Journal of Cellular Biochemistry 51:91–101 (1993)

ARTICLES

Okadaic Acid as an Inducer of the 78-kDa Glucose-Regulated Protein in 9L Rat Brain Tumor Cells

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Abstract Okadaic acid (OA), a potent inhibitor of protein phosphatases 1 and 2A, has been widely used as a tool for unravelling the regulation of cellular metabolic processes involving protein phosphorylation/dephosphorylation. It has recently been found that OA can induce reversible hyperphosphorylation of vimentin and reorganization of intermediate filaments [Lee et al., J. Cell. Biochem. 49: 378–393, 1992]. We report here that OA specifically induced the synthesis of a 78-kDa protein, which was identified as the 78-kDa glucose-regulated protein (GRP78) by two-dimensional sodium dodecylsulfate-polyacrylamide gel electrophoresis and peptide mapping. The induction of GRP78 by OA was dose-dependent and reversible. For 7 h treatments, GRP78 synthesis was initially enhanced under 50 nM OA and became the highest (about 6-fold) under 200 nM OA. Meanwhile, under 200 nM OA, GRP78 synthesis was initially enhanced after 4 h and reached its maximal level (about 8-fold) after 15 h of treatment. Subsequently, upon removal of OA, the level of OA-induced GRP78 was reduced to basal level after 12 h of recovery. Induction of GRP78 synthesis by OA was abolished in cells pretreated with actinomycin D and cycloheximide, indicating that it was regulated at the transcriptional level and its induction required de novo protein synthesis. Furthermore, OA suppressed protein glycosylation, and the result lent support to the hypothesis that suppression of protein glycosylation may correlate with induction of GRP78 synthesis.

Key words: okadaic acid, 78-kDa glucose-regulated proteins, brain tumor cells

Okadaic acid (OA) is a toxic polyether compound of a C-38 fatty acid first isolated from marine black sponges [Tachibana et al., 1981]. It has been demonstrated that OA is a potent inhibitor of protein phosphatases 1 and 2A [Bialojan and Takai, 1988] and the inhibitory effect is exerted through direct binding [Nishiwaki et al., 1990]. OA blocks the dephosphorylation of proteins that are substrates for multiple protein kinases [Suganuma et al., 1988], resulting in the apparent activation of the kinases [Issinger et al., 1988; Sassa et al., 1989], and subsequently induces a variety of cellular responses that are modulated by protein phosphorylation/dephosphorylation. For instance, OA "activates" the kinases and thus enhances the phosphorylation states of the regulatory enzymes of glycogen and lipid metabolism, glycolysis, and gluconeogenesis in hepatocytes and adipocytes [Haystead et al., 1989], "activates" cdc2/histone H1 kinase and transiently induces premature mitosis-like state in BHK21 cells [Yamashita et al., 1990], and induces hyperphosphorylation of epidermal growth factor receptor in A431 human epidermoid carcinoma cells [Hernandez-Sotomayor et al., 1991] and hyperphosphorylation of vimentin in primary human fibroblasts and 9L rat brain tumor cells [Yatsunami et al., 1991; Lee et al., 1992]. In addition, OA has been shown to inhibit protein synthesis at the translational level [Redpath and Proud, 1989; Schtonthal et al., 1991], and most recent evidence demonstrates that OA can block protein transport from the ER to the Golgi apparatus by enhancing protein phosphorylation [Lucocq et al., 1991; Davidson et al., 1992]. By Northern blotting analysis, OA has been further shown to function in regulating expression of a number of specific genes, including urokinase-type plasminogen-activator receptor gene, collagenase gene, *Erg-1*, and nuclear protooncogenes *c-fos* and *c-jun* [Kim et al., 1990; Nagamine and Ziegler, 1991; Schtonthal et al., 1991; Thevenin et al., 1991;

Abbreviations used: OA, okadaic acid; GRP78, 78-kDa glucose-regulated protein; PP1, protein phosphatases 1; PP2A, protein phosphatases 2A.

Received July 15, 1992; accepted August 12, 1992.

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Lund and Dano, 1992; Guy et al., 1992; Holladay et al., 1992].

Being cytotoxic, sublethal concentrations of OA must impose a certain degree of stress on cells in its presence. It has been well established that exposure of cultured cells to a variety of physiological stresses leads to the synthesis of a small group of proteins collectively known as the stress proteins which are usually subdivided into the heat-shock proteins (HSPs) and the glucose-regulated proteins (GRPs) [Hightower, 1991]. The GRPs are constitutively expressed and localized in the ER and the Golgi apparatus in higher eucaryotic cells. The GRPs were first identified as proteins whose syntheses were greatly enhanced in cells that were grown in medium depleted of glucose [Shiu et al., 1977]. Synthesis of the GRPs is greatly enhanced when cells in culture respond to other physiological stresses such as inhibition of protein glycosylation and calcium ionophores treatment [Hightower, 1991]. The most prominent member of the GRP family is GRP78/BiP, a protein with a molecular weight of 78 kDa [Munro and Pelham, 1986; Ting et al., 1987; Hendershot et al., 1988], which localizes in the lumen of the ER. GRP78 has been identified as the molecular chaperone protein that is able to associate with polypeptides and facilitate their proper assembly and transport [Gething et al., 1986; Hendershot et al., 1988; Rothman, 1989].

