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Induction of Stress Response and Differential Expression of 70 kDa Stress Proteins by Sodium Fluoride in HeLa and Rat Brain Tumor 9L Cells

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Abstract We herein demonstrate that sodium fluoride (NaF) acts as a stress response inducer on HeLa and 9L rat brain tumor cells. NaF is only slightly cytotoxic, and inhibitory to Ser/Thr-phosphatases but not to Tyr-phosphatases in both cell lines. After treatment with 5 mM NaF for 2 h, the phosphorylation levels of vimentin and an alkali-resistant 65-kDa phosphoprotein were enhanced, a common phenomenon detected in cells under a variety of stress conditions. Under an identical treatment protocol, in which the cells were treated with 5 mM NaF for 2 h and then allowed to recover under normal growing conditions for up to 12 h, NaF differentially induced the cytoplasmic/nuclear heat-shock protein70s (including both the inducible and the constitutively expressed members of this protein family) in HeLa cells and the endoplasmic reticulum residing heat-shock protein70 (the glucose-regulated protein with an apparent molecular weight of 78 kDa) in 9L cells. Electrophoretic mobility shift assays (EMSA) using probes containing well-characterized regulatory elements revealed the activation of the heat-shock factor in HeLa but not in 9L cells; this is in good agreement with the stress protein induction pattern. Additional differential induction of binding activities toward EMSA probes individually containing NF-κB, AP-2, and CRE-like elements were detected in NaF-treated cells. The possible involvement of these binding sites as well as the corresponding factors in the stress response are discussed. J. Cell. Biochem. 69:221–231, 1998. © 1998 Wiley-Liss, Inc.

Key words: sodium fluoride; stress response; stress proteins; heat shock proteins; rat brain tumor 9L cells

Sodium fluoride (NaF), a pharmacological agent, is commonly used for prevention of dental caries [Horowitz, 1990] and treatment for osteoporosis [Mamelle et al., 1988]. However, it has recently been demonstrated that, when used at high concentration and/or for prolonged duration, NaF is genotoxic [Zeiger et al., 1993], developmental toxic [Collins et al., 1995], and neurotoxic [Mullenix et al., 1995] in animal models. NaF is also a well-known serine/threonine (Ser/Thr)-phosphatase inhibitor and is widely used to investigate the role of protein phosphatases in various cellular processes [Chabre, 1990]. Moreover, NaF is used as a general stimulator of heterotrimeric G proteins [Strnad et al., 1986], which regulates several effector systems. The processes may comprise activation of PLC, elevation of $[Ca^{2+}]_i$, and subsequent activation of PKC [Bianca et al., 1988]. In addition, NaF causes the levels of cAMP and cGMP to increase [Hussain and Mustafa, 1993; Kagaya et al., 1996], which supposedly leads to the activation of PKA and PKG in the treated cells. Thus, NaF affects a number of essential cell signaling components. The effects of NaF may, however, vary depending on the type and physiological states of the cells studied, as well as the treatment protocols employed [Li et al., 1988]. Nevertheless, it is now clear that NaF disturbs normal cellular metabolism.

It is well established that cultured cells exposed to stressful conditions produces certain proteins collectively called stress proteins [Welch, 1992]. Among these, members of the 70-kDa family (referred to as the HSP70s) are ubiquitous, highly conserved, and the most ex-

Abbreviations: EMSA, electrophoretic mobility shift assay; GRP78, 78-kDa glucose-regulated protein; HSC70, constitutively expressed heat-shock protein70; HSP70, inducible member of 70-kDa heat-shock protein; PPase, protein phosphatase; SDS, sodium dodecyl sulfate.

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tensively studied. Mammalian cells contain two members of HSP70s, HSP70 and the HSC70, in the cytoplasm and the nucleus [Lee and Lai, 1995]. Whereas HSP70 is hardly expressed under normal conditions and is highly heat inducible, HSC70 is constitutively expressed but is only slightly heat inducible. GRP78, also a HSP70 protein, is located in the endoplasmic reticulum and is expressed in high amounts in basal level [Munro and Pelham, 1986]. GRP78 is not heat-inducible but is induced, rather, by physiological stress that affects protein glycosylation [Wooden et al., 1991] or $[Ca^{2+}]_{ER}$ [Li et al., 1993]. The induction of HSP70 and HSC70 mainly involves the binding of a trans-acting heat-shock transcription factor (HSF) to the cis-acting conserved sequence known as the heat-shock element (HSE) [Morimoto, 1993]. On the other hand, transactivation of GRP78 is accompanied by changes in binding activities of transcription factors for CCAAT and cAMP responsive elements (CREs) [Li et al., 1993, 1994]. It is well documented that activation of numerous transcription factors, including some of those mentioned above, requires protein phosphorylation/dephosphorylation steps that involve the regulation of a battery of protein kinases/phosphatases [Karin, 1994]. We reason that, NaF, being a potent Ser/Thr-phosphatase inhibitor, may be able to elicit a stress response by perturbing the intracellular protein phosphorylation/dephosphorylation balance.

We have shown in a previous study that NaF enhances the phosphorylation level of a 65-kDa phosphoprotein and induces the synthesis of GRP78 in 9L rat brain tumor cells [Lai et al., 1993]. In this report we demonstrate that NaF elicits a typical stress response comprising decreased cell survival, altered protein phosphorylation, and differential induction of the 70-kDa stress proteins in HeLa and 9L cells. In addition, by using recognition sequences of known transcription factors, we performed gel-shift assays to study probe-binding activities of the corresponding transcription factors activated in NaF-treated cells.

