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Modulation of Protein Phosphorylation and Stress Protein Expression by Okadaic Acid on Heat Shock Cells

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Abstract We have demonstrated that pretreatment but not post-treatment with okadaic acid (OA) can aggravate cytotoxicity as well as alter the kinetics of stress protein expression and protein phosphorylation in heat shocked cells. Compared to heat shock, cells recovering from 1 hr pretreatment of OA at 200 nM and cotreated with heat shock at 45°C for the last 15 min of incubation (OA → HS treatment) exhibited enhanced induction of heat shock proteins (HSPs) 70 and 110. In addition to enhanced expression, the attenuation of HSC70 and HSP90 after the induction peaks was also delayed in OA → HS-treated cells. The above treatment also resulted in the rapid induction of the 78 kDa glucose-regulated protein (GRP78), which expression remained constant in cells recovering from treatment with 200 nM OA for 1 hr, heat shocked at 45°C for 15 min, or in combined treatment in reversed order (HS → OA treatment). Enhanced phosphorylation of vimentin and proteins with molecular weights of 65, 40, and 33 kDa and decreased phosphorylation of a protein with a molecular weight of 29 kDa were also observed in cells recovering from OA → HS treatment. Again, protein phosphorylation in cells recovering from HS → OA treatment did not differ from those in cells treated only with heat shock. Since the alteration in the kinetics of stress protein expression and protein phosphorylation was tightly correlated, we concluded that there is a critical link between induction of the stress proteins and phosphorylation of specific proteins. Furthermore, the rapid induction of GRP78 under the experimental condition offered a novel avenue for studying the regulation of its expression.

Key words: glucose-regulated proteins, heat shock proteins, heat shock, okadaic acid, protein phosphorylation, vimentin

HSPs are a small set of proteins whose synthesis is enhanced or induced in cells coping with supraoptimal temperature [Lindquist and Craig, 1988; Schlesinger, 1990; Becker and Craig, 1994], whereas GRPs are proteins whose synthesis is enhanced by glucose deprivation [Munro and Pelham, 1986]. Together, they are frequently referred to as the stress proteins. Depending on the labeling methods, as well as based on their apparent molecular weights, four major families are commonly detected in mammalian cells, i.e., HSP110, 90, 70, and 32. The members of the HSP70 family are the most extensively studied and they are further distinguished by their levels of basal expression and subcellular localizations. In rodent cells, commonly detected HSP70s consist of two members residing in the cytoplasm, known, respectively, as HSP70 and HSC70 [Hightower and White, 1981; Lee et al., 1992a]. HSP70 is highly heat inducible and hardly detectable under normal conditions, whereas HSC70 is slightly heat inducible, constitutively expressed, and found at higher levels in growing cells than in resting cells [Pelham, 1986]. In addition, GRP78, which is located in the endoplasmic reticulum and exhibits a high level of basal expression, is also classified as a member of this family based on amino acid sequence homology [Munro and Pelham, 1986]. Interestingly, with only a few exceptions, induction of HSP70s and GRP78 are mutually exclusive [Watowich and Morimoto, 1988]. Furthermore, discriminatory induction of individual stress proteins is observed in different cell types under different stress conditions [Lai et al., 1993a]; therefore, differential transactivation of stress genes is apparently governed by distinct but overlapping pathways.

Transactivation of *hsp70* and *hsc70* genes during heat shock or heavy metal treatment is mediated by the activation of heat shock factor 1 (HSF1), a process that involves phosphorylation of the transcription factor [Baler et al., 1993;

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Morimoto, 1993; Sarge et al., 1993]. On the other hand, active transcription factors for CCAAT and cAMP-responsive elements are responsible for basal level expression and stress inducibility of the *grp78* promoter [Wooden et al., 1991; Alexandre et al., 1991; Li et al., 1993] which is further positively regulated by a transcription factor with a molecular mass of 70 kDa (p70CORE) [Li et al., 1994]. Although the exact mode of regulation of the CCAAT binding proteins and the p70CORE has not been fully elucidated, it has been shown that the major mode of regulation of cAMP-responsive element binding protein activity is phosphorylation of its activation domain [Gonalez et al., 1989]. Taken together, most of the stress genes, at least the members of the HSP70 family, are apparently upregulated by protein phosphorylation.

Reversible protein phosphorylation plays a central role in regulating cellular activities, and enhanced phosphorylation of a number of specific proteins has been identified in stressed cells [Duncan and Hershey, 1984, 1989; Maher and Pasquale, 1989; Lee et al., 1991; Lai et al., 1993a; Cheng and Lai, 1994]. In particular, enhanced phosphorylation of vimentin intermediate filament and a 65 kDa protein is commonly observed in cells subjected to heat shock and a variety of chemical stresses. We have suggested that these processes may be related to the induction of HSPs [Lee et al., 1991; Lai et al., 1993a; Cheng and Lai, 1994]. However, the exact molecular events underlying changes in protein phosphorylation and the initiation of stress response remains to be elucidated.