In the present study, we demonstrated that OA suppressed the synthesis of general proteins and concomitantly induced a 78-kDa protein which was identified as GRP78. The process of GRP78 induction was further characterized and the possible mechanisms were discussed.

MATERIALS AND METHODS

Materials

Okadaic acid was purchased from Gibco Laboratories (Grand Island, NY), dissolved in 10% dimethyl sulfoxide at a concentration of 0.5 mM, and stored in the dark at −20°C. It was diluted to appropriate concentrations with culture medium before use. All cultureware was purchased from Corning (Corning, NY) and culture medium components were purchased from Gibco Laboratories. [^{35}S]methionine (specific activity > 800 Ci/mmol) and [6-^{3}H]fucose (specific activity 72.7 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Chemicals for electrophoresis were purchased from BioRad (Richmond, CA). *Staphylococcus aureus* V8 protease was obtained from Boehringer-Mannheim (Mannheim, Germany). General chemicals were from Sigma (St. Louis, MO) or Merck (Darmstadt, Germany).

Cell Culture

The 9L rat brain tumor cells, originated from rat gliosarcoma, were a generous gift from Dr. M. L. Rosenblum, University of California at San Francisco [Weizsaecker et al., 1981]. Cells were maintained in Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin G, and 100 μg/ml streptomycin. The cells were kept in a humidified 37°C incubator with a mixture of 5% CO_2 and 95% air. Stock cells were plated in 25 cm^2 flasks or six-well plates at a density of 4 to 6 × 10^4 cells per cm^2. All experiments were performed using exponentially growing cells at 85–95% confluency.

Drug Treatments and [^{35}S]Methionine Labeling

To investigate the effects of OA, cells were treated with various concentrations of OA for various durations as indicated at 37°C. Moreover, for comparison, cells were also treated with 7 μM A23187 for 12 h. After treatment with OA, the media containing OA were removed and cells were labeled immediately with 50 μCi of [^{35}S]methionine per ml in methionine-free medium for 1 h. Alternatively, the treated cells were further incubated under normal growing conditions for various durations prior to labeling in the recovery experiments. For determination of the rate of protein synthesis, after labeling, the cells were washed thrice with ice-cold phosphate-buffered saline (PBS) and lyzed with 300 μl of sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% sodium dodecylsulfate, 5% β-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue). Incorporation of [^{35}S]methionine in acid-insoluble fractions were then determined by filter collection and scintillation counting as described by Lai et al. [1988]. The relative rate of protein synthesis of treated cells was referred to as the fraction of [^{35}S]methionine incorporation into proteins relative to that of untreated control cells.

Gel Electrophoresis

After labeling, cells were washed with ice-cold PBS and lyzed with 300 μl of sample buffer or 200 μl of lysis buffer (9.5 M urea, 2% Nonident

P-40, 2% ampholytes, and 5% β-mercaptoethanol), depending on the electrophoresis system employed. One-dimensional sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [1970]. The samples for SDS-PAGE were heated in boiling water for 5 min and then microfuged (Eppendorf, full speed) for 3 min before loading. They were applied to 10% SDS-polyacrylamide gels on the basis of equal amounts of cell lysates. For molecular weight calibration, a subset of the following molecular standards (Sigma) was included in each gel: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), and egg albumin (45 kDa). After electrophoresis, the gels were removed and stained for 1 h in staining solution (0.1% Coomassie brilliant blue R250 in 10% acetic acid and 50% methanol). The gels were then destained and dried under vacuum. Autoradiography was performed at room temperature using Fuji RX X-ray film. The optical densities of the protein bands of interest on autoradiographs were quantitated by scanning the resulting autoradiographs on an Ultroscan laser densitometer (LKB, GSXL software). Two-dimensional PAGE was performed according to the method of O'Farrell [1975]. Equal amounts of cell lysates were loaded onto the pre-run isoelectrofocusing (IEF) gels and run for 16 h at 400 V and then 1 h at 800 V. Subsequently, the IEF gels were loaded onto 10% SDS-polyacrylamide slab gels with a 4.75% stacking gel for electrophoresis in the second dimension. After electrophoresis, the gel slabs were processed for autoradiography as described above. The pH gradient formed was measured from slices obtained from replicate IEF.

Peptide Mapping

One-dimensional peptide mapping after limited proteolysis was performed as described by Cleveland et al. [1977] with minor modifications. The radioactive samples were first separated by 10% SDS-polyacrylamide gels as described above. The gels were stained and destained briefly. The 78-kDa protein bands generated by OA or A23187 were individually cut out and equilibrated again in Buffer A (0.125 M Tris/HCl, pH 6.8, 0.1% SDS, and 1 mM EDTA). The gel slices were then applied to the sample wells of a second SDS-polyacrylamide gel together with 100 ng of *Staphylococcus aureus* V8 protease. In the procedure, 14.5% separating gels were used. The gels were processed for autoradiography as described above.