MATERIALS AND METHODS

Materials

All cultureware was purchased from Corning (Corning, NY) and culture medium components were from Gibco Laboratories (Grand Island, NY). [^{35}S]methionine (specific activity >800 Ci/mmol) and [γ-^{32}P]ATP (5,000 Ci/mmol) were obtained from Amersham (Buckinghamshire, England), and [^{32}P]orthophosphate (specific activity 8,500–9,120 Ci/mmol) was from New England Nuclear (Boston, MA). Chemicals for electrophoresis were purchased from Bio-Rad (Richmond, CA) and reagents for gel shift assay were from Stratagene (LaJolla, CA) or Promega (Madison, WI). All other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO).

Cell Culture

HeLa (ATCC CCL 2) and 9L rat brain tumor cells [Weizsaecker et al., 1981] were routinely grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) and MEM, respectively, and supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C under a 5% CO_2 atmosphere. Cell survival after drug treatment was determined by colony formation technique as previously described [Lai et al., 1993]. Exponentially growing cells at 80–90% confluency were used for all experiments.

Chemical Treatment and Metabolic Labeling

NaF was dissolved in Milli-Q water at a concentration of 1 M and stored at −20°C in dark. The cells were treated with the desired concentration of NaF, for up to 2 h, by directly diluting the stock to the required final concentration. Phosphorylation of intracellular proteins and synthesis of stress proteins in the NaF-treated cells was analyzed by [^{32}P]orthophosphate and [^{35}S]methionine labeling, respectively. For protein phosphorylation analysis, the cells were prelabeled with 1 mCi/ml of [^{32}P]orthophosphate for 1 h and then treated with 5 mM NaF in the presence of the isotope for another 1 to 2 h before harvesting. To monitor *de novo* synthesis of stress proteins, the cells were treated with 5 mM NaF for 2 h and then allowed to recover under normal growing conditions for up to 12 h. The cells were labeled with 20 μCi/ml of [^{35}S]methionine for 1 h prior to harvesting.

Determination of Protein Phosphatases Activities

Cellular Ser/Thr- and Tyr-phosphatase activities were determined by the free phosphate detection kit from Promega (Madison, WI) according to the manufacturer's instruction. After treatment, the cells were homogenized in Buffer A (5 mM Tris-HCl, pH 7.4, 1 mM EDTA) at 4°C and then centrifuged at 100,000 *g* at 4°C for 10 min to obtain cell lysates. The cell lysates were

applied to a Sephadex G-25 columns (2.5-ml bed volume), pre-equilibrated in Buffer A, to remove endogenous free phosphates. After determining the protein concentration by the Bradford method [Bradford, 1976], the cell lysates were incubated with a substrate peptide (RRA(pT)VA) of Ser/Thr-phosphatases, or a substrate peptide (END(pY)INASL) of Tyr-phosphatases, in a total volume of 50 μl. The reactions were allowed to proceed at 30°C for 5 min and then stopped by adding 50 μl molybdate dye/additive mixture. The optical density of each sample was measured by using a plate reader with a 630-nm filter. Phosphate standards were made by diluting 1 mM standard phosphate solution with phosphate-free water.

Gel Electrophoresis and Autoradiography

SDS-PAGE was performed according to the method of Laemmli [1970]. Sample preparation and other related experimental procedures were carried out as previously described [Lai et al., 1993]. For detection of alkali-resistant phosphoproteins, the gels were heated at 55°C in 1 M KOH for 2 h before autoradiography [Lai et al., 1993]. Bands of interest on the autoradiographs were quantified by densitometric scanning set at 2-D mode (Molecular Dynamics, Sunnyvale, CA). For phosphoprotein quantitation, one unit of the relative phosphorylation levels represents the absolute numbers of pixels of phosphoprotein, in which background levels has been subtracted, in control cells. For quantitation of *de novo* protein synthesis, the amount of actin synthesis was used as a relative control and the value means the division of pixels of interested proteins into that of actin for each sample.

Nuclear Protein Extraction and Electrophoretic Gel Mobility-Shift Assay (EMSA)

Nuclear protein extracts were prepared according to Dignam et al. [1983]. Briefly, nuclear proteins were typically obtained from 6 × 10^6 cells by two-step extraction, using lysis buffer I (10 mM Hepes-KOH, pH 7.9, 0.5% Triton X-100, 0.1 mM EDTA, 0.5 M sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF) for cell lysis and lysis buffer II (lysis buffer I containing 5 mM NaCl and 5% glycerol) for nuclear protein extraction. The nuclear protein extracts were kept frozen at −70°C at a concentration of 1 mg/ml until used.

The binding activities towards the consensus binding sites of several regulatory elements in the nuclear extracts were determined by EMSA [Hennighausen and Lubon, 1987] using double-stranded oligonucleotides. Shown below are the sequences of one strand of the respective oligonucleotides labeled from 5′ to 3′ and the core consensus sequence is underlined.

