Inhibition of Translational Initiation by Activators of the Glucose-regulated Stress Protein and Heat Shock Protein Stress Response Systems

ROLE OF THE INTERFERON-INDUCIBLE DOUBLE-STRANDED RNA-ACTIVATED EUKARYOTIC INITIATION FACTOR 2α KINASE*

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Depletion of endoplasmic reticulum (ER) Ca²⁺ perturbs protein folding and processing within the organelle while inhibiting translational initiation through activation of the double-stranded RNA-activated eukaryotic initiation factor (eIF)-2α kinase (PKR) (Prostko, C. R., Dholakia, J. N., Brostrom, M. A., and Brostrom, C. O. (1995) J. Biol. Chem. 270, 6211–6215). The glucose-regulated stress protein (GRP) chaperones are subsequently induced. We now report that sodium arsenite, a prototype for stressors fostering cytoplasmic protein misfolding, also inhibits translational initiation through activation of PKR while subsequently inducing the heat shock protein (HSP) chaperones. Arsenite neither mobilized ER-associated Ca²⁺ nor slowed peptide chain elongation. Various HSP-inducing chemicals caused rapid phosphorylation of eIF-2α. When incubated with double-stranded RNA, extracts derived from arsenite-treated cells displayed greater degrees of phosphorylation of PKR and eIF-2α than did control extracts. Cells overexpressing a dominant negative PKR mutation resisted translational inhibition and eIF-2α phosphorylation in response to ER or cytoplasmic stressors. Induction of either the HSP or GRP chaperones was accompanied by development of translational tolerance to either Ca²⁺-mobilizing agents or arsenite. Following induction of the HSPs by arsenite, cells remained susceptible to induction of the GRPs by Ca²⁺-mobilizing agents. Conversely, cells possessing induced GRP contents in response to Ca²⁺-mobilizing agents readily induced the HSPs in response to arsenite. It is concluded that the two chaperone systems function independently except for their mutual suppression of PKR.

Chemicals and conditions that damage proteins, cause protein misfolding, or inhibit protein processing trigger the onset of protective homeostatic mechanisms resulting in “stress responses” in mammalian cells (reviewed in Refs. 1–3). Included in these responses are an acute inhibition of mRNA translation, a subsequent induction of various protein chaperones, and the recovery of mRNA translation. Separate, but closely related, stress response systems exist for the endoplasmic reticulum (ER), relating to the induction of specific glucose-regulated stress proteins (GRPs) and for the cytoplasm (cytosol and mitochondria), pertaining to the induction of the heat shock proteins (HSPs). The most prominent GRPs are chaperones of 78 and 94 kDa that share sequence homology with HSP70 and HSP90, respectively but localize to the ER lumen. GRP78, also termed BiP, is hypothesized to function in early ER protein folding and assembly, in the translocation of proteins from the cytosol to the ER for processing, and in the retention and accumulation of improperly folded proteins within the ER lumen when processing is distressed (4, 5). GRP94 is a high capacity Ca²⁺-binding glycoprotein thought to chaperone partially oxidized or folded intermediates (6). The ER functions critically in the early processing of newly synthesized secretory, lysosomal, and integral membrane proteins. Ca²⁺-sequenced by the ER supports the oligomerization, folding, and trimming of mannose residues of glycoproteins as well as the degradation of various incompletely assembled or abnormal proteins (7–14). The oxidizing environment of the lumen is required for the processing of proteins with disulfide-bonded intermediates (15). Agents such as Ca²⁺-ionophores, thapsigargin, and extracellular chelators that deplete ER Ca²⁺ or those that act as luminal reductants induce GRP78 and GRP94 (reviewed in Refs. 4 and 5). Induction of these GRPs is also associated with viral infections, overproduction of incomplete secretory proteins, and inhibition of protein glycosylation from such agents as tunicamycin. GRP genes possess highly conserved promoter regions that confer ER stress inducibility and that bind specific nuclear factors during stress. An ER transmembrane kinase appears key for signaling of the ER stress response in yeast (16). Initiation on grp78 mRNA can occur by a cap-independent, internal ribosome-binding mechanism (17). GRP78 and GRP94 maintain viability, and induction appears necessary for survival during persistent ER stress (18).

Induction of the HSPs follows the production of damaged or misfolded cytoplasmic proteins in response to elevated temperature, oxidative free radicals, and heavy metals or from the synthesis of aberrant cytoplasmic proteins in response to amino acid analogs (reviewed in Refs. 1–3). Proteins containing sulfhydryl groups are particularly sensitive to modification. Sodium arsenite, a prominent inducer of the HSPs that pro-

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duces minimal suppression of viability, is thought to act largely by inactivating sulfhydryl groups (19). Mammalian HSPs induced by these stressors include proteins of 110, 90, 72, 70, 60, and 30 kDa of differing subcellular distribution and chaperone function (3). The depletion of preexisting HSPs by binding to eccentric protein structures permits the trimerization of latent monomeric heat shock factor to a form that binds to heat shock response elements on DNA such that induction of heat shock mRNAs ensues (20, 21). These mRNAs, like that for grp78, possess structural features that permit their selective translation during stress. The chaperone activity of the HSPs is exemplified by HSP70 and a closely related form of the protein expressed in nonstressed cells, HSC72. Both prevent incorrect folding of polypeptides during synthesis, permitting delivery to organelles in an unfolded state for translocation. These chaperones may also solubilize or refold denatured or aberrant proteins and/or deliver them to a degradative system.