Antibiotic Experiments

For study of the effects of antibiotics on induction of GRP78 synthesis by OA, cells were pretreated with 0.1 μM actinomycin D or 50 μg/ml cycloheximide for 30 min and then incubated with 200 nM OA for 7 h. For comparison, cells were also pretreated with the same concentration of actinomycin D or cycloheximide and then incubated with 7 μM A23187 for 7 h. After treatment with OA, cells were labeled with 50 μCi [^{35}S]methionine per ml in methionine-free medium for 1 h and then harvested. Further processing of the samples was the same as described above.

Determination of Rate of Protein Glycosylation

To determine the rate of protein glycosylation, cells were treated with various concentrations of OA for various durations as indicated and then labeled with 6 μCi of [6-^{3}H]fucose per ml in MEM for 1 h. The untreated cells were washed thrice with ice-cold PBS and lyzed with SDS sample buffer. For determination of acid-precipitation counts, 10% trichloroacetic acid was added to an aliquot of cell lysates for 4°C. After 30 min, the precipitate was collected on Whatman GF/A filter and washed thrice with 8% trichloroacetic acid as described by Chang et al. [1987] with minor modifications. Radioactivity on the filters was measured in a scintillation counter. The rate of protein glycosylation of treated cells was referred to as the fraction of incorporations of [6-^{3}H]fucose into proteins relative to that of untreated control cells.

RESULTS

Effects of OA on Synthesis of Cellular Proteins in 9L Cells

To investigate whether OA affects the synthesis of cellular proteins, cells were treated with 200 nM OA for 7 h and then labeled with [^{35}S]methionine for 1 h in the absence of OA. It was found that the relative rates of protein synthesis were suppressed in the presence of OA and the effect was concentration- and time-dependent (Fig. 1). The relative rates of protein synthesis decreased to about 10% and 18% of the control levels in cells that were treated with 600 nM OA for 7 h and 200 nM OA for 10 h, respectively.

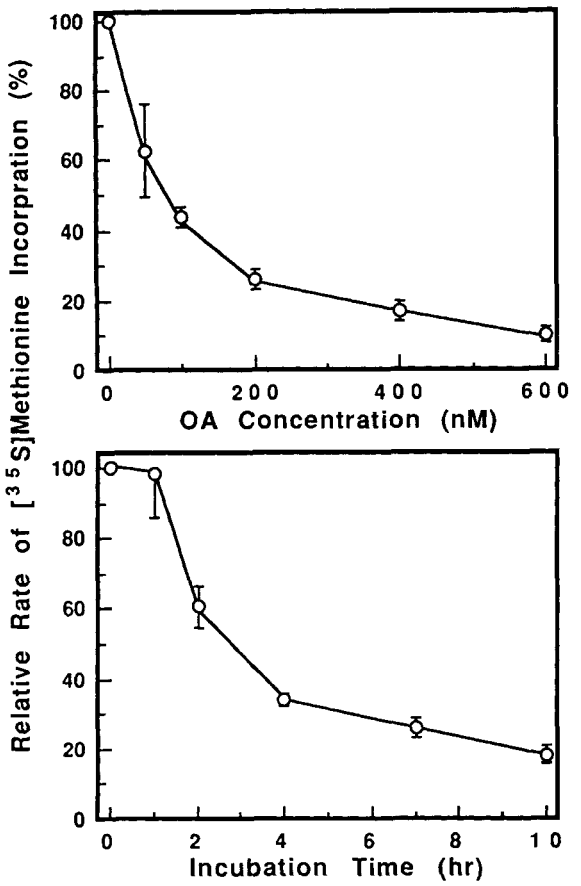

Fig. 1. Effect of OA on rate of synthesis of cellular proteins in 9L cells. After OA treatment, cells were labeled with [^{35}S]methionine for 1 h and changes in rate of protein synthesis were monitored by [^{35}S]methionine incorporation. The data are from three independent experiments.

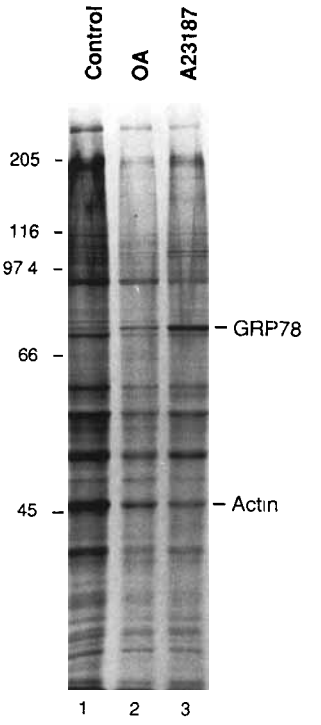

Fig. 2. Effect of OA on de novo synthesized proteins in 9L cells. After treatment of cells with OA and A23187, cells were labeled with [^{35}S]methionine and then harvested. The protein samples were analyzed by 10% SDS-PAGE and autoradiography was shown. **Lane 1:** Untreated control cells; **lane 2:** OA-treated cells; **lane 3:** A23187-treated cells as a positive control. Numbers on the left denote molecular makers.

Furthermore, for comparison, cells were also treated with 7 μM A23187, an inducer for GRP78, for 12 h and then labeled with [^{35}S]methionine for 1 h in the absence of A23187. The patterns of total cellular proteins were analyzed. Figure 2 shows that a protein with an apparent molecular weight of 78 kDa was significantly induced in OA- and A23187-treated cells.