HSE 5′-CTAGAAGCTTCTAGAAGCTTCTA-3′

NF-κB 5′-AGTTGAGGGGACTTTCCCAGGC-3′

SP1 5′-ATTCGATCGGGGCGGGGCGAGC-3′

AP2 5′-GATCGAACTGACCGCCCGCGGCCCGT-3′

CRE-like element

5′-CGCATGGTCACTGCACTCAACGCCTCC-3′

The HSE- and CRE-like elements were synthesized according to the sequences published by Wu et al. [1987] and Li et al. [1994], respectively, and the others obtained from Promega. The oligonucleotides were radioactively labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (Stratagene) and further purified on spin-columns. The assays were carried out according to the method of Wu et al. [1987] for HSE binding or Hennighausen and Lubon [1987] for other sequences. For each assay, 2 μg nuclear protein was mixed with 2 μg poly(dI-dC) (Pharmacia, Uppasla, Sweden) in GS buffer (4 mM Tris-HCl, pH 8.0, 12 mM Hepes, pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 12% glycerol, and 300 μg/ml BSA) in a volume of 12 μl. After 10 min of preincubation at 30°C, specific ^{32}P-labeled oligonucleotide probes (0.3 ng, 5,000–10,000 cpm) were added and the final volume was adjusted to 15 μl. The reaction was allowed to proceed for another 30 min, after which, the samples were analyzed by native pre-run 10% polyacrylamide gel in 0.5 × TBE (22 mM Tris-Borate, pH 8.0, 0.5 mM EDTA) and subsequent processing of the gels was performed as described above.

RESULTS

Effects of NaF on Cell Survival and Cellular Protein Phosphatase Activities

Shown in Figure 1A are the concentration-dependent survival curves of HeLa and 9L cells after exposure to NaF for 2 h. The viability of both cell lines dropped sharply in a concentration-dependent manner upon exposure to low

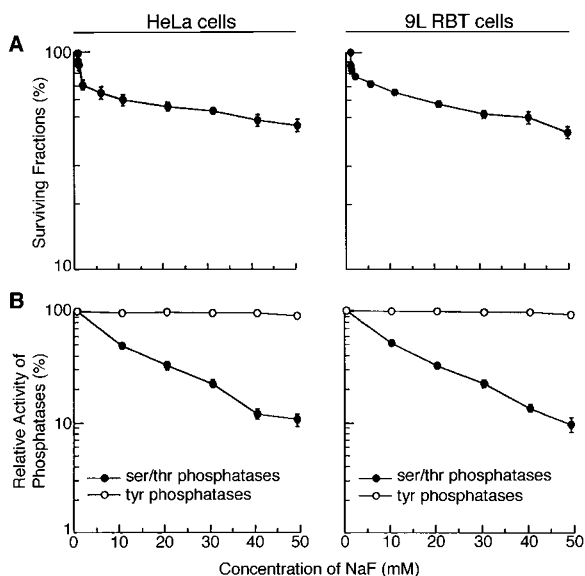

Fig. 1. Effects of NaF on the survivals (A) and cellular protein phosphatases activities (B) of HeLa and 9L RBT cells. Cells were incubated with the indicated concentrations of NaF at 37°C for 2 h. After treatment, changes in cell survival and protein phosphatases activities were monitored by colony formation assays and free phosphate detection method, respectively, as described in Materials and Methods. All assays were performed as duplicate and the data are presented as the mean ± S.D. from three independent experiments.

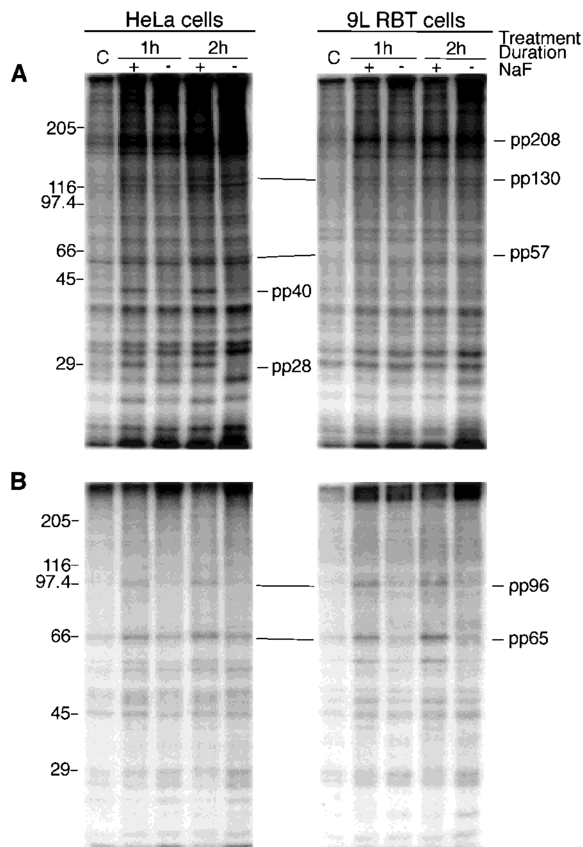

Fig. 2. Time-dependent phosphorylation of cellular proteins in NaF-treated HeLa and 9L cells. Cells were prelabeled with [^{32}P]orthophosphate (1 mCi/ml) for 1 h followed by treatment with 5 mM NaF for 1 or 2 h in the presence of the isotope. The cells were then lysed with SDS sample buffer and the cellular proteins were resolved by 10% SDS-PAGE and subjected autoradiography (A). Duplicate gels were heated in 1 M KOH at 55°C for 2 h and then processed for autoradiography (B). Molecular weight standards are shown at the left and several phosphoproteins are marked at the right with their apparent molecular weight indicated (in kDa). Lane C: control cells.

concentrations of NaF ($\leq$1 mM); however, further increase in NaF concentration did not cause similar decrease in cell viability. Although the decreases in cell survival rate were correlated linearly to concentrations between 10 to 50 mM, cell death upon treatment with 50 mM NaF was small (survival fraction > 0.4) in either cell line. The LD_{50}, calculated for both cell lines, was extremely high (between 40 to 50 mM), indicating that NaF is only weakly cytotoxic to these cells for exposure time below 2 h. The effect of NaF on the activity of cellular protein phosphatases was investigated using the same treatment protocol. The activity of Ser/Thr-phosphatases in untreated HeLa and 9L cells was similar (17.04 ± 0.45 and 15.05 ± 0.45 pmol phosphate released/min/μg protein, respectively) while that in the treated cells was found to be inhibited in a concentration-dependent manner and the inhibitory effect was almost identical in both cell lines. Approximate 10% residual enzyme activity was detected in both cell lines after treatment with 50 mM NaF for 2 h (Fig. 1B). In contrast, the activity of Tyr-phosphatases in HeLa and 9L cells remained unchanged following NaF treatment. The activity of cellular Tyr-phosphatases was determined to be 9.74 ± 0.30 and 9.65 ± 0.19 pmol phosphate released/min/μg protein in HeLa and 9L cells, respectively, and the decrease in enzyme activities after treatment with 50 mM NaF for 2 h is negligible (Fig. 1B).