Okadaic acid (OA), a potent inhibitor of protein phosphatases 1 and 2A, has been widely used as a tool to study protein phosphorylation in relation to the regulations of cellular activities [Cohen, 1990]. Being cytotoxic, sublethal doses of OA must impose a certain degree of stress on cells in its presence. During the inflicted disturbance of protein phosphorylation in OA-treated cells, intermediate filament proteins are found to be rapidly hyperphosphorylated [Yatsunami et al., 1991; Lee et al., 1992; Lai et al., 1993b]. Furthermore, it has been demonstrated that OA can markedly enhance the synthesis of GRP78 [Hou et al., 1993] and potentiate the heat-induced *hsp70* promoter activity [Chang et al., 1993]. In this study, the effects of combined OA and heat shock treatments on the induced synthesis of stress proteins were investigated and the possible involvement of protein phosphorylation in differential induction of the stress genes is discussed.

MATERIALS AND METHODS

Materials

OA 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 (Grand Island, NY). ^{35}S-methionine (specific activity >800 Ci/mmole) was obtained from Amersham (Buckinghamshire, England), and ^{32}P-orthophosphate ($^{32}P_i$, specific activity 8,500–9,120 Ci/mmole) was from New England Nuclear (Boston, MA). Chemicals for electrophoresis were purchased from BioRad (Richmond, CA). Other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO).

Cell Culture and Determination of Cell Survival

The 9L rat brain tumor (RBT) cells, originating from a rat gliosarcoma, were a generous gift from Dr. M.L. Rosenblum, University of California at San Francisco [Weizsaecker et al., 1981]. Routine maintenance of cells was performed as previously described [Lee et al., 1992; Hou et al., 1993]. Prior to each experiment, stock cells were seeded in 25 cm^2 flasks or six-well plates at a density of 4–6 × 10^4 cells per cm^2. Exponentially growing cells at 80–90% confluency were used. Cell survivals were determined by colony formation technique. Briefly, cells were trypsinized, serially diluted, and counted with a hemocytometer. The plating efficiency was determined by seeding in duplicated dishes at appropriate density of cells per 60 mm dish containing 4 ml of culture medium. The plated cells were then allowed to grow for 8–10 days. Subsequently, the samples were rinsed with phosphate-buffered saline (PBS), stained with 1.5% Methylene blue in PBS, drained, and rinsed gently under running water. The colonies formed with more than 50 cells were scored. The surviving fraction of the treated cells was referred to as the fraction of plating efficiency relative to that of untreated controls.

Heat Shock and OA Treatment

In the heating experiments, the flasks or plates were sealed with Parafilm and submerged in a water bath preset at 45 ± 0.1°C. The designated temperature of the medium in the heating protocol was reached within 3 min and the time required for equilibrium was included in the treatment duration. For OA treatment, stock solution of OA was diluted with culture medium to the specified concentrations before adding to the cells, which were treated at 37°C for 1 hr. To study the combined effects of OA and heat shock, cells were sequentially treated with OA and heat shock in alternated sequences. In OA → HS treatment, cells were incubated with OA for 45 min before they were heat shocked for 15 min, which was performed in the presence of OA. Alternatively, cells were incubated with OA for 1 hr, washed, and then heat shocked for 15 min (results obtained were indistinguishable between these two experimental protocols). In HS → OA treatment, cells were heat shocked at 45°C for 15 min and then incubated with OA at 37°C for 1 hr.

Isotopic Labeling, Gel Electrophoresis, and Immunoblot Analysis

Synthesis of stress proteins in the treated cells was revealed by ^{35}S-methionine labeling. After treatments, the cells were washed twice and incubated with fresh medium at 37°C for different periods (which were defined as the recovery durations) before labeling with 20 μCi of ^{35}S-methionine in 1 ml of medium for 1 hr. After labeling, the cells were washed, lysed, and the cell lysates subjected to electrophoresis as previously described [Hou et al., 1993; Lai et al., 1993]. Sodium dodecylsulfate–polyacrylamide gel

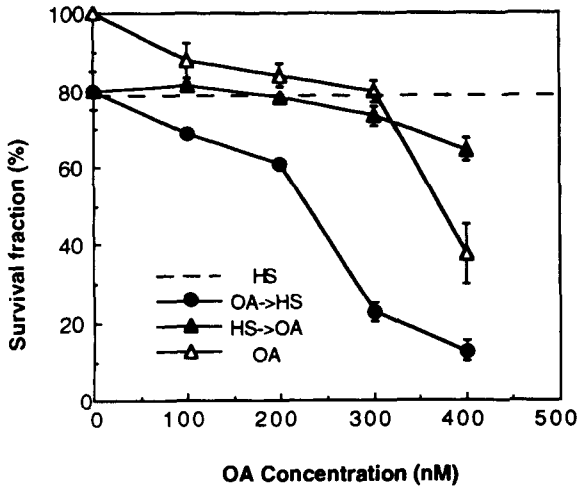

Fig. 1. Cell survival of 9L cells treated with heat shock and OA. Cells were treated with 0–400 nM OA or at 45°C for 15 min and with 0–400 nM OA for 1 hr in reciprocal orders. After treatment, changes in cell survival were monitored by colony formation assays. Data represent the means ± SD of three independent experiments.

