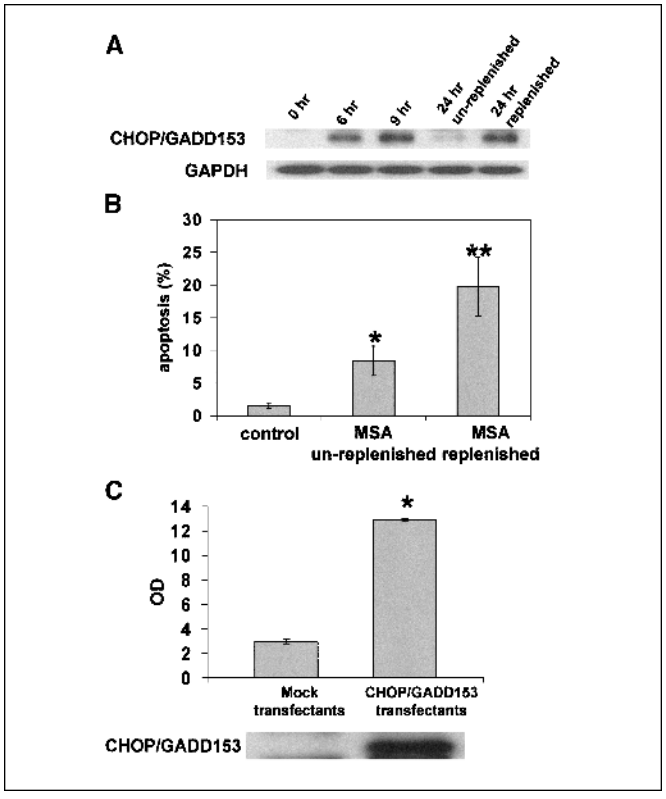

Figure 3. Sustained CHOP/GADD153 induction by MSA replenishment correlates with increased apoptosis. *A* and *B,* MSA replenishment experiments. Cells were treated with 10 μmol/L MSA for 9 hours. After centrifugation to pellet the cells, half of the culture was given fresh medium containing 10 μmol/L MSA; the other half was given back the old medium. Both cultures were incubated for an additional 15 hours before harvesting for CHOP/GADD153 Western blot (*A*) or apoptosis analysis by Annexin V staining (*B*). *C,* induction of apoptosis by CHOP/GADD153 overexpression. Cells were not treated with MSA. Apoptosis was determined with the Cell Death Detection ELISA Kit at 24 hours. *, significantly different from control value; **, significantly different from * value.

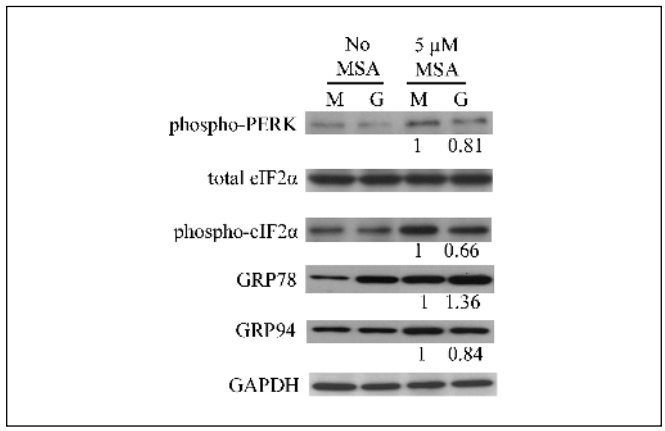

Figure 4. GRP78 overexpression reduces ER stress by MSA. *M,* mock transfection with empty vector; *G,* GRP78 transfection. Transfected cells were grown for 18 hours before treatment with 5 μmol/L MSA for 15 hours. Representative immunoblots are shown. The numbers beneath the bands denote the average fold of change relative to the mock transfection value ($n = 3$). All the GRP78 transfection data are significantly different from the mock transfection data ($P < 0.05$).