Alteration in Protein Phosphorylation in NaF-Treated Cells

Although the Ser/Thr-phosphatases in both cell lines were significantly inhibited upon exposure to NaF, the general pattern of protein phosphorylation in these cells was not drastically affected (Fig. 2A and B). However, the phosphorylation level of several proteins was found to be increased upon treatment of the cells with 5 mM NaF. During the 2-h incubation, the phosphorylation levels of two phosphoproteins with apparent molecular weights of 40

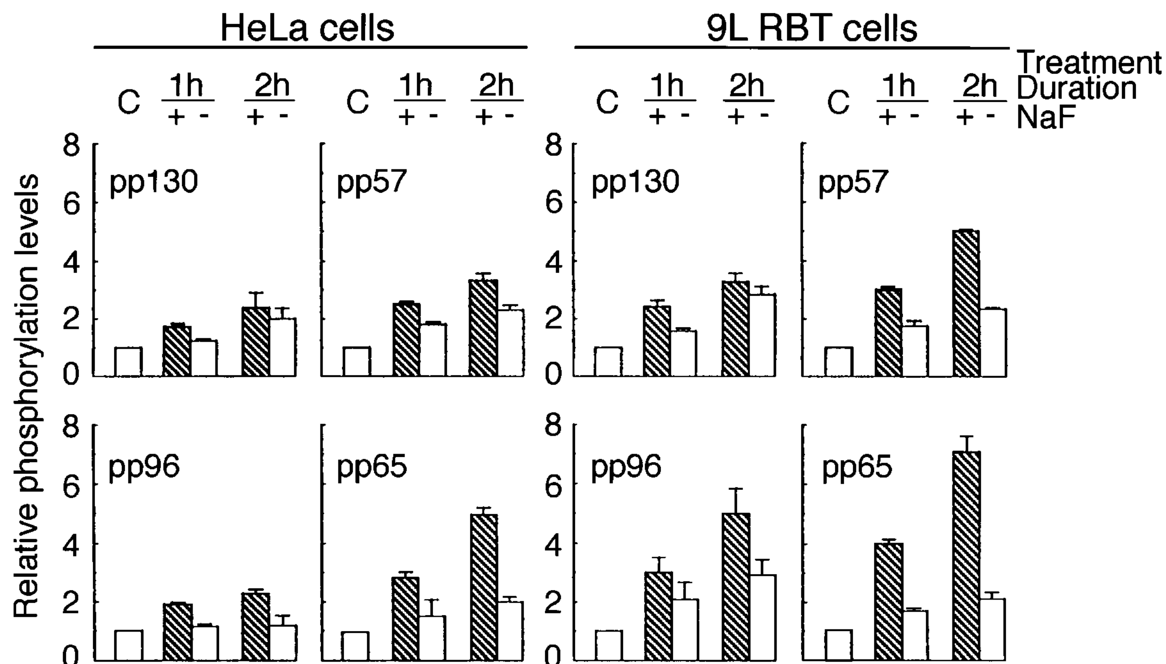

Fig. 3. Increase in phosphorylation levels of pp208, 130, 96, 65, and 57 by NaF in HeLa and 9L cells. Relative phosphorylation levels of pp208, 130, 96, 65, and 57 (vimentin) at the indicated time intervals as shown in Figure 2 were quantified by densitometry. Bars represent the ratio of the phosphorylation level of each phosphoprotein from the treated or untreated cells to that from the cells prelabeled for 1 h. Values represent the mean ± S.D. from three independent experiments.

and 28 kDa (designated as pp40 and pp28) were increased specifically in HeLa cells, accompanied with increased phosphorylation levels of two phosphoproteins, pp130 and pp57, in both cell lines, while increase in pp208 was specifically observed in 9L cells (Fig. 2A). Phosphorylation was enhanced in two additional phosphoproteins, pp96 and pp65, as prominently detected by alkaline digestion to detach phosphate groups from alkali-unstable phosphoserines and phosphothreonines (Fig. 2B). The pp57 was identified to be vimentin by Western blotting analysis (data not shown). Comparison of the treated with the untreated cells showed that the phosphorylation of vimentin in HeLa cells increased only by 50% after 2 h of treatment, whereas it showed a time-dependent increase and reached 2.7-fold difference in 9L cells (Fig. 3). We have previously reported that the alkali-resistant phosphorylation of pp65 was involved in a number of stress responses elicited by a variety of chemical inducers [Lai et al., 1993]. The changes in phosphorylation level of this protein were found to differ in the two cell lines. In HeLa cells, phosphorylation of pp65 increased 1- and 3.4-fold after 1 and 2 h of treatment, respectively, whereas phosphorylation increased only by 0.8- and 1.6-fold in 9L cells for identical treatment. Quantitative analysis of the alterations in phosphorylation levels is shown in Figure 3. Taken together, our results show that protein phosphorylation in these two cell lines is specifically affected despite the apparent inhibition of general Ser/Thr-phosphatases and a lack of effect on Tyr-phosphatases in the NaF-treated cells. These results indicate that the activity of certain protein kinase(s) is affected by NaF treatment and that the effect is species- and/or cell type-specific.