The acute depression of mRNA translation occurring in response to ER stress results specifically from the phosphorylation of eIF-2 α and initiation of translation (22–27). Recently reported that the dsRNA-activated, interferon-inducible protein kinase (PKR) mediates eIF-2α phosphorylation occurring in NIH-3T3 cells in response to mobilization of ER-sequestered Ca2+ (27, 28). While eIF-2α phosphorylation has been reported to occur in response to stressors that selectively induce HSPs (reviewed in Ref. 29), it was unclear whether translation was inhibited primarily at initiation or peptide chain elongation (30–32). We now report that PKR mediates the phosphorylation of eIF-2α in response to sodium arsenite without evident effects on translational elongation. The potential role of PKR as a general eIF-2α kinase regulating translational initiation is discussed.

**EXPERIMENTAL PROCEDURES**

**Materials—**Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgGs, alkaline phosphatase-conjugated rabbit IgG, and protein molecular weight standards were purchased from Bio-Rad. Enhanced chemiluminescence (ECL) kits, [32P]methionine, and [γ-32P]ATP (3000 Ci/mmol) were purchased from Amersham Corp. Ampholines of pH range 3.5–10 (Resolzyme) and range 4.5–8 were obtained from BDH and Sigma, respectively. Protein A-agarose (Life Technologies, Inc.) and ultrapure urea were obtained from Boehringer-Mannheim. [3H]leucine and [14C]leucine were purchased from ICN. The following reagents graciously provided reagents used in this study: Dr. Lynn O'Brien, University of Rochester (monoclonal anti-eIF-2α); Dr. Sidney Ahearn, University of Washington (rabbit anti-human PKR); Dr. Sidney Pestka, Robert Wood Johnson Medical School (interferon-α A/D Bgl); and Dr. Aaron Shatkin, Rutgers-CAMB (reovirus dsRNA). Purification of eIF-2α has been described previously (33). All other materials were of reagent grade.

**General Methodology—**Murine NIH-3T3 cells were cultured in dishes or multiwell plates in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and were utilized at confluence (1.5 × 107/cm²). NIH-3T3 cells expressing a dominant negative mutant PKR (K296P) in catalytic subdomain II, clone KP-A were derived as described previously (28). KP-A cells were cultured as above in the presence of 1 mg/ml G418 and utilized at confluence. Rat GH³ pituitary cells were maintained in suspension and utilized as described (34). Prior to treatments, cells were equilibrated for 5–15 min with serum-free Ham's F-10 modified to contain 25 μM leucine and 0.2 mM Ca²⁺. Amino acid incorporation was measured as described (35) for 30-min incubations of 3 × 10⁶ cells/experimental condition. Incubations were conducted in triplicate, and results are presented as the average or average ± range of values obtained. Findings were reproduced on at least two separate occasions. [32P]Sodium labeling (2 × 10⁶ cpm/experimental condition), one-dimensional 10% polyacrylamide gel electrophoresis (PAGE) of detergent-solubilized extracts of methionine-labeled cells, and autoradiography were conducted as described previously (25). Ribosomal and polyribosomal size distributions were measured by density gradient centrifugation as described previously (34). Cell-associated Ca²⁺ was measured as described (36).

**Activation of PKR by Arsenite**

**Determination of Average Ribosomal Transit Times—**Transit times were determined for cells in monolayer culture by a modification of the procedure of Bedford and Davis (37). Growth media were removed by aspiration from cells grown to confluence in 76-cm² dishes (2 × 10⁵ cells). The cells were washed with modified Ham's F-10 adjusted to contain 100 μM leucine, 33 μM methionine, 200 nM Ca²⁺ chloride, and 0.6 μM phorbol 12-myristate 13-acetate (PMA) and then equilibrated with 38 ml of the medium for 30 min at 37°C with various experimental additives. Controls (blanks) were adjusted to 2 mM leucine and 100 μM cycloheximide to prevent incorporation. Incorporations were initiated by the addition of 50 μl [35S]leucine (5 μCi). After 45 min the samples were adjusted with 50 μl [3H]leucine (20 μCi), and the incubation continued for 15 min. Samples (50 μl) were taken for determination of slopes, the incubation media were removed, and the cultures were adjusted with 40 ml of ice-cold 125 μM cycloheximide plus 2 mM leucine in isotonic saline to terminate incorporation. This medium was removed, and the cells were dislodged into 2.5 ml of lysis buffer containing 50 mM Tris, pH 7.6, 25 mM KCl, 1% Triton X100, 1% sodium deoxycholate, 10 μg/ml cycloheximide, 2 mM leucine, 1 mg/ml heparin, and 5 mM MgCl₂. The cells were homogenized with a Dounce glass homogenizer equipped with a tight fitting pestle (20 strokes), and the lysates were transferred to 15-ml Corex centrifuge tubes. The supernatants were removed into 5-ml vials on ice. The remaining of each fraction was centrifuged at 100,000 × g for 1 h. The postribosomal supernatant (PMS) fraction was decanted, and four 500-μl samples were taken as both. Both sets of samples were then precipitated with 10% trichloroacetic acid and analyzed for radioactivity by the procedure used for conventional incorporations (35). Samples taken for slope determination were adjusted to comparable 14C and 3H counting efficiencies to those for conventional incorporation samples. Samples were analyzed for each isotope in a multichannel scintillation counter, and the data were corrected for channel crossover. Slopes were calculated from the relationship,