and dampening the activation of the transducers (34). The method of overexpression largely increases the availability of free GRP78. Transiently GRP78-overexpressing cells were cultured for 24 hours before treatment with 5 μmol/L MSA. The empty expression vector was used as the mock transfection control. Cells were treated for 15 hours before harvesting for Western blot analysis. The results are shown in Fig. 4. In the mock- and GRP78-transfected cells that were not treated with MSA, the basal levels of phospho-PERK and phospho-eIF2α were the same, suggesting that GRP78 overexpression itself did not change the phosphorylation status of these two proteins. MSA-induced PERK phosphorylation was ~20% less in the GRP78-transfected cells than in the mock-transfected cells. In line with the decreased PERK phosphorylation, eIF2α phosphorylation in the GRP78-transfected cells was also decreased by ~30%. A tempered phosphorylation status of PERK and eIF2α was indicative of reduced ER stress because these transducer molecules are involved in helping cells cope with the insult. The level of GRP78 in the GRP78-transfected cells was comparable to that in the mock-transfected cells treated with MSA. This was to be expected because endogenous induction of GRP78 is a survival response. The amount of overexpressed GRP78 was apparently enough to reduce MSA induction of ER stress to a degree that only required a more muted signaling of PERK and eIF2α. GRP94 is an ER-resident chaperone known to be induced in ER stress. The levels of GRP94 were the same in the mock- and GRP78-transfected cells that were not treated with MSA. The data suggest that GRP78 overexpression did not interfere with the regulation of other ER chaperones. MSA treatment increased GRP94, but produced ~20% less in the GRP78-transfected cells than in the mock-transfected cells. The results are consistent with the interpretation that GRP78 overexpression protects against ER stress caused by MSA.

Overexpression of GRP78 reverses methylseleninic acid induction of apoptosis. If MSA stimulates apoptosis through ER stress, lowering ER stress should reduce apoptosis. To address this question, GRP78- and mock-transfected cells were treated with 5 μmol/L MSA for 24 hours before apoptosis analysis by Annexin V staining. The results are shown in Fig. 5. MSA induced apoptosis in both groups; however, the magnitude of increase was less in the

GRP78-transfected cells (4-9%) than in the mock-transfected cells (6-14%), suggesting that ER stress response is an important mechanism in mediating the apoptotic effect of MSA.

GRP78 overexpression reduces the responsiveness of endoplasmic reticulum stress apoptotic markers to methylseleninic acid. The responsiveness of ER stress apoptotic markers was also studied in GRP78-transfected cells treated with 5 μmol/L MSA (Fig. 6*A*). The levels of CHOP/GADD153, cleaved caspase-12, and PARP cleavage were the same in the mock- and GRP78-transfected cells that were not treated with MSA. In contrast, the change of these markers in response to MSA was quite different between the two groups. CHOP/GADD153 expression, cleaved caspase-12, cleaved caspase-7, and PARP cleavage were reduced by ~44%, ~40%, ~60%, and ~30%, respectively, in the GRP78-transfected cells compared with the mock-transfected cells after 15 hours of MSA treatment. The amount of full-length caspase-7 was not changed by MSA. It has been reported that during ER stress, caspase-7 dissociates from GRP78 so that it can be released from the ER to the cytosol (35, 36). Coimmunoprecipitation with the full-length caspase-7 antibody confirmed that GRP78 was no longer bound to caspase-7 in MSA-treated cells (Fig. 6*B*). The question was whether the loss of GRP78 was due to cleavage of the full-length caspase-7 or to an actual dissociation from the caspase. From the results in Fig. 6*A*, it was apparent that cleaved caspase-7 represented only a very small fraction of the total because there was no detectable change of total caspase-7 despite the substantial increase of cleaved caspase-7 in the presence of MSA. Therefore, the decrease of GRP78 in the caspase-7 coimmunoprecipitation complex was most likely due to the release of GRP78 from caspase-7. This interpretation makes sense because GRP78 is needed urgently to rescue the misfolded proteins.

Induction of endoplasmic reticulum–resident chaperones and CHOP/GADD153 by methylseleninic acid in other human prostate cancer cell lines. To show that the ER stress response to MSA is not unique to PC-3 cells, we also looked at four other human prostate cancer cell lines treated with 10 μmol/L MSA and determined the changes in GRP94, GRP78, and CHOP/GADD153 by Western blot analysis. The results are shown in Fig. 7. LNCaP cells have a very low basal level of GRP94 and GRP78 expression. In contrast, LNCaP-LN3, DU145, and LAPC-4 cells have noticeably more GRP94 and GRP78 even without MSA treatment. All four cell lines responded to MSA with very robust induction of both ER-resident chaperones. Basal expression of CHOP/GADD153 is very low in prostate cancer cells not treated with MSA. This is generally true based on our experience with breast or lung cancer cells. On exposure to MSA, there was a healthy increase of CHOP/GADD153, as shown in Fig. 7. Based on the above data, we believe that ER stress response to selenium is a universal phenomenon.