Differential Induction of HSP70s in NaF-Treated HeLa and 9L Cells

To investigate if NaF acts as a stress inducer, we monitored the pattern of *de novo* protein synthesis in HeLa and 9L cells after they were treated with 5 mM NaF for 2 h. We found that the incorporation of [^{35}S]methionine in NaF-treated HeLa cells was normal and there was no synthesis of stress proteins immediately following the treatment. Extension of the recovery period, however, resulted in enhanced synthesis of HSP70 in this cell line (Fig. 4, left panel); the synthesis increased initially after 4 h and peaked after 8 h of recovery (Fig. 5, left panel). The induction of HSP70 was more pronounced

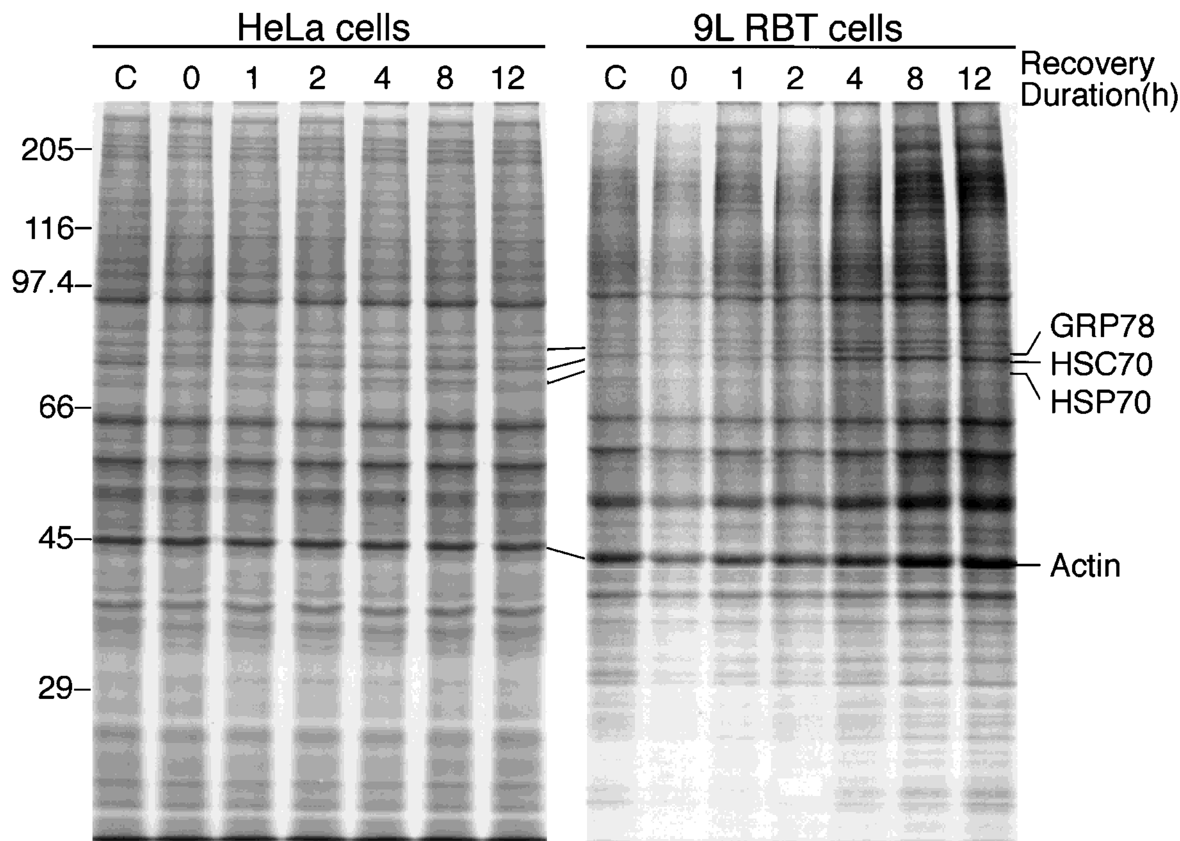

Fig. 4. Induction of stress proteins in NaF-treated HeLa and 9L cells. Cells were treated with 5 mM NaF for 2 h and allowed to recover for the indicated time points. The cells were labeled with [^{35}S]methionine for 1 h prior to harvesting. The cells were lysed with SDS sample buffer and the cellular proteins were resolved by 10% SDS-PAGE. After electrophoresis, the gels were processed for autoradiography. Molecular weight standards are shown at the left and the stress proteins induced by NaF are marked at the right. **Lane C:** control cells.

compared with that of HSC70 although the rate of synthesis of HSC70 was higher than that of HSP70. The synthesis of GRP78 was essentially the same after NaF treatment in the HeLa cells (Figs. 4 and 5, left panels). The response of 9L cells to similar treatment with NaF was completely different. The general protein synthesis in NaF-treated 9L cells was significantly inhibited immediately following the treatment and appeared to be gradually restored during the recovery period, although the amount of total proteins make no prominent differences. Moreover, the synthesis of neither HSP70 nor HSC70 was affected in the treated or recovering 9L cells. Interestingly, the synthesis of GRP78 was significantly enhanced in the recovering cells (Figs. 4 and 5, right panels). Enhanced synthesis of GRP78 was detected as early as 1 h, peaked at 4 h, and abruptly dropped to normal level after 8 h of recovery (Fig. 5, right panel). These results clearly demonstrate that treatment with NaF differentially induces the synthesis of HSC70 as well as HSP70 in HeLa and GRP78 in 9L cells.