\[ \text{Slope} = (\text{H} / \text{C}) / \text{(Incubation time)} \]

(Eq. 1)

Average ribosomal transit times were calculated by the relationship,

\[ \text{Transit time} = (2 / \text{slope}) \times (\text{H} / \text{C}) \times \text{PMS} / (\text{H} / \text{C}) \times \text{PRS} \]

(Eq. 2)

**Determination of the Phosphorylation State of the α-Subunit of eIF-2A—**Cells were cultured with a tight-fitting pestle (20 strokes), and the lysates were transferred to 15-ml Corex centrifuge tubes. The supernatants were removed into 5-ml vials on ice. The remaining of each fraction was centrifuged at 100,000 × g for 1 h. The postribosomal supernatant (PMS) fraction was decanted, and four 500-μl samples were taken as both. Both sets of samples were then precipitated with 10% trichloroacetic acid and analyzed for radioactivity by the procedure used for conventional incorporations (35). Samples taken for slope determination were adjusted to comparable 14C and 3H counting efficiencies to those for conventional incorporation samples. Samples were analyzed for each isotope in a multichannel scintillation counter, and the data were corrected for channel crossover. Slopes were calculated from the relationship,

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(Eq. 2)

**Measurement of eIF-2α Kinase Activity—**The preparation of extracts of variously treated NIH-3T3 cells, the methodology for measuring the phosphorylation of endogenous PKR and of exogenous, purified eIF-2α in vitro, and the procedures for immunoprecipitation of PKR were each described previously (27).

**RESULTS**

**Effects of ER and Heat Shock Stressors on Amino Acid Incorporation and Ca²⁺ Contents of NIH-3T3 Cells—**Acute exposure of various cultured cells to either thiol-reducing or Ca²⁺-mobilizing agents inhibits amino acid incorporation in conjunction with eIF-2α phosphorylation and depressed eIF-2B activity, lowered 43 S prepolypeptide complexes for the disappearance of polyribosomal contents (22, 23). NIH-3T3 cells were chosen for the current investigation in view of their strong induction of

² Developed at the National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161, PB95-500195GEE.
PKR in response to interferon-α and the activation of the enzyme by ER stressors (27). On acute challenge, protein synthesis in NIH-3T3 cells was remarkably sensitive to inhibition by such ER stressors as the Ca\(^{2+}\) ionophore ionomycin, to thapsigargin, an irreversible inhibitor of ER Ca\(^{2+}\) accumulation, and to dithiothreitol, a sulfhydryl-reducing agent. All of these agents on longer term exposures (several hours) induce expression of GRP78 and GRP94 but not the HSPs. Protein synthesis in NIH-3T3 cells was also readily inhibited by sodium arsenite and cadmium ion, both of which induce the HSPs but not the GRPs.

During longer term exposures suitable for inducing either HSPs or GRPs, most cell types recover approximately 50–100% of their original rates of amino acid incorporation. These recoveries depend on new mRNA synthesis. NIH-3T3 cells, however, were unable to restore amino acid incorporation when challenged by ER stressors such as thapsigargin unless fetal bovine serum, PMA, or epidermal growth factor was included in the incubation (not shown). Amino acid incorporation by NIH-3T3 cells also declined sharply (84%) during a 3-h exposure to actinomycin D. When either serum or PMA was added, actinomycin D blocked recovery from thapsigargin inhibition in the predicted fashion. Recovery of amino acid incorporation in cells challenged with arsenite, however, did not depend on the addition of promoters such as PMA. The addition of PMA was necessary, however, to demonstrate that actinomycin D prevented recovery of incorporation on longer-term arsenite treatment. The beneficial effects of PMA in stabilizing rates of amino acid incorporation in longer term incubations of NIH-3T3 cells prompted its judicial use in subsequent experiments.