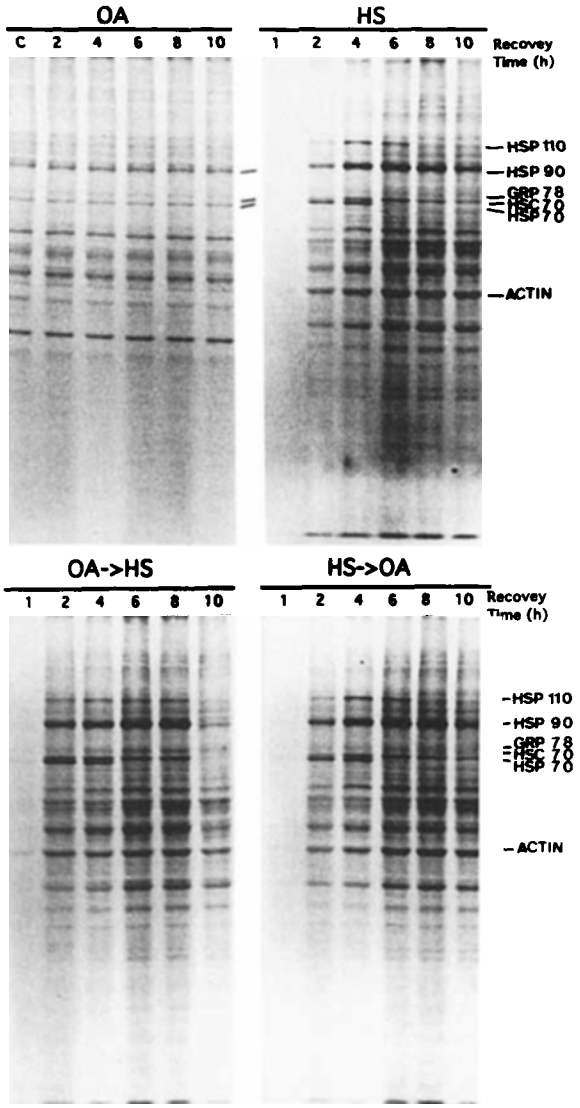

Fig. 2. Induction of stress protein synthesis in 9L cells treated with heat shock and OA. Cells were treated with 200 nM OA (OA), heat shocked at 45°C for 15 min (HS), or treated at 45°C for 15 min and with 200 nM OA for 1 hr in reciprocal orders (HS → OA and OA → HS, respectively). After treatment, the cells were allowed to recover under normal culture conditions for 0–8 hr and labeled with ^{35}S-methionine for 1 hr before lysing. Equal amounts of cell lysates were electrophoresed on SDS-PAGE followed by autoradiography. Molecular weight standards are shown at the left (in kDa). Stress proteins and actin are marked at the right.

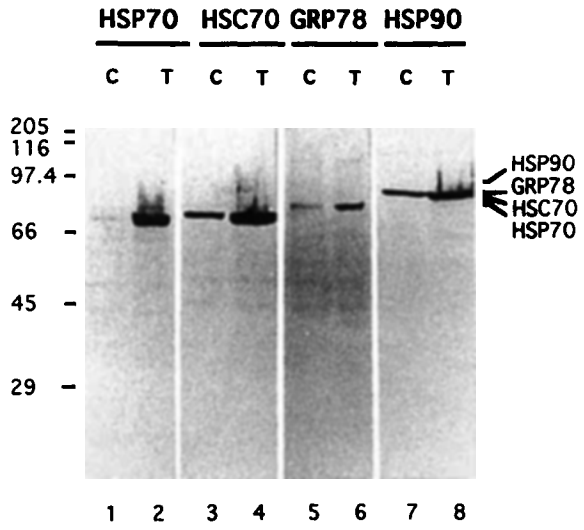

Fig. 3. Western blot analysis for the identification of the four stress proteins, HSP70, HSC70, GRP78, and HSP90, in 9L cells. Cells were treated with 200 nM OA for 1 hr followed by 45°C heat shock for 15 min. After treatment, the cells were allowed to recovered under normal culture conditions for 8 hr before being lysed. Cell lysates were separated on 10% SDS-polyacrylamide gels using a minigel apparatus. After electrophoresis, proteins were transferred to nitrocellulose membrane and analyzed by immunoblot assay. Molecular weight standards are shown at the left (in kDa). Stress proteins are marked at the right. C, untreated cells; T, treated cells.

electrophoresis was performed according to the method of Laemmli [1970], and followed by autoradiography. Bands of interest on the X-ray films were quantitated by densitometric scanning in 2-D mode (Molecular Dynamics). The relative synthesis rate of each stress protein was referred to as the band intensity relative to that of actin, which served as an internal control. For immunoblot analysis, cell lysates were resolved using a mini-gel apparatus (Bio-Rad). After electrophoresis, the proteins were electro-transferred onto a nitrocellulose membrane (Hybond-C super, Amersham) and probed with antibodies against HSP70, HSC70, GRP78, and HSP90, separately. Procedures for immunoreactions and protein visualization were performed as described [Lee et al., 1991, 1992a].

Protein phosphorylation in the treated cells was studied by in vivo ^{32}P-phosphate labeling. Cells were prelabeled with 1 mCi of ^{32}P-phosphate in 1 ml labeling medium (phosphate-free DMEM containing 10% FBS) before treatment, which was performed in the presence of ^{32}P-phosphate. Cells were harvested at the end of the treatment and the total labeling time was 2 hr. Visualization of the total and alkali-resistant phosphoproteins was performed as previously described [Cheng and Lai, 1994]. Bands of interest were quantitated by densitometric scanning, and alterations of the phosphorylation levels of the phosphoproteins were represented by changes in band intensity relative to that of phosphoactin.