Effects of NaF on Binding Activities of the Nuclear Extracts From HeLa and 9L Cells to Specific Transcription Elements

In a first step to characterize the molecular mechanisms underlying the differential induction of HSP70s, which is transcriptionally regulated, in HeLa and 9L cells, we analyzed the binding activities of nuclear proteins extracted from the NaF-treated cells to several DNA probes encompassing consensus sequences of known transcription elements including HSE, NF-κB, AP-2, CREB-like, as well as SP-1. Treatment with NaF induced the appearance of two shifted bands in HeLa cell extracts but not in 9L cell extracts when a nucleotide containing HSE was used as a probe (Fig. 6A and B). The DNA-protein complexes appeared immediately following NaF treatment and persisted at a high level for at least 4 h (Fig. 6A). This result

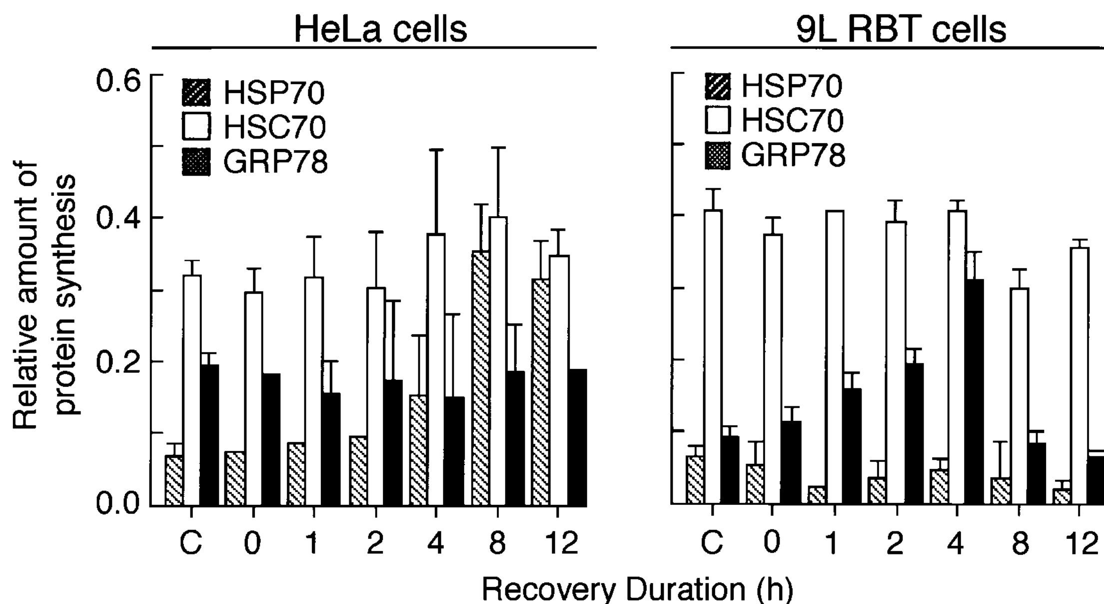

Fig. 5. The relative rate of synthesis of HSP70, HSC70, and GRP78 in NaF-treated HeLa and 9L cells. Autoradiographs as shown in Figure 4 were analyzed by computer densitometry. Data represent the mean ± S.D. from three independent experiments.

suggests an activation of HSF and explains the subsequent transactivation of HSP70 and HSC70 in HeLa cells but not in 9L cells upon treatment with NaF. Activation of NF-κB binding was observed in the NaF-treated HeLa cells and the binding activity rose and fell abruptly, detectable only at 0.5 h after treatment (Fig. 6C). On the other hand, NF-κB binding activity in 9L was constitutively high and appeared to be unaffected by NaF (Fig. 6D). In contrast to the weak and almost constant AP-2 binding activity in HeLa cells, a time-dependent enhancement of AP-2 binding activity was observed in the NaF-treated 9L cells (Fig. 6E and F). The binding activity of nuclear extracts from HeLa cells to the CRE-like element containing probe was not well defined. The untreated cells showed only one shifted band, whereas, the gel shift pattern became very complicated when the cells were treated with NaF (Fig. 6G). On the other hand, the binding activity toward the same probe was simply significantly higher in the NaF-treated 9L cells (Fig. 6H). Finally, the binding activity to the SP-1 probes was gradually increased in HeLa cells but not affected by NaF in both cell lines (Fig. 6I and J). These results demonstrate that NaF activates a number of transcription element binding activities in the treated cells and, thus, these factors may be involved in the transactivation of the affected genes.

DISCUSSION

We have herein demonstrated that NaF acts as an effective chemical stress inducer on HeLa and 9L cells. This slightly cytotoxic compound alters cellular protein phosphorylation and induces synthesis of stress proteins in the cell lines studied. Together, the above changes in cellular activities are generally considered to be a typical stress response in mammalian cells [Welch, 1992]. Such effects of NaF have apparently escaped the attention of researchers, although many biological effects of this compound have been extensively investigated [Whitford, 1990].