The identity of the 78-kDa protein induced by OA was further examined by two-dimensional SDS-PAGE and peptide mapping. It was found that the 78-kDa proteins induced by OA and A23187, respectively, migrated to the same position on two-dimensional SDS-polyacrylamide gels (Fig. 3) and that the patterns of proteolytic fragments of these 78-kDa proteins were indistinguishable (Fig. 4). These data strongly indicated that OA can induce the synthesis of GRP78.

Effects of Various OA Incubation Conditions on Induction of GRP78 Synthesis

To assess the kinetics of induction of GRP78 synthesis, 9L cells were treated with various concentrations of OA for various incubation durations and the rates of GRP78 synthesis were monitored. For 7 h treatments, the level of GRP78 synthesis was initially enhanced about twofold when cells were treated with 50 nM OA but could be enhanced 5- to 6-fold, and reached its maximal level when OA concentration was increased to 200 nM. At higher concentrations, enhanced synthesis of GRP78 was found to level off (Fig. 5). Enhanced synthesis of GRP78 in OA-treated cells was time-dependent. Under 200 nM OA, GRP78 synthesis was not significantly affected within 2 h but enhanced by 7- and 8-fold after 10 and 15 h of treatments, respectively (Fig. 6). Prolonged incubation resulted in cell detachment (not shown). Subsequently, to investigate the effect of OA removal on the synthesis of GRP78 in OA-treated cells, cells were treated with 200 nM for 7 h and allowed to recover

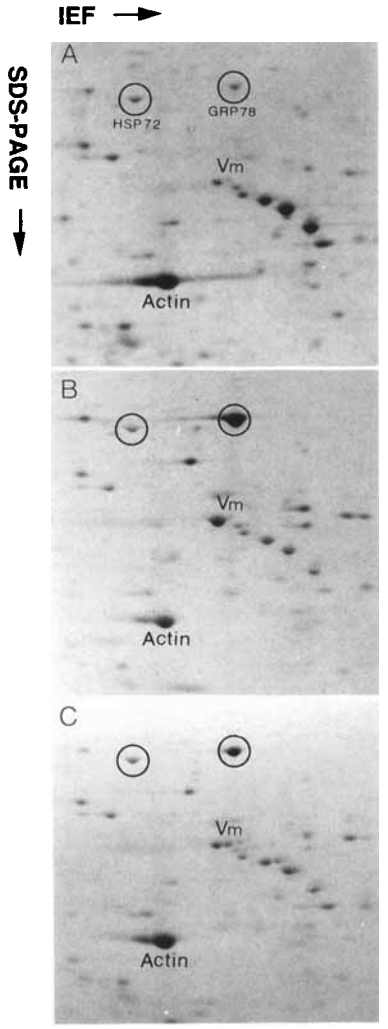

Fig. 3. Positions of proteins induced by calcium ionophore A23187 and OA in 9L cells. Cells were treated with either 200 nM OA for 7 h or 7 μM A23187 for 12 h and then labeled with [^{35}S]methionine for 1 h. The treated cells were then lyzed with lysis buffer and the protein samples were analyzed by two-dimensional PAGE. Following electrophoresis, the gels were processed for autoradiography. **A:** Untreated control cells. **B:** A23187-treated cells. **C:** OA-treated cells. Circles indicate the positions of the proteins induced by A23187 or OA with molecular weights shown in kDa. The pH ranges of the IEF gels are shown at the bottom of the autoradiograms.

under normal growing conditions. The level of GRP78 synthesis was then monitored at different time intervals. As shown in Figure 7, the level of GRP78 synthesis continued to increase and reached its maximum after 2 h of recovery. Subsequently, the synthesis of GRP78 returned to the basal level after 12 h of recovery in a time-dependent manner. The above results demonstrated that the induction of GRP78 synthesis by OA was dose-dependent and reversible.

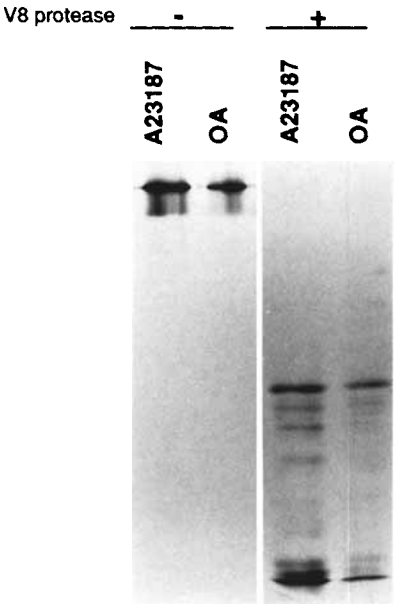

Fig. 4. Limited digestion with V8 protease of 78-kDa proteins induced by A23187 and OA in 9L cells. GRP78, induced by A23187 or by OA, was obtained from cells treated with either 7 μM of A23187 for 12 h or 200 nM OA for 7 h, and then labeled with [^{35}S]methionine for 1 h. The radioactive protein bands, identified by SDS-polyacrylamide gels, were cut from the gels and subjected to limited proteolysis with 100 ng of *Staphylococcus aureus* V8 protease and then analyzed using 14.5% SDS-polyacrylamide gel electrophoresis. Following electrophoresis, the gel was processed for autoradiography and the autoradiogram is shown.