RESULTS

Cell Survival of 9L RBT Cells Treated With OA and Heat Shock

OA was found to be cytotoxic in a concentration-dependent manner (Fig. 1). After 1 hr of OA treatment at 300 nM, the cell survival rate decreased to 0.80 ± 0.03, which was similar to that of heat shocked at 45°C for 15 min (SF = 0.82 ± 0.05). Subsequently, cells were sequentially treated with OA and heat shocked in reciprocal orders. If the cells were heat shocked and then treated with various concentration of OA (HS → OA treatment), cell survivals decreased in a concentration-dependent manner. However, the survival curve obtained does not significantly differ from that of the OA-treated or heat shocked cells, providing the concentration of OA was kept under 300 nM. In contrast, cells were much more susceptible to the combined treatment if the cells were first incubated with OA before they were subjected to the heat shock treatment (OA → HS treatment). These data showed that OA was highly cytotoxic at relative high concentrations (above 300 nM) and that OA, if added before the heat shock treatment, could potentiate the cell-killing effect of heat shock. Most importantly, since the survival curve of the OA → HS treatments differed greatly from that of the HS → OA treatments, the results demonstrated that the potentiation of the cytotoxic effect of heat shock could be obtained by pre-treatment, but not post-treatment, with OA.

Induction of Stress Protein Synthesis in OA-Treated and Heat Shocked 9L RBT Cells

The kinetics of stress protein synthesis in cells recovering from OA and heat shock treatments were monitored by ^{35}S-methionine incorporation. Treatments of the cells were carried out as described above, except that the concentration of OA was kept at 200 nM to avoid excessive cell killing. No stress protein was synthesized in cells recovering from the treatment with 200 nM OA for 1 hr. On the other hand,

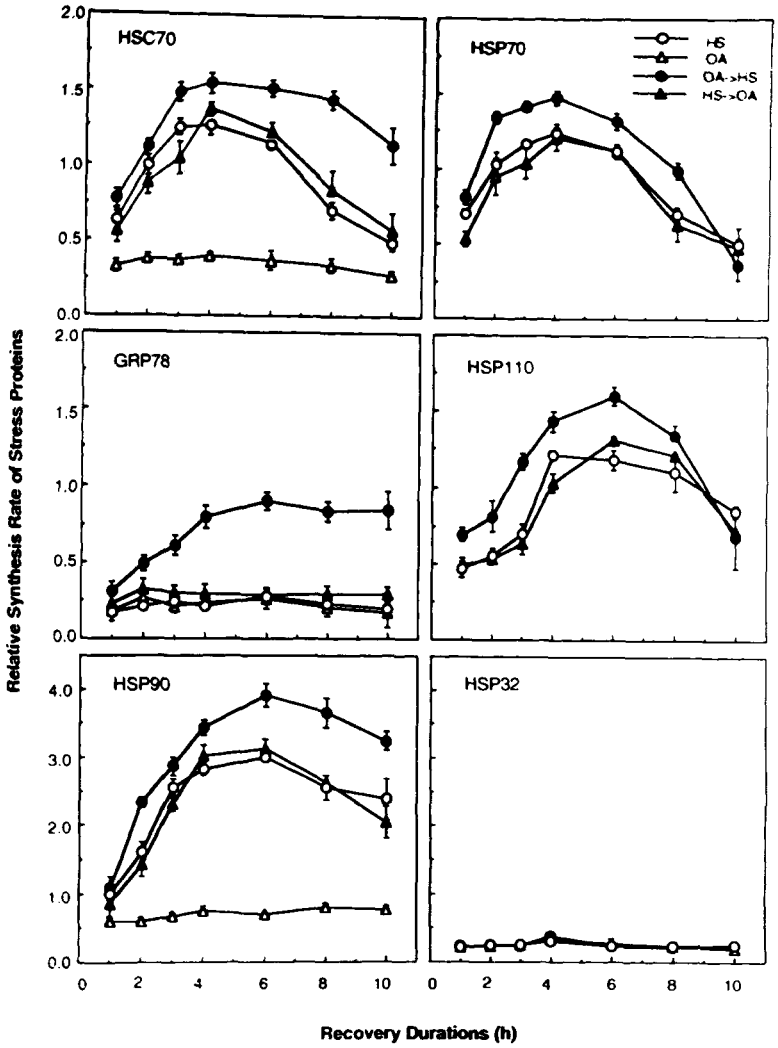

Fig. 4. Relative rate of stress protein synthesis in 9L cells treated with heat shock and OA. Autoradiographs as shown in Figure 2 were scanned and the relative protein synthesis rate of each stress protein was referred to as the fraction of band intensity relative to that of actin (internal control). The data are means ± SD from three independent experiments.

enhanced synthesis of HSC70 and HSP90 as well as induction of HSP70 and HSP110 were detected in cells recovering from heat shock (Fig. 2). In cells recovering from HS → OA treatments, the expression of GRP78 was greatly enhanced, in addition to the induction of the above HSPs (Fig. 2). Besides their apparent molecular weights, the stress proteins HSP70, HSC70, GRP78, and HSP90 were further identified by Western blot analysis (Fig. 3).