The ability of sodium arsenite to release NIH-3T3 cell-associated Ca\(^{2+}\) was compared with that of two established releasers, ionomycin and thapsigargin (not shown). Both agents released approximately 40% of cell-associated Ca\(^{2+}\) during incubation periods ranging from 90 min to 4 h. Arsenite did not release Ca\(^{2+}\) or alter the release of Ca\(^{2+}\) occurring in response to either thapsigargin or ionomycin. Cells that were pretreated with arsenite under conditions that would induce HSPs were not altered either in their Ca\(^{2+}\) contents or in their responses to either thapsigargin or ionomycin.

**Development by NIH-3T3 Cells of Translational Cross-tolerance to ER and Cytoplasmic Stressors—Cross-tolerance to translational inhibition by ionomycin in NIH-3T3 cells that were accommodated to arsenite. Amino acid incorporation was compared for cells that were either pretreated for 90 min with 150 μM sodium arsenite followed by a 2-h recovery period without drug or carried as untreated controls. Leucine pulse labeling during the recovery period indicated that protein synthesis was gradually returning in the arsenite-treated samples after a nearly complete inhibition (Fig. 1, inset, filled circles). After 30 min in fresh medium, the two sets of samples were challenged with increasing concentrations of sodium arsenite or ionomycin (Fig. 1). Leucine incorporation by cells not previously exposed to arsenite was strongly inhibited by arsenite concentrations ranging from 25 to 100 μM and by ionomycin ranging from 30 to 100 nM (Fig. 2, open circles). Cells previously exposed to ionomycin were almost completely tolerant to translational inhibition by either stressor at these respective concentration ranges and markedly tolerant at higher concentrations as well (filled circles). Both sets of cells exhibited comparable degrees of leucine incorporation when incubated without stressors.

It was of interest to ascertain whether NIH-3T3 cells retain responsiveness to ER stressors after the induction of the HSPs and, conversely, whether inducers of the HSPs are active following induction of GRPs. In an initial experiment cells were pretreated with sodium arsenite for 90 min (stage 1), washed and exposed to either thapsigargin or ionomycin for 3.5 h (stage 2), and subsequently exposed to pulse labeling with \(^{35}\)S methionine (Fig. 3A). Controls without drug were also included for each of the two stages of the incubation, and some samples during the second stage were incubated with actinomycin D to block potential induction of stress proteins during this period. Cells treated with arsenite (lanes g–l) during the first stage of the treatment displayed the typical induction of HSPs of 110, 90, 72, 70, 60, and 30 kDa. These inductions were equivalent for cells treated in the second stage either without Ca\(^{2+}\) mobilizer (lane g), or with thapsigargin (lane h) or ionomycin (lane i) but were reduced sharply by actinomycin D during that period (lanes, j, k, and l, respectively). Overall protein synthesis was lowered by actinomycin D in these samples but not abolished. Controls that were not treated with arsenite (lanes a–f) did not induce any detectable HSPs either without Ca\(^{2+}\) mobilizer (lane a) or with thapsigargin (lane b) or ionomycin (lane c). Actinomycin D added to comparable respective samples (lanes d, e, and f) effaced almost all methionine incorporation in the thapsigargin (lane e) or ionomycin (lane f)–treated cells in contrast to the control without Ca\(^{2+}\) mobilizer (lane d), which was not perceptibly affected. Comparable inductions of GRP78, which were found for cells exposed to either thapsigargin (lanes b and h) or ionomycin (lanes c and i) for cells in the second stage of the incubation irrespective of previous arsenite treatment. No inductions of GRP78 were observed in cells treated with actino-

![Fig. 1. Induction by sodium arsenite of tolerance to translational inhibition by ionomycin.](image)

**Activation of PKR by Arsenite**

**Fig. 1. Induction by sodium arsenite of tolerance to translational inhibition by ionomycin.** NIH-3T3 cells were pretreated for 90 min in the absence (○) or presence (●) of 150 μM sodium arsenite and then allowed to recover for 2 h in arsenite-free medium. After a 30-min reequilibration in fresh medium, cultures were challenged for 30 min with arsenite or ionomycin at the indicated concentrations and analyzed for pulse incorporation of \(^{3}\)Hleucine into protein. The inset (right panel) indicates pulse incorporation into proteins at the indicated times of the recovery period.
mycin D under these respective conditions (lanes e, f, k, and l) or in cells that were never treated with a Ca\textsuperscript{2+} mobilizer (lanes a, d, g, and j).

The converse experiment was performed in which cells were treated with thapsigargin for 3 h to induce the GRPs (stage 1) and then exposed to sodium arsenite or CdCl\textsubscript{2} for 2.5 h (stage 2) and analyzed for pulse labeling with \[^{35}\text{S}\]methionine. As shown in Fig. 3A, drug-free samples for each stage and actinomycin D controls were included. Cells treated in stage 1 with thapsigargin (lanes g–l) displayed the strong induction of GRP78 and modest induction of GRP94, a later appearing protein. Pulse labeling of these proteins was not altered from the stage 2 control (lane g) by either arsenite (lane i) or Cd\textsuperscript{2+} (lane k). Actinomycin D (lanes h, j, and l, respectively) reduced, but did not eliminate, pulse labeling of these proteins. No perceptible induction of the HSPs was observed in any sample in response to thapsigargin. Exposure of thapsigargin-pretreated cells (lane g) during stage 2 to either arsenite (lane i) or Cd\textsuperscript{2+} (lane k) induced the full range of HSPs. Qualitatively comparable HSP inductions were noted for similarly treated control cells that had not received thapsigargin during stage 1 (lanes a, c, and e, respectively). Actinomycin D blocked the stage 2 induction of the HSPs by either arsenite (lanes d and j) or by Cd\textsuperscript{2+} (lanes f and l). It seems clear from these results (Figs. 3A and B) that, despite the cross-tolerance of protein synthesis developing in response to ER and heat shock stressors (Figs. 1 and 2), the HSP and GRP stress proteins remain independently inducible.