Cytotoxic effects of NaF on mammalian cells *in vitro* have been described for human fibroblasts, lymphoblastoid cells, and fibroblastoid EUE cells, as well as in Syrian hamster embryonic cells and Chinese hamster V79 cells [Slamenova et al., 1992]. We focused on 2-h treatment while other investigations focused on prolonged incubation (20–24 h). The LD_{50} of NaF in long-term treatments fell between 3.5 to 5 mM. This is consistent with our conclusion that NaF is only slightly cytotoxic. NaF is a well-known Ser/Thr-phosphatase inhibitor. In addition, NaF inhibits Tyr-phosphatase activity in bone-forming cells and, thus, mimics the growth factors. Tyr-phosphatase activity, however, was unaffected by NaF, at least in human

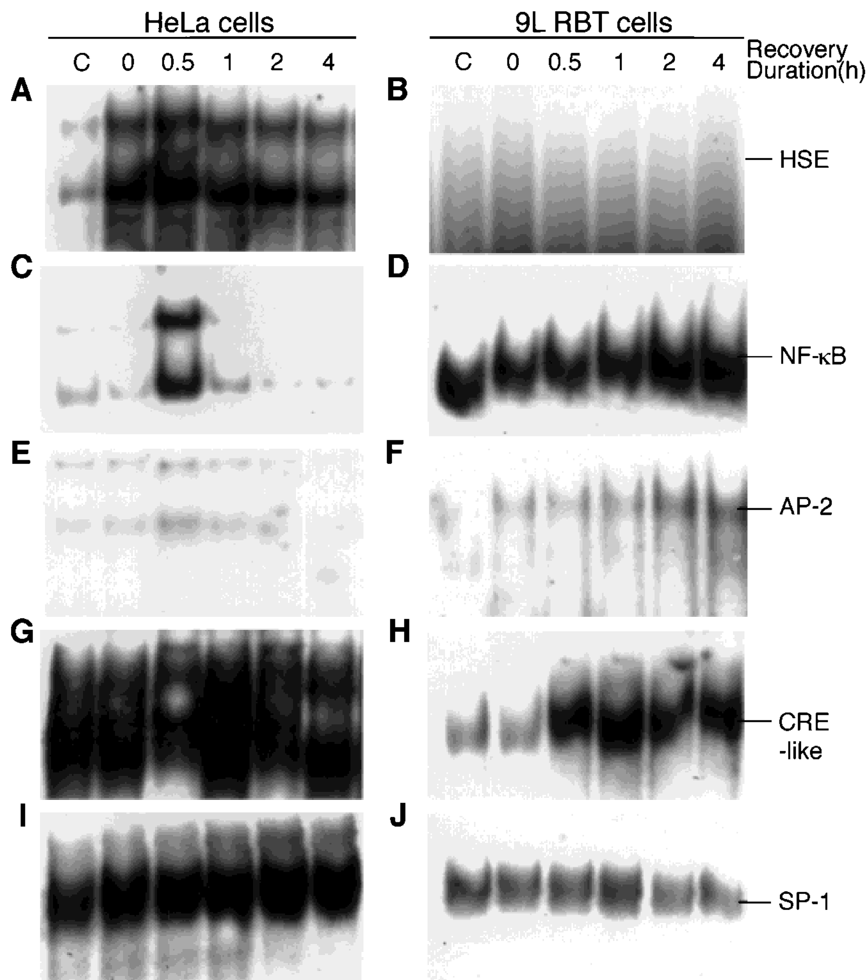

Fig. 6. Effects of NaF on the binding activities of the nuclear proteins in HeLa and 9L cells toward EMSA probes containing HSE (**A** and **B**), NF-κB (**C** and **D**), AP2 (**E** and **F**), the CRE-like element (**G** and **H**), and SP1 (**I** and **J**), after treatment with 5 mM NaF for up to 2 h. At the indicated time points, nuclear proteins were extracted and incubated with ^{32}P-labeled oligonucleotides. The resulting protein-oligonucleotide complexes were resolved by 10% PAGE. After electrophoresis, the gels were processed for autoradiography and only the regions containing the bound probes were shown. **Lane C:** control cells.

skin fibroblasts [Lau et al., 1988]. We found that NaF inhibits the activity of general Ser/Thr-phosphatases but fails to inhibit Tyr-phosphatases in HeLa and 9L cells. Only END(pY)INASL was employed in the present study. Therefore, the possibility that NaF may act on certain Tyr-phosphatases cannot be entirely excluded.

We also delineated inhibition of cellular PPases and direct/indirect activation of protein kinases by NaF by monitoring the changes in phosphorylation levels of cellular phosphoproteins in treated cells. Protein phosphorylation has long been recognized to play important roles in cell signaling and gene expression [Karin, 1994]. We previously showed that enhanced phosphorylation of vimentin (the pp57) and pp65 is a common phenomenon occurring simultaneously with the onset of stress response elicited by heat-shock and several cytotoxic chemicals [Lee et al., 1992; Cheng and Lai, 1994]. Therefore, we suggested that the processes may be essential for regulating stress genes [Lai et al., 1993]. This phenomenon was also detected in HeLa and 9L cells after treatment with NaF. These results substantiate our finding that the disturbance of cellular protein phosphatases/kinases, which leads to enhanced phosphorylation of vimentin and p65, is a prominent effect when cells were in a stressful environment.