Quantitative analysis of the autoradiographs further revealed the differences in the kinetics of the stress protein synthesis resulting from the above treatments. As shown in Figure 4, the synthesis of the inducible stress proteins (i.e., HSP32, 70, and 110) was not detected and the synthesis of the constitutive stress proteins (i.e., HSC70, HSP70, and GRP78) was not affected in OA-treated cells. It was also found that the synthesis of HSP32 was barely detected in heat shocked and HS → OA-treated cells, but its expression remained relatively constant during the recovery period. Besides, the expression of HSC70, HSP70, HSP90, and HSP110 (but not GRP78) was enhanced in heat shocked and HS → OA-treated cells, and the kinetics of expression of these HSPs were virtually identical between these two treatments. Enhanced expression of these HSPs was observed as early as 2 hr into treatment, attained maxima at 4–6 hr after treatment, and then subsided. However, the kinetics of stress protein induction in OA → HS-

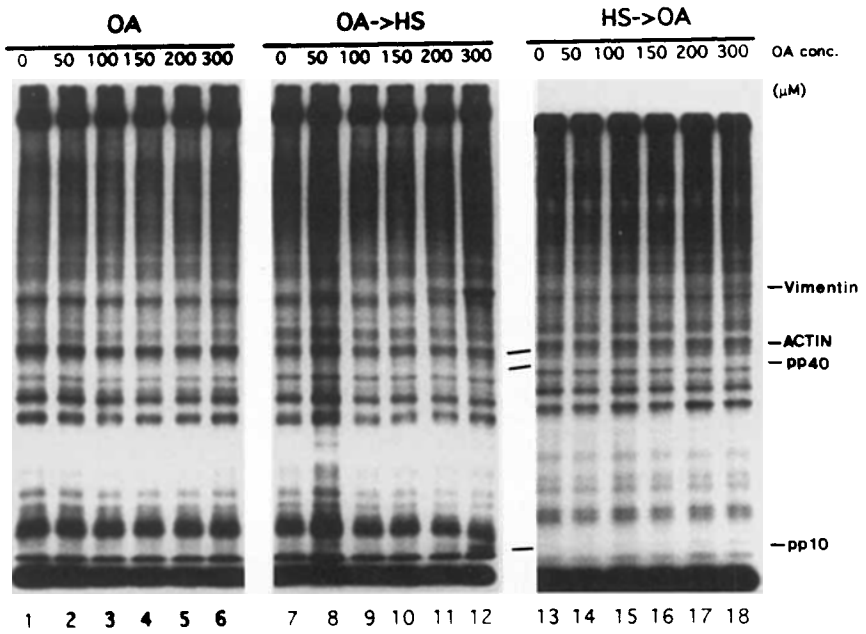

Fig. 5. Changes of phosphorylation levels of intracellular phosphoproteins in 9L cells treated with OA and heat shock. Cells were prelabeled with ^{32}P-orthophosphate and then treated with 0–300 nM OA for 1 hr (control). Additionally, cells were heat shocked at 45°C during the last 15 min of the OA treatment (OA → HS) or prior to incubation with OA (HS → OA). For all treatments, cells were labeled for a total of 2 hr before harvest. Immediately after treatment and labeling, the cells were lysed and equal amounts of cell lysates were electrophoresed on SDS-PAGE followed by autoradiography. Vimentin and a phosphoprotein with a molecular weight of 40 kDa (pp40) are marked at the right.

treated cells differed significantly from the above treatments. The expression of the HSPs was further upregulated and the levels of maximal expression of the HSPs were generally higher than those of heat shocked or HS → OA-treated cells. Although the downregulation of HSP70 and HSP110 (the inducible HSPs) after the peak of induced synthesis in OA → HS-treated cells was similar to those of heat shocked or HS → OA-treated cells, enhanced expression of HSC70 and HSP90 (the constitutive HSPs) was sustained further into the recovery period. Additionally, although the expression of GRP78 remained to be constant in heat shocked, OA- or HS → OA-treated cells, it was unexpectedly upregulated in the OA → HS-treated cells. Most importantly, the enhanced synthesis of GRP78 in cells recovering from OA → HS treatment was rather rapid. Upregulation of GRP78 was detected almost immediately after the treatment, attained a plateau within 4–6 hr, and lasted through at least 10 hr of recovery (Fig. 4).

Modulation of Protein Phosphorylation in 9L Cells Immediately After Combined OA and Heat Shock Treatments

Alteration of protein phosphorylation was studied in cells sequentially treated with OA and heat shock in reciprocal orders. Cells were prelabeled with ^{32}P-phosphate and then subjected to the treatments as described in the cell survival studies; the labeling period was extended to 2 hr, including the treatment duration. Immediately after labeling and treatment, cellular phosphoproteins were resolved by gel electrophoresis and visualized by autoradiography (Fig. 5). Overall, protein phosphorylation in heat shocked cells was slightly increased immediately after treatment but the general pattern remained the same (Fig. 5, compare lane 1 to lane 7). In OA-treated cells, the phosphorylation level of phosphovimentin was enhanced in a concentration-dependent manner and the incorporation of ^{32}P-phosphate in vimentin was increased threefold in cells treated with 300 nM OA for 1 hr, compared to that of the untreated cells (Fig. 6, top). Further enhancement of vimentin phosphorylation was observed in OA → HS-treated cells, and the process also occurred in a concentration-dependent manner (Fig. 6, top). In contrast, the phosphorylation levels of phosphovimentin remained constant in cells subjected to HS → OA treatments. In addition, the phosphorylation of two phosphoproteins with molecular weights of 40 and 10 kDa (pp40 and pp10) was drastically increased in cells under the OA → HS treatment