Effects of Actinomycin D and Cycloheximide on GRP78 Synthesis Induced by OA

Induction of GRP78 synthesis by most inducers was regulated at the transcriptional level and required de novo protein synthesis. To examine the role of de novo protein synthesis in OA-induced GRP78 synthesis, 9L cells were pretreated with cycloheximide for 30 min to block new protein synthesis prior to addition of OA. For comparison, cells were also treated with 7 μM A23187 in parallel experiments. The cells were harvested after 7 h of incubation and 1 h of labeling, and total cellular proteins were analyzed by SDS-PAGE. Figure 8 shows that GRP78 synthesis was reduced to basal level in the presence of cycloheximide in OA- and A23187-induced cells. Meanwhile, we also examined the effects of actinomycin D on OA- and A23187-induced GRP78 synthesis, and found that the induction of GRP78 synthesis was inhibited in the presence of actinomycin D. These results indicated that OA-induced GRP78 synthesis was regulated at the transcriptional level and its mediation required de novo protein synthesis.

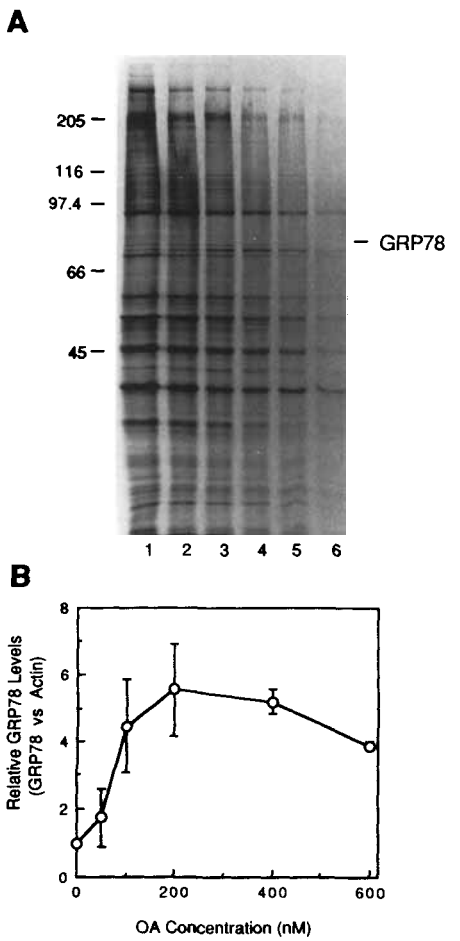

Fig. 5. Concentration-dependent effect of OA on the induction of GRP78 synthesis in 9L cells. Cells were treated with OA at the concentrations as indicated for 7 h and labeled with [^{35}S]methionine for 1 h. The treated cells were lyzed with SDS sample buffer and the protein samples were analyzed by 10% SDS-PAGE. Following electrophoresis, the gels were processed for autoradiography (**A**) in which the relative levels of GRP78 were determined by densitometry (**B**). Background levels of optical density were subtracted and the amounts of GRP78 were presented as O.D. units calculated from the peak areas. Lane 1: control; lanes 2–6: cells were treated with 50, 100, 200, 400, and 600 nM OA, respectively.

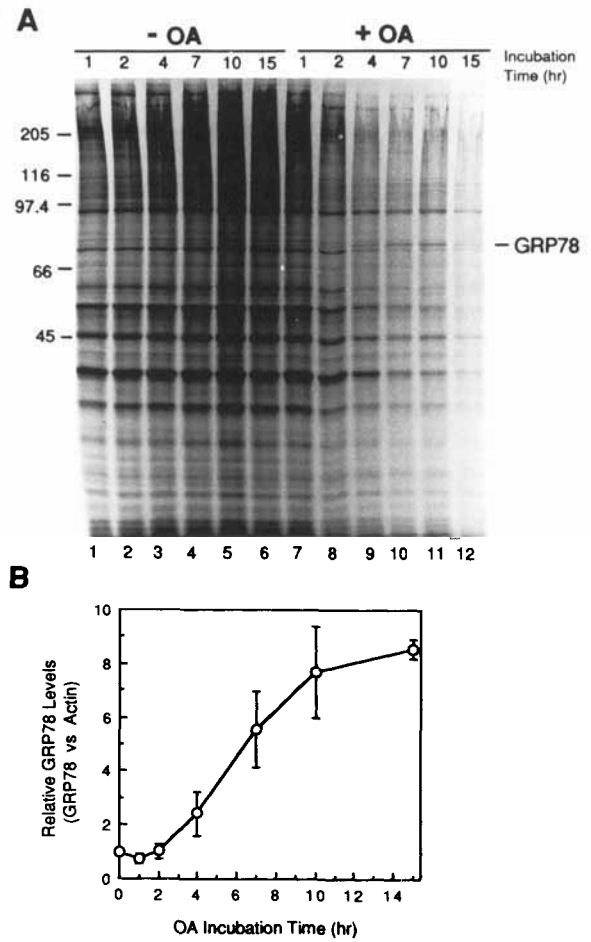

Fig. 6. Time-dependent effect of OA on the induction of GRP78 synthesis in 9L cells. Cells were treated with 200 nM OA for various incubation durations as indicated. Other experimental conditions were as described in the legend to Figure 4. **A:** Autoradiography of the GRP78 synthesis after treatments with OA. **B:** Quantitation of the relative levels of GRP78 by densitometry.