Interestingly, we found that the same treatment protocol (5 mM NaF for 2 h) induces differential synthesis of different members of

the HSP70s in HeLa and 9L cells. Differential transactivation of the stress genes might be caused by differences in intrinsic signaling pathways in the two cell lines. It is established that protein phosphorylation also plays a central role in the induction of stress response [Lai et al., 1993, and references therein). Recently, numerous protein kinases have been found to be activated upon stress [Kyriakis and Avruch, 1996]. Noteworthy are PKA [Marchler et al., 1993], MAPK, SAPK, SEK, MEKK, MAPKAP-kinase-2, and kinase F_A/GSK-3 [Yang et al., 1996]. These may be responsible for phosphorylation of HSP25, c-jun, and other proteins [Lai et al., 1993]. Moreover, the transactivation of *hsp70* and *grp78* appeared to be affected by a variety of kinase or phosphatase inhibitors. Staurosporine and H-7, PKC inhibitors, and genistein, a tyrosine kinase inhibitor, all exert inhibitory effects on *hsp70* induction [Yamamoto et al., 1994]. Using okadaic acid as a specific inhibitor for Ser/Thr-phosphatase activities, it has been suggested heat-induced transcriptional activation of the heat shock genes is regulated by phosphorylation of component(s) of the transcriptional complex and that PP2A was implicated in this induction process [Mivechi et al., 1994]. Thapsigargin was found to exert a mild stimulative effect on induction of *grp78* transcription, and PP1, as opposed to PP2A, seems to play a major role in this process. Furthermore, the transcriptional activation of *grp78* by thapsigargin is almost totally eliminated by genistein, a tyrosine kinase inhibitor [Cao et al., 1995]. Therefore, although *hsp* and *grp* genes are both stress-inducible genes and share some common induction conditions, their corresponding signaling pathways differ. The transactivation of *hsp* and *grp* gene are, thus, regulated by different effectors.

Differences in protein expression possibly reflect species-specific differences in transcription function. The DNA-binding factors either possibly are present at different levels or have different activities in human and rat lines. It is known that human HSP70 is constitutively expressed by CCAAT or SP1 boxes [Morgan, 1989; Williams et al., 1989]. In addition, TFIID, MYC, and an ATF/AP1-like factors are known to bind the promoter region of the human *hsp70* gene and to contribute possibly to constitutive expression of HSP70 [Morgan, 1989; Williams et al., 1989]. The inductive expression of HSP70 is mainly caused by the binding of HSF to HSE and the coordinate action of other transcription factors since HSE-HSF binding alone is insufficient to induce the expression of HSP70 [Hensold et al., 1990]. Recently, Shopland et al. [1995] proposed that a complicated promoter architecture, established by multiple interdependent factors including GAGA factor, TFIID, and RNA polymerase II, is critical for HSF binding *in vivo.* On the other hand, the regulatory elements of *grp78* promoter consist of two major domains, CCAAT [Wooden et al., 1991] and CRE-like element; furthermore, the gene is positively regulated by a transcription factor of molecular mass 70 kDa (p70CORE) [Li et al., 1994]. Both regions confer basal expression and stress-inducibility by using transient transfection experiments.

In examining the changes in probe-binding activity of nuclear extracts from NaF-treated cells, we detected the binding of NF-κB binding sites after 0.5-h recovery in addition to the expected HSF-HSE binding in HeLa cells. The NF-κB transcription activator is a multiprotein complex, activated in response to many stimuli, most of which represent pathologic stressors [Baeuerle, 1991]. During its cytoplasmic activation, specific inhibitor of NF-κB, IκB-α, is phosphorylated and proteolytically degraded. This exposes nuclear localization sequences in the remaining NF-κB heterodimer, leading to nuclear translocation and subsequent binding of NF-κB to DNA regulatory elements. PKC, PKA, and heme-regulated kinase have been reported to possibly regulate the process of NF-κB activation [Baeuerle, 1991]. Recently, PP2A also has been found to influence the phosphorylation of IκB-α and the ensuring activation of NK-κB [Sun et al., 1995]. There is increasing evidence that NK-κB/Rel family is involved in cellular stress. It has not been reported that activation of NK-κB is involved in the regulated expression of HSP70s during stress; however, involvement of NK-κB in the induction of stress genes is conceivable for the following reasons. First, a common intracellular reaction elicited by most, if not all, stimuli activating NF-κB is accompanied by an increased production of reactive oxygen intermediates (ROIs) leading to oxidative stress [Baeuerle, 1991], which would also induce the synthesis of HSP [Tacchini et al., 1995]. Second, Ras GTP protein and Raf-1 kinase, the upstream effectors of activation of NF-κB [Devary et al., 1993], were proved to be involved in the signal transduction pathway upon irradiation stress and to control the transcription of

heat shock genes [Engelberg et al., 1994]. Third, TNF-α and interleukins, commonly used activators of NF-κB, would induce phosphorylation of small HSP and excite some signaling pathways similar to that during heat-shock treatment [Westwick et al., 1995]. In this scenario, it is logical to predict NF-κB would play some roles in the stress response. Whether NF-κB is related to the signal transduction pathway during stress or NF-κB is involved in the regulation of expression of stress genes needs to be further clarified. In addition, we detected alterations in AP2- and CRE-like-binding activity in 9L RBT cells. AP2 probably acts independently as a cAMP- and TPA-inducible element being related to protein kinase A and C. However, it is shown *grp78* lacks AP2-like binding sites and the stress inducibility of *grp78* gene has been conferred to CCAAT and CRE-like sites. Unfortunately, the result obtained by using an EMSA probe containing the CRE-like site is too complicated at this point. Activation of CREB is thought to be mediated through PKA activation, a phenomenon occurring in treatment, and subsequent phosphorylation of CREB [Yamamoto et al., 1988]. Due to the multiplicity and versatility of CREB, it is difficult to define its role(s) in the signaling pathway of a stress response. Therefore, the activation of AP2-like and CRE-like binding activity may or may not be involved in the signaling pathways in the stress response elicited by NaF.

Further explanation of the EMSA data is impossible without the identification of the protein factors and detailed functional analysis of the putative transcription elements in the promoter regions of the stress genes. Nevertheless, the present experimental system presents an additional challenge for unraveling the coordinated transactivation of stress genes in cells under stress.

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