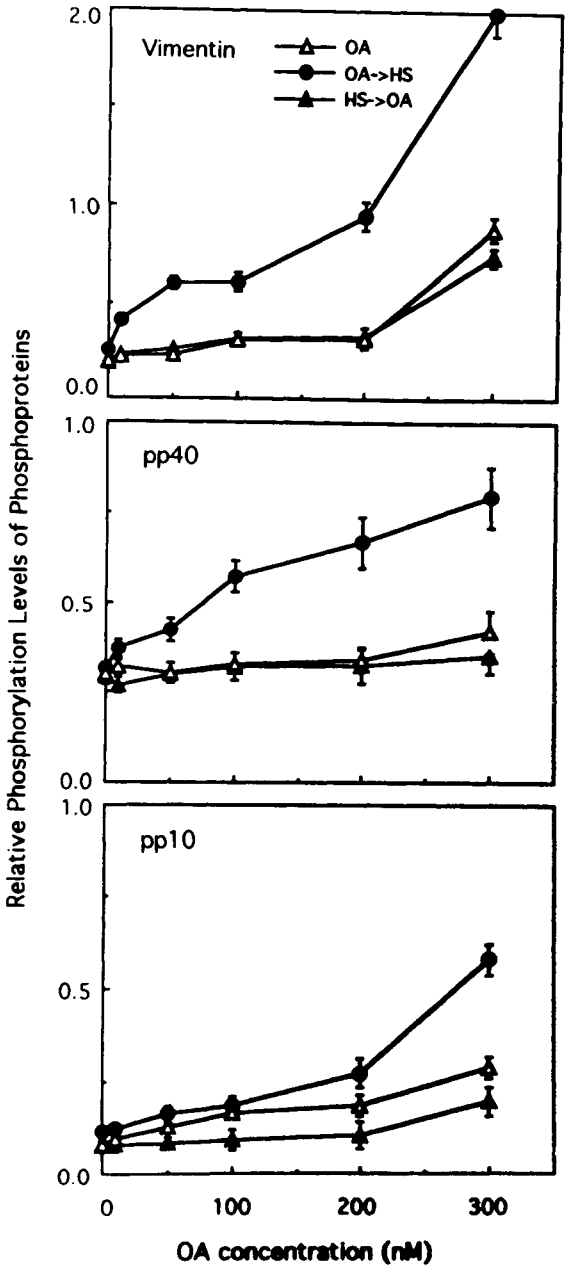

Fig. 6. Concentration-dependent effects of OA on phosphorylation of vimentin and pp40 in OA-treated and heat shocked 9L cells. Autoradiographs as shown in Figure 5 were scanned and the phosphorylation levels of phosphovimentin and pp40 were determined by densitometry. Levels of phosphorylation were presented as the sums of the pixel values after background subtraction. The data are means ± SD from three independent experiments.

but not under OA or HS → OA treatment (Fig. 6, middle).

Figure 7 shows the alkaline-resistant phosphoproteins in cells treated as described above. The phosphorylation levels of three phosphoproteins with molecular weights of 65, 33, and 29 kDa (pp65, pp33, and pp29, respectively) were significantly changed in cells treated with OA and heat shock. Increased phosphorylation of pp65 and pp33 was found in OA-treated cells in a concentration-dependent manner (Fig. 8, top and middle). Most interestingly, further increase of phosphorylation of pp65 and pp33 was observed in OA → HS-treated cells, but not in the HS → OA-treated cells (Fig. 8). On the other hand, phosphorylation of pp29 increased slightly in heat shocked cells, remained constant in OA-treated cells, and decreased in cells subjected to combined treatments (both of HS → OA and OA → HS) (Fig. 8, bottom).

DISCUSSION

The function of a stress response is to allow the stressed cells to cope with the physiological insults; thus, cell survival can be deemed a general indicator for the severity of the stressful conditions. The present studies demonstrate that heat shock–inflicted cell killing is potentiated by pretreatment, but not post-treatment, with OA. The mechanism whereby OA potentiates the cytotoxicity of heat shock is not known at this time. However, the inability of OA to act additively or synergistically with heat shock when the drug was added after the heat shock is consistent with the subsequent studies on the induction of stress proteins and the alteration of protein phosphorylation. The reciprocal sequential treatments of OA and heat shock thus provide particular insights in studying the roles of protein phosphorylation–dephosphorylation involved in the signal transduction pathway(s) of a heat shock response, as well as in the regulation of stress protein expression.