Selective and Reversible Inhibition of Translational Initiation by ER Ca\textsuperscript{2+}-mobilizing Drugs and Sodium Arsenite—The ability of sodium arsenite to inhibit translational initiation was compared with that of thapsigargin and ionomycin. Lysates derived from NIH-3T3 cells treated acutely for 45 min with 300 nM ionomycin (Fig. 4, upper panel, lane b), 30 nM thapsigargin (lane c), or 150 \(\mu\text{M}\) sodium arsenite (lane d) displayed a comparable accumulation of ribosomal subunits and a disappearance of polysomes as was seen with untreated control (lane a). These alterations were completely reversible for each substance following the addition of an inhibitor of polypeptide chain elongation, cycloheximide, for an additional 15 min (Fig. 4, lower panel). Longer term incubations with either 150 \(\mu\text{M}\) sodium arsenite or with 0.5 \(\mu\text{M}\) ionomycin resulted in

**Fig. 2.** Induction by ionomycin of tolerance to translational inhibition by sodium arsenite. NIH-3T3 cells in medium containing 0.6 \(\mu\text{M}\) phorbol 12-myristate 13-acetate were pretreated for 3.5 h with (●) or without (○) 0.5 \(\mu\text{M}\) ionomycin. Monolayers were washed twice with medium containing 2 mg/ml fatty acid-free bovine serum albumin and reincubated for 15 min in fresh medium without albumin. Cells were then challenged for 30 min with arsenite or ionomycin at the indicated concentrations and analyzed for pulse incorporation of \[^{3}H\text{]leucine into protein. The inset (right panel) indicates pulse incorporation into protein at the indicated times during the pretreatment period.**

**Fig. 3.** Independent inductions of GRPs and HSPs in response to ER and heat shock stressors. A, induction of the synthesis of GRP78 by thapsigargin or ionomycin superimposed on HSP induction by sodium arsenite. NIH-3T3 cells in F-10 medium containing 10% fetal bovine serum were incubated for 90 min (stage 1) with 150 \(\mu\text{M}\) sodium arsenite (lanes g–l) or as untreated controls (lanes a–f). The media were removed by aspiration and replaced with serum-free medium containing 0.6 \(\mu\text{M}\) phorbol 12-myristate 13-acetate and no further additions (lanes a and g), 1 \(\mu\text{g/ml}\) actinomycin D (lanes d–f and j–l), 0.1 \(\mu\text{M}\) thapsigargin (lanes b, e, h, and k), or 0.5 \(\mu\text{M}\) ionomycin (lanes c, f, i, and l). The incubation was continued for 3.5 h (stage 2). Proteins were then pulse-labeled for 30 min with \[^{35}\text{S}\]methionine and sampled for SDS-PAGE (10%) and autoradiography. Filled arrows indicate migration positions of the heat shock proteins, and the asterisk indicates the migration position of GRP78. B, induction of HSP synthesis by sodium arsenite or cadmium chloride superimposed on GRP induction by thapsigargin. NIH-3T3 cells pretreated for 5 min in medium containing 0.6 \(\mu\text{M}\) phorbol 12-myristate 13-acetate were adjusted to 0.1 \(\mu\text{M}\) thapsigargin (lanes g–l) or not adjusted (lanes a–f). After 3 h of incubation (stage 1), cells received fresh medium containing no additions (lanes a and g), 1 \(\mu\text{g/ml}\) actinomycin D (lanes b, d, f, h, j, and l), 150 \(\mu\text{M}\) sodium arsenite (lanes c, d, i, and j), or 15 \(\mu\text{M}\) cadmium chloride (lanes e, f, h, and l). The incubation was continued for 2.5 h (stage 2). Proteins were then pulse-labeled for 30 min with \[^{35}\text{S}\]methionine, and samples were analyzed by SDS-PAGE (10%) and autoradiography. Filled arrows indicate migration positions of the heat shock proteins, and the asterisk indicates the migration position of GRP78.