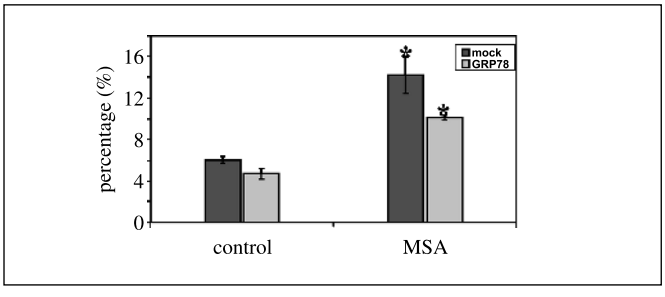

Figure 5. GRP78 overexpression desensitizes cells to apoptosis induction by MSA. Cells were treated with 5 μmol/L of MSA for 24 hours before harvesting for apoptosis analysis by flow cytometry. *, significantly different from the respective control value ($n = 3$).

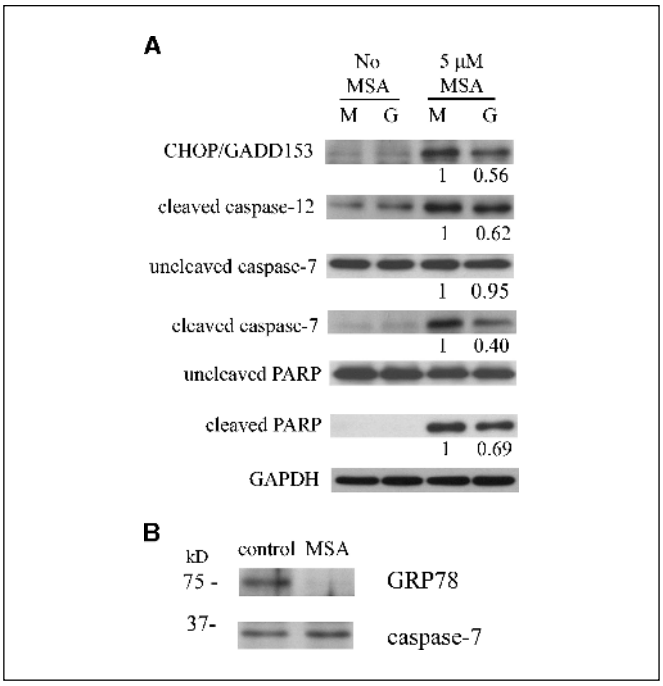

Figure 6. GRP78 overexpression reduces ER stress proapoptotic markers and PARP cleavage in cells treated with MSA. *A,* mock- (*M*) and GRP78- (*G*) transfected cells were grown for 18 hours before exposure to 5 μmol/L MSA for 15 hours. Representative immunoblots are shown. The numbers beneath the bands denote the average fold of change relative to the mock transfection value ($n = 3$). *B*, MSA treatment caused dissociation of GRP78 from caspase-7. Coimmunoprecipitation was carried out by using caspase-7 antibody. The immunoprecipitate was analyzed by immunoblotting with GRP78 antibody or caspase-7 antibody.

Discussion

The present study provides strong evidence to support the role of ER stress in mediating the apoptotic effect of selenium. Treatment with selenium induces a number of signature ER stress markers in a time-dependent manner. These markers could be categorized into two groups: (*a*) the survival/rescue molecules such as phospho-PERK, phospho-eIF2α, GRP78, and GRP94; and (*b*) the apoptotic molecules such as caspase-12, caspase-7, and CHOP/GADD153. The increase of phospho-PERK and phospho-eIF2α happens within 1 hour after exposure to selenium, suggesting that the activation of ER stress response is almost instantaneous. The acuteness is consistent with our previous finding that selenium causes measurable thiol/disulfide interchange in proteins (therefore protein misfolding or unfolding) as quickly as 30 minutes (28). Most of the damaged proteins recover within a short time, but a small fraction retains the redox changes for at least 12 to 24 hours. Cells clearly have a calibrated capacity to manage stress. If the stress level is too severe and exceeds the resource for repair, the signal for apoptosis will be triggered (1). It is thus reasonable to find that low doses of selenium preferentially activate the rescue arm of the ER stress response, whereas high doses of selenium lead to the assembly of the apoptotic machinery.