Similar to those cytotoxic studies, post-treatment incubation of the heat shocked cells with OA at nontoxic doses (200 nM, 1 hr) does not result in any apparent changes in the synthesis of HSPs and levels of protein phosphorylation compared to those in cells treated only with heat shock. We have demonstrated that heat shock, regardless of the presence and order of addition of OA, elicits the induction of the four major HSPs, including HSP110, 90, 70, and HSC70. The expression kinetics of these HSPs are significantly affected when the heat shocked cells were pretreated with OA. Furthermore, after the peaks of stress protein synthesis, the downregulation of the constitutive HSPs, i.e., HSC70 and HSP90, is particular delayed, whereas downregulation of the inducible HSPs, i.e., HSP70

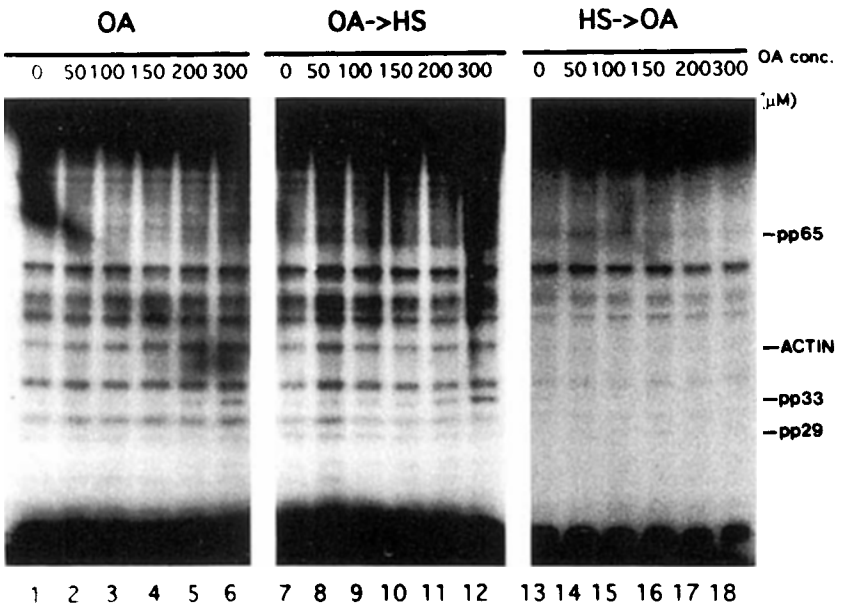

Fig. 7. Changes of phosphorylation levels of alkali-resistant phosphoproteins in 9L cells treated with OA and heat shock. Cells were prelabeled with ^{32}P-orthophosphate and then treated with 0–300 nM OA for 1 hr (control). Additionally, cells were heat shocked at 45°C during the last 15 min of the OA treatment *(OA → HS) or prior to incubation with* OA (HS → OA). For all treatments, cells were labeled for a total of 2 hr before harvest. Immediately after treatment and labeling, the cells were lysed and equal amounts of cell lysates were electrophoresed on SDS-PAGE. After electrophoresis, the gels were heated at 55°C in the presence of 2 M KOH for 1 hr and then processed for autoradiography. Alkali-resistant phosphoproteins with molecular weights of 65, 33, and 29 kDa (i.e., pp65, pp33, and pp29, respectively) are marked at the right.

and 90, is not affected. These observations clearly indicate that protein phosphorylation–dephosphorylation is involved in the transactivation as well as attenuation of the heat shock genes, and that the expression of constitutive HSPs and inducible HSPs is governed by distinct but overlapping mechanisms.

Protein phosphorylation has long been recognized to have important roles in signal transduction and gene expression. Changes in the phosphorylation level and thus the activity of phosphoproteins can be caused by changes in the relative activities of protein kinases and protein phosphatases, as well as changes in conformation of the substrate proteins. Alterations of phosphorylation of several phosphoproteins are detected in the present treatment protocols, and the results are consistent with the data on cell survivability and induction of stress proteins. That is, cells recovering from OA → HS, but not from HS → OA, treatment exhibit significant changes in phosphorylation of a number of proteins. We have previously shown that enhanced phosphorylation of vimentin and pp65 is a common phenomenon occurring simultaneously with the onset of a stress response elicited by heat shock and a variety of cytotoxic compounds; therefore, we have suggested that the processes may play an essential role in regulating stress genes [Lee et al., 1991, 1993; Lai et al., 1993a; Cheng and Lai, 1994]. The present studies, in addition, show that the phosphorylation levels of pp40, pp33, pp29, and pp10 are modulated by OA → HS treatment. Under the experimental conditions, pp40, pp33, and pp10 are phosphorylated but pp29 is dephosphorylated compared to those observed in the heat shocked cells. The data imply that the changes of the presumptive phosphorylation events of the treated cells are due to modulations of protein kinase(s) and/or phosphatase(s) by OA-sensitive dephosphorylation reactions. Furthermore, the changes in phosphorylation kinetics of the above phosphoproteins are correlated to the changes in stress protein induction. These observations lead us to conclude that the phosphorylation events are directly related to the regulation of the stress genes. In fact, induction of heat shock genes is shown to be mediated mainly by the HSF, which in turn may be regulated by phosphorylation [Sarge et al., 1993; Mivechi et al., 1994]. Using constructed plasmids containing reporter genes driven by *hsp70* promoter, it has been shown that OA is able to