Effect of OA on Glycosylation of Proteins

Since perturbation in protein glycosylation may be one of the common stimuli involved in transcriptional activation of GRP78, we investigated whether OA treatment would have the similar effect. It was found that protein glycosylation was blocked in OA-treated cells and this process was also dose-dependent (Fig. 9). The relative rates of radioactive fucose incorporation were dropped to about 20% and 25% of the control levels when cells were subjected to 600 nM OA for 7 h and 200 nM OA for 10 h, respectively. The above results demonstrated that OA could inhibit glycosylation of proteins, supporting the notion that inhibition of protein glycosylation may correlate with induction of GRP78 synthesis.

DISCUSSION

In the present study, we have demonstrated that OA suppressed synthesis of general cellular proteins and induced synthesis of GRP78 in 9L rat brain tumor cells. This provided the first example that OA, a widely used tool to unravel cellular metabolic processes mediated by protein phosphorylation/dephosphorylation, can elicit stress response. Moreover, we further identified that GRP78 synthesis induced by OA was dose-dependent and virtually reversible, that GRP78

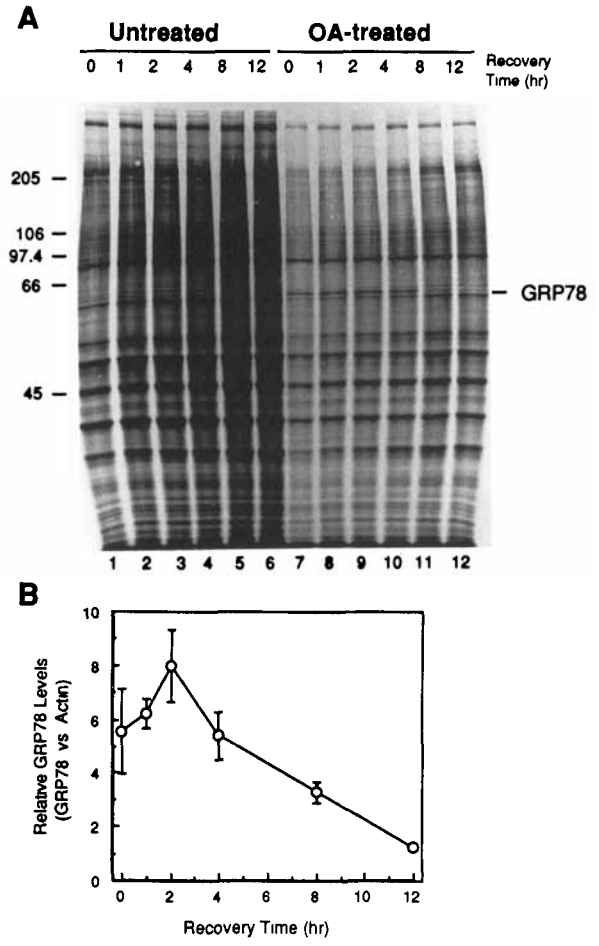

Fig. 7. Time-course decay of the level of OA-induced GRP78 after removal of OA from treated 9L cells Cells were treated with 200 nM OA for 7 h, washed, and allowed to recover under normal growing conditions for various durations as indicated (**lanes 7–12**). The cells were labeled with [^{35}S]methionine for 1 h and then processed as described in the legend to Figure 4 Untreated cells were also labeled and processed simultaneously (**lanes 1–6**). **A:** Autoradiograph of GRP78 synthesis after treatment with OA **B:** Quantitation of the relative levels were determined by densitometry