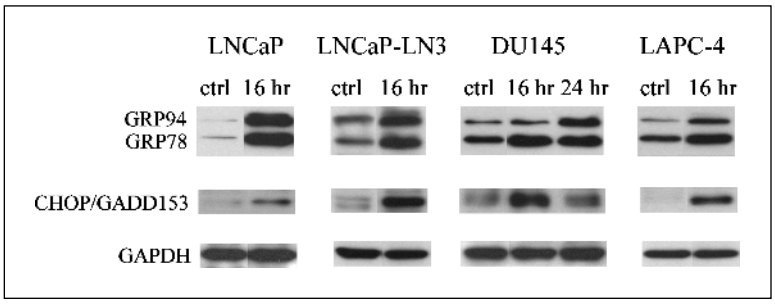

Figure 7. Induction of GRP94, GRP78, and CHOP/GADD153 by 10 μmol/L MSA in LNCaP, LNCaP-LN3, DU145, and LAPC-4 human prostate cancer cells.

GRP78 is like the rheostat of the ER stress transducer circuit board. Our experiments with GRP78 overexpression reinforce the idea that selenium acts through ER stress, at least in part, to induce apoptosis. We found that the ability of selenium to up-regulate apoptosis and apoptotic markers (caspase-12, caspase-7, and CHOP/GADD153) was significantly muted in GRP78-transfected cells. This could be explained by the reasoning that a generous supply of free GRP78 allows cells to cope better with ER stress, thereby improving the odds for survival. The inference is that, depending on how well cells are able to marshall the rescue effort, different cell types may have different thresholds for the apoptosis tripwire. The molecular mechanism underlying ER stress–associated apoptosis is far from clear. Caspase-12 has been implicated in mice. It is known to be cleaved by the Ca^{2+}-dependent protease m-calpain (13). It can also be activated through IRE1α recruitment of tumor necrosis factor receptor–associated factor 2 to the ER membrane (13, 14, 37). In human cells, caspase-12 may not be functional. The only caspase-12 homologous gene identified in the human genome has several stop codons in the exons (33). This peculiarity may prevent the production of a full-length and functional caspase-12 although the caspase-12 antibody can still recognize a cleaved band in Western blot. Other caspases that may be involved in ER stress include caspase-7 (15), caspase-4 (16), and a caspase-8 like isoform (17). As shown in previous studies (35, 36) and in Fig. 6, GRP78 and caspase-7 can bind to each other. We discovered that selenium stimulates their dissociation. To our knowledge, this is a new mechanism by which selenium recruits caspases, in addition to the more traditional way of activating the intrinsic and extrinsic death signaling pathways (38–40).

The robust expression of CHOP/GADD153 by the classic ER stress inducers (e.g., tunicamycin, thapsigargin, or DTT) has been well documented (18). However, the downstream effectors of CHOP/GADD153 are poorly understood. Two observations from the present study suggest that CHOP/GADD153 could be important in selenium induction of apoptosis. First, when the transient induction of CHOP/GADD153 was upgraded to a constant high level of expression by selenium replenishment, the above condition produced an augmentation in apoptotic response. Second, in GRP78-overexpressing cells, the induction of CHOP/GADD153 and apoptosis by selenium was diminished in parallel. Further studies will be needed to elucidate the molecular targets which are under the transcriptional control of CHOP/GADD153.

ER stress has been studied for a long time, but mainly in neuropathology, such as Parkinson's disease and Alzheimer's disease (41). Scanty information is available about ER stress and cancer. As far as we are aware, this is the first report describing the effect of a cancer chemopreventive agent on ER stress. Based on the results in Fig. 7, it is clear that selenium induction of ER stress response is a universal phenomenon and is not cell type specific. Different cells have different abilities to manage and cope with various forms of stress. Many factors may affect ER stress response to selenium. A recent study reported that selenium sensitizes tumor cells to a number of therapeutic drugs and increases the resistance of normal tissues to the toxic effects of these drugs (42). The above finding implies that selenium may have a dichotomous effect: it favors survival response in normal cells and facilitates apoptotic response in cancer cells. Indeed, the protection from drug toxicity by ER stress preconditioning has been reported previously in several noncancer cell models (43, 44). Potential differences in ER stress effector response between normal and cancer cells need to be studied more critically.

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