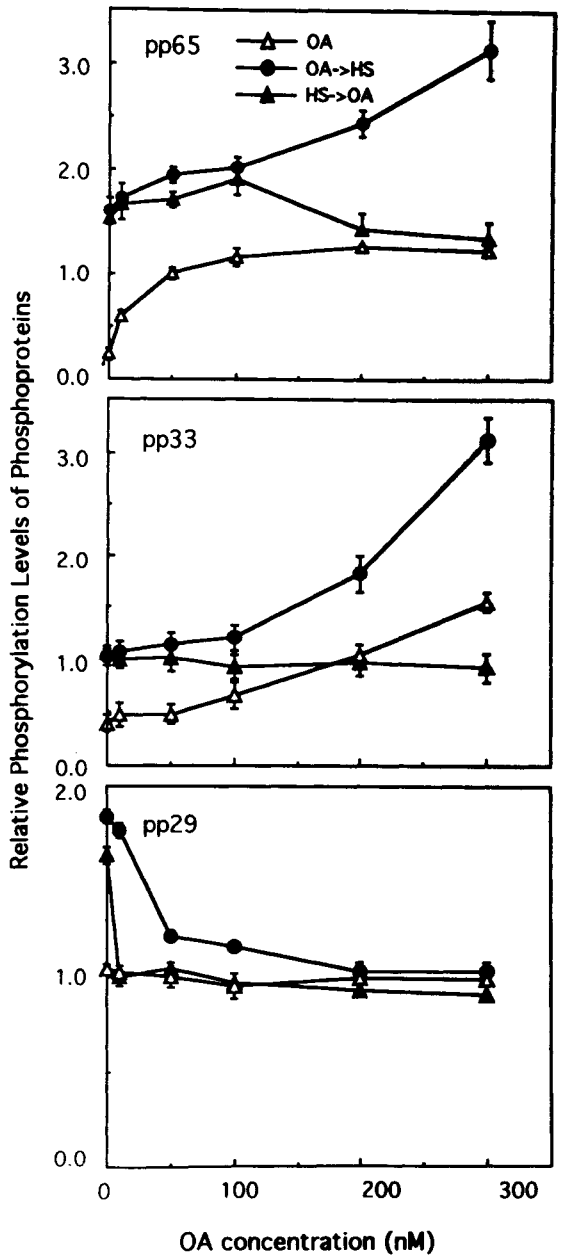

Fig. 8. Concentration-dependent effects of OA on phosphorylation of alkali-resistant phosphoproteins pp65, pp33, and pp29 in OA-treated and heat shocked 9L cells. Autoradiographs as shown in Figure 7 were scanned and the phosphorylation levels of pp65, pp33, and pp29 were determined by densitometry. Levels of phosphorylation were presented as the sums of the pixel values after background subtraction. The data are means ± SD from three independent experiments.

potentiate the heat-induced promoter activity [Chang et al., 1993; Mivechi, 1994]. It is logical to hypothesize that HSF is normally dephosphorylated by ser/thr phosphatases such as PP1 and PP2A which are inhibited by OA [Mivechi et al., 1994]. However, the data from the HS → OA treatment argue against this simple explanation. In all experiments, incubation of the cells with OA after heat shock results in effects that do not differ from those treated only with heat shock. The phosphorylation events required for further regulation of HSP expression in heat shocked cells must occur rather rapidly, and once initiated can no longer be affected, even in the presence of a potent phosphatase inhibitor. Alternatively, the effect of OA in HS → OA treatment may be inhibited or masked by heat shock via other unknown mechanisms. Nevertheless, our findings are in agreement with the notion that the heat-induced transcriptional activation of heat shock genes is associated with the phosphorylation of a component(s) of the transcription complex [Chang et al., 1993] in which HSF–HSE interaction is only one of them. Preliminary analysis of the available sequence data on the promoter regions of the heat shock genes indicates the existence of multiple and sometimes redundant putative regulatory elements. Further study on the functionality and cooperativity of the involved cis-acting elements and trans-acting protein factors is therefore warranted.

The rapid enhancement of GRP78 synthesis in cells recovering from OA → HS treatment is rather surprising and interesting. GRP78 has not been known to be induced by heat and its induction by OA cannot be detected after 1 hr of treatment at 200 nM. The expression of GRP78 is found to be enhanced by treatment with glucose deprivation [Munro and Pelham, 1986; Kozutsumi et al., 1988], 2-deoxyglucose [Winning et al., 1989], OA [Hou et al., 1993], A23187 [DeJong et al., 1986; Li et al., 1993], thapsigargin [Li et al., 1993], and brefeldin-A [Price et al., 1992; Liu et al., 1992; Chao et al., 1993]. However, in all of the above induction conditions, at least several hours of sustained treatment is deemed necessary for the enhanced expression of GRP78. Under our experimental conditions, enhanced synthesis of GRP78 is detected almost immediately after the OA → HS treatment, reaches its plateau within 4–6 hr after treatment, and lasts for up to 10 hr of recovery. Again, the expression of this protein is not affected by HS → OA treatment. Although it has been found that the rat *grp78* promoter contains multiple arrays and redundant functional regulatory elements which would attribute to the basal and induced expression of the protein [Wooden et al., 1991; Alexandre et al., 1991], the

involvement and cooperativity of the multiple transcription factors under various induction conditions are not fully elucidated. Nevertheless, the rapid induction of GRP78 by the treatment of the OA-exposed cells with heat shock may represent a novel regulatory process for the transactivation of GRP78.

In summary, we have demonstrated that pretreatment, but not post-treatment, with OA can aggravate cytotoxicity, modulate protein phosphorylation, and potentiate stress protein induction in heat shocked cells, and that the above events are tightly correlated. The observations indicate that normal-functioning protein phosphatases represent an early parameter during the events of cellular stress response. Further delineation of the phosphorylation events and clarification of involved kinases/phosphatases are necessary for the elucidation of the molecular mechanisms underlying the differential regulation of the stress genes.

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