The inhibition of translational initiation in response to Ca\textsuperscript{2+}-mobilizing agents has been previously found to depend on the phosphorylation of eIF-2\(\alpha\) (22). NIH-3T3 cells and GH\textsubscript{3} pituitary cells were therefore compared for phosphorylation of eIF-2\(\alpha\) in response to ionomycin or to various agents that induce the HSPs, including arsenite, two heavy metal cations,
and two agents that generate oxidative free radicals (Fig. 5). Each of these agents promoted eIF-2\(\alpha\) phosphorylation to varying extents that were sufficient for substantial inhibition of protein synthesis. While fairly high degrees of eIF-2\(\alpha\) phosphorylation can be achieved from one inhibitor to another, it should be noted that only 20–30%, depending on cell type, is ordinarily required for complete inhibition of translational initiation (22, 26, 38, 39). This relationship is highlighted by an experiment detailing the increasing degrees of phosphorylation of eIF-2\(\alpha\) and of translational inhibition in NIH-3T3 cells in response to increasing concentrations of ionomycin or sodium arsenite (Table I). Maximal phosphorylation was achieved with a combination of ionomycin and arsenite. NIH-3T3 cells that were pretreated with either arsenite or ionomycin became refractory to eIF-2\(\alpha\) phosphorylation upon rechallenge with either agent. For example, cells pretreated with 150 \(\mu\)M sodium arsenite for 2 h followed by a 2-h recovery period were refractory to eIF-2\(\alpha\) phosphorylation in response to the concentrations of ionomycin or arsenite employed above (Table II). It was clear that the recovery from the initial arsenite treatment was incomplete, since all of the samples retained some residual phosphorylation and displayed reduced protein synthesis with respect to the untreated control. The effect of ionomycin pretreatment on subsequent eIF-2\(\alpha\) phosphorylation in response to rechallenge with arsenite or ionomycin was examined in a separate experiment (Table III). Acute treatment with ionomycin, arsenite, or both agents increased the phosphorylation of eIF-2\(\alpha\) with respect to untreated controls. In contrast, cells that were pretreated with ionomycin, washed, and allowed to recover were resistant to eIF-2\(\alpha\) phosphorylation and translational suppression upon comparable rechallenge.

Activation of PKR by Sodium Arsenite—We previously reported that depletion of ER Ca\(^{2+}\) from NIH-3T3 cells by either Ca\(^{2+}\) ionophore or thapsigargin activates an eIF-2\(\alpha\) kinase identified as PKR (27). This activation occurs in intact cells but is retained upon subsequent homogenization; it is not generated by treatment of lysates with these agents. Activation is particularly prominent after pretreatment of the cells with interferon-\(\alpha\), a well-established inducer of PKR. Extracts of NIH-3T3 cells, when incubated with \(\gamma\)\(^{32}\)P\(\cdot\)ATP, display autophosphorylation of PKR and phosphorylation of eIF-2\(\alpha\), each of which is amplified by the addition of viral dsRNA and purified eIF-2 to the incubation. NIH-3T3 cells cultured with interferon were therefore used to test the possibility that sodium arsenite causes PKR to become activated (Fig. 6). Cells were exposed to 150 \(\mu\)M sodium arsenite for 15 (lanes b and e) or 30 min (lanes c and f) or incubated for 30 min as untreated controls (lanes a and d). Extracts derived from arsenite-challenged cells were found to phosphorylate both PKR, as determined following immunoprecipitation with an antibody to the enzyme (Fig. 6A), and eIF-2\(\alpha\) (Fig. 6B). The phosphorylation of each of the two proteins was greater in cells treated with arsenite for 30 min than for 15 min. While some phosphorylation of each protein was evident without the addition of dsRNA to the incubation (lanes a–c), phosphorylation was greatly increased by this addition (lanes d–f) and was evident to some extent even for lysates derived from cells that were not exposed to arsenite. When cells cultured without interferon were subjected to identical protocols, phosphorylations of PKR or eIF-2\(\alpha\) in response to dsRNA addition or arsenite treatment were barely detectable (not shown).

Effects of ER and Heat Shock Stressors on Amino Acid Incorporation and eIF-2\(\alpha\) Phosphorylation in Cells Expressing a Dominant Negative Mutation in PKR—NIH-3T3 cells that overexpress a dominant negative human PKR mutant K296P
Effect of sodium arsenite pretreatment on eIF-2α phosphorylation in response to subsequent ionomycin or arsenite challenge

| Stressor                  | Nonpretreated cells | Leucine incorporation | Arsine-pretreated cells | Leucine incorporation |
|---------------------------|---------------------|-----------------------|-------------------------|----------------------|
|                           | eIF-2α(P) %         | nmol/10^6 cells       | eIF-2α(P) %             | nmol/10^6 cells       |
| None                      | 5                   | 0.88                  | 13                      | 0.46                 |
| Ionomycin (30 nM)         | 15                  | 0.32                  | 12                      | 0.39                 |
| Ionomycin (100 nM)        | 18                  | 0.15                  | 10                      | 0.38                 |
| Ionomycin (300 nM)        | 23                  | 0.10                  | 10                      | 0.37                 |
| Ionomycin (1 μM)          | 30                  | 0.04                  | 9                       | 0.40                 |
| Arsinite (50 μM)          | 24                  | 0.12                  | 10                      | 0.44                 |
| Arsinite (150 μM)         | 30                  | 0.07                  | 12                      | 0.43                 |
| 1 μM ionomycin + 150 μM arsenite | 41            | 0.02                  | 10                      | 0.42                 |