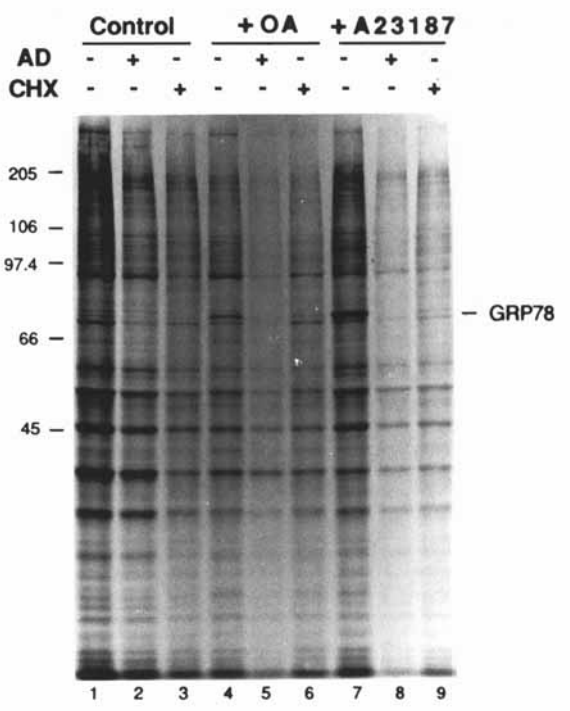

Fig. 8. Effects of actinomycin D and cycloheximide on OA-induced GRP78 synthesis in 9L cells Cells were pretreated with 0 1 μM actinomycin D or 50 μg/ml cycloheximide for 30 min prior to the addition of 200 nM OA, and then harvested after 7 h of incubation and 1 h of labeling For comparisons, cells were also pretreated with 0 1 μM actinomycin D or 50 μg/ml cycloheximide and further incubated with 7 μM A23187 for 7 h The cell lysates were analyzed by SDS-PAGE and processed for autoradiography **Lane 1:** Untreated control cells, **lane 2:** 0 1 μM actinomycin D was added for 7 5 h, **lane 3:** 50 μg/ml cycloheximide was added for 7 5 h, **lane 4:** 200 nM OA was added for 7 h, **lane 5:** 0 1 μM actinomycin D was added for 0 5 h prior to the addition of 200 nM OA for further incubation for 7 h, **lane 6:** 50 μg/ml cycloheximide was added for 7 5 h, **lane 7:** 7 μM A23187 was added for 7 h, **lane 8:** 0 1 μM actinomycin D was added for 0 5 h prior to the addition of 7 μM A23187 for further incubation for 7 h, **lane 9:** 50 mg/ml cycloheximide was added for 0 5 h prior to the addition of 7 μM A23187 for further incubation for 7 h

synthesis induced by OA was regulated at the transcriptional level and its initiation required newly synthesized proteins, and that OA suppressed protein glycosylation.

The effects of OA on general protein synthesis appear to be complicated. It has been shown that PP1 and PP2A are involved in positive and negative regulation of protein synthesis at the translational level [for review, see Hershey, 1989]. PP1 dephosphorylates the ribosomal protein S6 while PP2A dephosphorylates S6 kinase [Ballou et al., 1988a,b; Olivier et al., 1988; Jeno et al., 1988]; both of these events are thought to result in reduction of protein synthesis. Thus, it would be expected that inhibition of PP1 and PP2A by OA would enhance protein synthesis. However, since both PP1 and PP2A dephosphorylate and hence activate the α subunit of initiation factor 2 [Hershey, 1989], it might be expected that OA could inhibit protein synthesis. Furthermore, it has been demonstrated that OA decreases the activity of elongation factor 2 in vitro, which would also result in the inhibition of protein synthesis [Redpath and Proud, 1989]. On the other hand, OA has been shown to induce hyperphosphorylation and aggregation of vimentin [Lee et al., 1992] and it was also suggested that inhibition of protein synthesis by

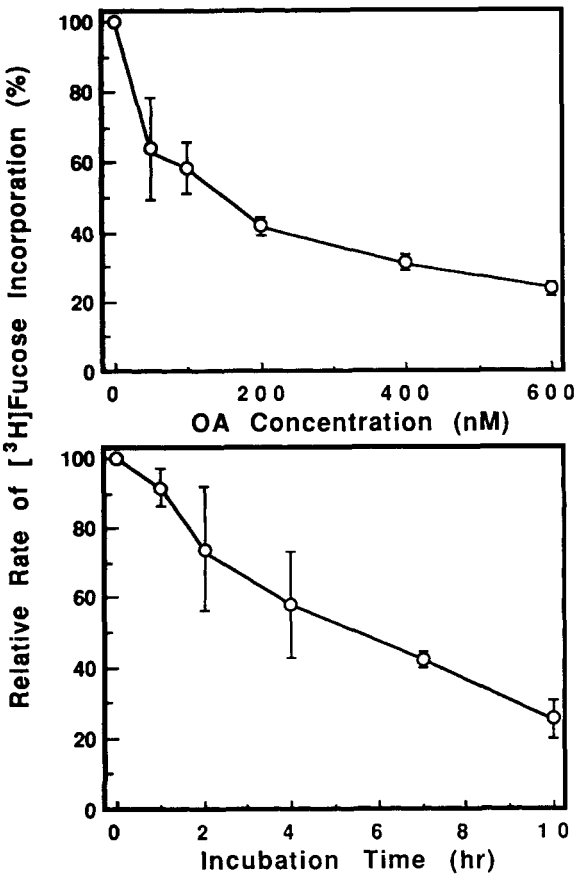

Fig. 9. Effect of OA on protein glycosylation in 9L cells Cells were treated with various concentrations of OA for various incubation durations as indicated and then labeled with [6-^{3}H]fucose for 1 h in the absence of OA After labeling, the relative rate of protein glycosylation was monitored by [6-^{3}H]fucose incorporation The data are from three independent experiments

heat shock might be associated with aggregation of vimentin filaments [Biessmann et al., 1982]. This implied that OA-induced inhibition of protein synthesis may be related to the aggregation of vimentin filaments. Since OA causes a decrease in protein synthesis, the results indicate that the net effect on translation is inhibitory.