Effect of ionomycin pretreatment on eIF-2α phosphorylation in response to subsequent ionomycin or arsenite challenge

| Pretreatment additive | Stressor                  | eIF-2α(P) %         | Leucine incorporation | nmol/10^6 cells |
|-----------------------|---------------------------|---------------------|-----------------------|----------------|
| None                  | None                      | 6                   | 0.92                  |                |
| None                  | Ionomycin                 | 30                  | 0.04                  |                |
| None                  | Arsinite                  | 15                  | 0.24                  |                |
| None                  | Ionomycin + arsenite      | 56                  | 0.02                  |                |
| Ionomycin             | None                      | 5                   | 0.90                  |                |
| Ionomycin             | Ionomycin                 | 10                  | 0.45                  |                |
| Ionomycin             | Arsinite                  | 5                   | 0.85                  |                |
| Ionomycin             | Ionomycin + arsenite      | 15                  | 0.30                  |                |

Fig. 7. Induction of expression of endogenous PKR by interferon-α and phosphorylation of eIF-2α by ER and heat shock stressors in wild-type NIH-3T3 cells and cells expressing a dominant negative mutant PKR. A, effect of interferon on expression of PKR. Wild-type cells and those that overexpress a dominant negative human PKR mutant (K296P in catalytic subdomain II, clone KP3A) were cultured for 24 h with interferon-α (IFNα, 1000 units/ml). Lysates were subjected to SDS-PAGE (12.5%) followed by immunoblotting with a polyclonal antibody recognizing both murine and human PKR. Arrows indicate the migration positions of the murine and human kinases. The asterisk indicates an unidentified species present in all samples and that serves as substrate for the enzyme conjugated to the secondary antibody. B and C, eIF-2α phosphorylation in NIH-3T3 and KP3A cells following treatments with ER and heat shock stressors. NIH-3T3 (B) and KP3A (C) cells were treated for 30 min without drug (1), with 0.1 or 0.3 μM ionomycin (2 and 3), with 0.2 or 0.6 mM dithiothreitol (4 and 5), with 50 or 150 μM sodium arsenite (6 and 7), or with 2 or 6 mM t-butylhydroperoxide (8 and 9). Lysates were subjected to slab gel isoelectric focusing followed by immunoblotting for eIF-2α. Arrows indicate the migration positions of the phosphorylated (eIF-2α(P)) and nonphosphorylated (eIF-2α) subunits.
HSP family in view of the close sequence homologies that exist between GRP78 and HSP70/HSC72 and between GRP94 and HSP90 (3–5). The two sets of stress proteins, however, are induced in response to different chemicals and conditions in mammalian cells and localize to different subcellular compartments. In this report we have chosen to view ER stressors as those perturbants that inhibit translation and ER protein folding or processing while subsequently inducing the ER resident chaperones, GRP78 and GRP94. The ER stress response system is activated by Ca\(^{2+}\)-mobilizing or thiol-reducing agents. In contrast, those perturbants that inhibit translation and protein folding in the cytoplasm while inducing the HSPs are viewed as cytoplasmic or “heat shock” stressors. The heat shock stress response system is activated by oxidative chemicals and heavy metal ions. Interdigitation of these two closely related systems is of considerable interest. In this report we have utilized Ca\(^{2+}\) ionophore and thapsigargin as representative activators of the ER stress system. As activators of the cytoplasmic stress system we employed the sulfhydryl-inactivating agents sodium arsenite and, to a lesser extent, cadmium ion. Sodium arsenite offered the advantage of producing reproducible effects while subsequent rechallenge of the cells with arsenite did not induce detectable amounts of the GRPs (Fig. 3).

Previous reports have emphasized that eIF-2\(\alpha\) phosphorylation is increased by a variety of HSP inducers to uneven degrees, ranging from pronounced phosphorylations with arsenite to low to high phosphorylations with heat shock to marginal phosphorylations with iodoacetamide and various amino acid analogs (29–32). The relative contributions of initiation and elongation blockade to the overall degree of translational inhibition by these chemicals has been unclear. Translation is also suppressed in some cell types by serum removal in conjunction with eIF-2\(\alpha\) phosphorylation (29). Serum depletion almost certainly also slows translational elongation, since this condition is associated with dephosphorylation of various elongation factors (40).

It is evident from the present data and those of previous reports (27, 28) that perturbants of either the ER/GRP or cytoplasmic/HSP chaperone systems produce an immediate activation of the eIF-2\(\alpha\) kinase, PKR, and the phosphorylation of eIF-2\(\alpha\) in conjunction with the inhibition of translational initiation. Phosphorylation of eIF-2\(\alpha\) is well established to inhibit eIF-2B, the GTP/GDP exchange factor required for recycling of eIF-2 during initiation (29). Once sufficient eIF-2\(\alpha\) is phosphorylated to complex eIF-2B, additional degrees of phosphorylation are superfluous, although in extreme circumstances almost all of the eIF-2\(\alpha\) pool can be phosphorylated (24). Sodium arsenite inhibited translational initiation without affecting peptide chain elongation. Polyribosomal contents almost totally disappeared from NIH-3T3 cells, and ribosomal subunits were correspondingly increased (Fig. 4). The addition of cycloheximide such that elongation once again became rate-limiting restored polyosomal contents. This reversibility pointed to a slowing of initiation without the occurrence of physical damage to the translational apparatus. More importantly, average ribosomal transit times were not lengthened detectably by sodium arsenite (Table I), allowing us to conclude that elongation was not affected even by relatively high arsenite concentrations that abolished amino acid incorporation. All of these effects were faithfully reproduced by ionomycin and thapsigargin, which have been found repeatedly to inhibit translational initiation selectively in a variety of cell types (23, 25).

The induction of either the GRPs or the HSPs over several hours in NIH-3T3 cells was accompanied by a reduced phosphorylation of eIF-2 (Tables II and III) and a partial resumption of mRNA translation (Figs. 1 and 2). These data provided further evidence that the acute inhibition of initiation derived from the phosphorylation of eIF-2\(\alpha\). Induction of either class of stress proteins was associated with the development of translational tolerance to subsequent rechallenge of the cells with either ER or heat shock stressors. Tolerance was observed in terms of continued amino acid incorporation (Figs. 1 and 2), maintenance of polyribosomal contents, and the lack of increased eIF-2\(\alpha\) phosphorylation (Tables II and III). Translational recovery from inhibition by Ca\(^{2+}\) ionophores is partly overturned by antisense oligonucleotides directed against grp78 mRNA (41). Presumably, various GRP and HSP chaperones inhibit PKR through complexing with a critical component(s) of the enzyme or with other protein(s), which affect its activity. If so, the sequence homology of GRP78 and HSP70 may be important to the putative dual input. PKR is thought to be a complex enzyme that interacts with various inhibitory proteins, responds to various activators and inducers, and possesses at least one other substrate, I-\(\kappa\)B (42, 43). A closely related eIF-2\(\alpha\) kinase of erythroid cells (heme-regulated eIF-2\(\alpha\) kinase) is reported to exist in association with HSP70 and HSP90 (44). Not all stresses affect PKR comparably. PKR is found in the soluble fraction of lysates derived from cells treated with sodium arsenite but in the insoluble fraction of heat-shocked cells (45). In our experiments with arsenite- and ionomycin-treated cells, PKR was found in the soluble fraction of cell lysates containing Triton X-100.

With the exception of their mutual abilities to influence the activity of PKR and translational initiation, the two stress systems appear to operate independently. For example, sodium arsenite did not induce detectable amounts of the GRPs (Fig. 3), nor did it affect ER function in NIH-3T3 cells. The cells maintained their Ca\(^{2+}\) contents during extended treatment with arsenite and, upon Ca\(^{2+}\) depletion with either ionomycin or thapsigargin, readily induced GRP78 superimposed upon
the preexisting Hsp induction by arsenite (Fig. 3). Similarly, following the induction of GRP78 with Ca\(^{2+}\)-mobilizing agents, the cells remained responsive to Hsp induction by subsequent arsenite treatment. It should be recognized that the phosphorylation of eIF-2\(\alpha\) and inhibition of translation do not appear mandatory for the subsequent induction of stress proteins even in unstressed cells. Low concentrations of ER stressors clearly induce GRP78 in GH\(_3\) cells in the absence of eIF-2\(\alpha\) phosphorylation or inhibition of translation (26).

The body of available information pertaining to eIF-2\(\alpha\) kinase activities suggests that PKR may function as a common focal point for governing rates of translational initiation in response to a variety of stimuli including, but not limited to, viral infections, ER stress, and cytoplasmic proteotoxic stress. Currently only two mammalian eIF-2\(\alpha\) kinases are known to exist: heme-regulated eIF-2\(\alpha\) kinase, which is expressed selectively by erythroid cells (46), and PKR, which is ubiquitous to all mammalian cell types. The lack of other eIF-2\(\alpha\) kinase activities and the activation of PKR by stressors suggests that the enzyme may mediate most, if not all, eIF-2\(\alpha\)-dependent inhibitions of translational initiation in higher eukaryotes. Putative additional stimuli for PKR activation could include hormonal or nutritional alterations or treatments that damage the plasma membrane. The possibility that PKR possesses multiple substrates and/or serves more broadly in cellular control mechanisms is also supported by various recent findings. For example, PKR activates specific gene transcription through NF-\(\kappa\)B-dependent (41, 42) and -independent (47) mechanisms and is strongly implicated in the control of differentiation and growth (48). The emerging picture suggests that the biochemical structure and regulation of the enzyme should prove both interesting and informative.

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