GRP78 is the most prominent member of the GRP family and localizes in the lumen of the ER [Bole et al., 1986; Munro and Pelham, 1986]. Many potent inducers for GRP78 have been identified, such as 2-deoxyglucose, which causes depletion of glucose [Watowich and Morimoto, 1988], calcium ionophore A23187, which perturbs intracellular calcium ion concentration [Resendez et al., 1986; Drummond et al., 1987], tunicamycin, which blocks N-linked glycosylation [Watowich and Morimoto, 1988], and brefeldin A, which inhibits protein transport from the ER to the Golgi apparatus [Liu et al., 1992]. In our present study, the most effective dose for the induction of GRP78 synthesis was 200 nM OA for 7 h, and the initiation of GRP78 induction was observed at 4 h after the addition of OA. Moreover, OA-induced GRP78 synthesis was aborted in cycloheximide-treated cells, suggesting that newly synthesized proteins were required for the transcriptional activation by OA. The results suggested that induction of GRP78 synthesis may be a secondary response, implying that the new synthesis of one or more protein factor(s) are required to stimulate the increase in GRP78 synthesis, hence the delay in GRP78 activation. Furthermore, OA-induced GRP78 synthesis is inhibited in the presence of actinomycin D, suggesting that the process is regulated at the transcriptional level. The observation is consistent with that of other GRP78 inducers [Watowich and Morimoto, 1988; Resendez et al., 1986; Wooden et al., 1991]. However, recent evidence demonstrates that induction of GRP78 synthesis regulated by brefeldin A utilized both transcriptional and post-transcriptional mechanisms, depending on the cell types [Liu et al., 1992]. We do not exclude the possibility that expression of GRP78 gene induced by OA may also be modulated at the post-transcriptional level.

It has been suggested that the presence of malfolded and underglycosylated proteins in the ER is the primary signal for induction of GRP78 [Kozutsumi et al., 1988; Liu et al., 1992]. Recently, brefeldin A, a blocker of protein transport from the ER to the Golgi apparatus [Misumi et al., 1986; Fujiwara et al., 1988], was shown to induce GRP78 synthesis [Liu et al., 1992]. It is suggested that brefeldin A introduced physiological stress to the ER by disrupting the normal protein efflux from the ER to the Golgi apparatus and resulted in the accumulation of non-ER-resident proteins in the lumen of the ER [Wang et al., 1991]. Most recently, OA was shown to inhibit transport of newly synthesized proteins and the process involved protein phosphorylation in mammalian cells [Lucocq et al., 1991; Davidson et al., 1992]. In Davidson's study, several lines of evidence indicate that protein phosphorylation, rather than a lack of protein dephosphorylation per se, is actually responsible for the inhibition of protein transport. This implied that in addition to inhibition of protein phosphatases, protein kinase(s) is also involved in the process. It is also suggested that an early

event in protein transport, related to the formation or budding of vesicles, is inhibited by protein phosphorylation [Davidson et al., 1992]. Furthermore, it has been suggested that organelles move along microtubules but must associate with intermediate filaments in order to maintain proper positions [Eckert, 1986]. Therefore, it is possible that OA may affect protein transport from the ER to the Golgi apparatus via hyperphosphorylation and disruption of vimentin, again resulting in the accumulation of malfolded proteins in the ER.

On the basis of the above evidence that OA blocked protein transport from the ER to the Golgi apparatus, the processing of protein glycosylation was correspondingly inhibited in the Golgi apparatus. On the other hand, the ER is a major organelle for calcium ion storage and it has been reported that calcium ionophores A23187 blocked protein glycosylation and induced GRP78 synthesis by disrupting the calcium ion pool [Chang et al., 1987; Lee, 1987], and that high calcium ion concentration seemed to be necessary for the processing of glycoproteins in this organelle [Lodish and Kong, 1990]. Furthermore, OA was shown to be an ionophore-like substance [Shibata et al., 1982]. In combination with these studies, it is likely that OA may interact with the ER causing calcium ion release which would suppress protein glycosylation. In fact, the process of protein glycosylation is very complicated; which step of the glycosylation process is affected by OA remains to be elucidated.

In summary, our plausible explanation about the signal of OA-induced GRP78 synthesis is as follows. OA directly inhibited protein phosphatases which led to the "activation" of protein kinases. Concomitant with the activation of these kinases, the level of hyperphosphorylation of vimentin was increased and the formation or budding of vesicles was inhibited, resulting in the block of protein transport. Subsequently, the processing of protein glycosylation was inhibited in the Golgi apparatus and non-resident/malfunctioned proteins accumulated within the ER, complexed stably with GRP78, and sequentially induced the transcription of GRP78 gene. Furthermore, the reversibility of this process indicated that once the cells are relieved of stress, the non-resident/malfunctioned proteins can quickly dissociate themselves from GRP78 and recover to continue their normal processing, including refolding into proper conformation and transport to the Golgi apparatus.

Taken together, it is apparent that the cellular metabolic processes including protein translation, protein phosphorylation, protein transport, organization of intermediate filaments (or cytoskeleton), gene expression, and calcium ion concentration are interconnected. Further dissection of the present experimental system may enable researchers to unravel these interconnections.

ACKNOWLEDGMENTS

This work was supported by grants from the National Science Council of R.O.C. to Y.-K.L. (NSC 81-0211-B-007-04, NSC 82-0211-B-007-04). The authors thank Dr. S.-D.Y. and Dr. K.-Y.J. for their valuable discussions.

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