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Okadaic Acid, Sphingosine, and Phorbol Ester Reversibly Modulate Heat Induction on Protein Kinase FA/GSK-3α in A431 Cells

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Abstract Exposure of A431 cells to a rapid and sudden increase from 37°C to 46°C for 30 min could induce an increase in protein level and cellular activity of protein (kinase FA/GSK-3α) up to ~200% of control level. However, when cells were first treated with 500 nM tumor promoter phorbol ester TPA at 37°C for 30 min to activate cellular protein kinase C (PKC) or with 400 nM okadaic acid at 37°C for 30 min to inhibit cellular protein phosphatases followed by heat shock at 46°C for another 30 min, the heat induction on kinase FA/GSK-3α was found to be completed blocked. In sharp contrast, when cells were first treated with 1 μM TPA at 37°C for 24 h or with 5 μM sphingosine at 37°C for 30 min to down-regulate cellular PKC, the heat induction on kinase FA/GSK-3α was found to be reversely promoted up to ~250% of control level, demonstrating that kinase FA/GSK-3α may not represent a constitutively active/mitogen-inactivated protein kinase as previously conceived. Taken together, the results provide initial evidence that TPA/sphingosine and okadaic acid could reversibly modulate the heat induction on kinase FA/GSK-3α in A431 cells, suggesting that phosphorylation/dephosphorylation mechanisms are involved in the regulation of the heat-shock induction of kinase FA/GSK-3α, representing a new mode of signal transduction for the regulation of this multisubstrate protein kinase and a new mode of signaling pathway modulating the heat-induction process. © 1996 Wiley-Liss, Inc.

Key words: protein kinase C, heat-induction process, phosphorylation/dephosphorylation

Protein kinase FA was originally identified as an activating factor of Mg·ATP-dependent type-1 protein phosphatase but has subsequently been demonstrated as a protein kinase identical to glycogen synthase kinase-3α (GSK-3α) [Yang et al., 1980; Vandenheede et al., 1980; Hemmings et al., 1981; Woodgett, 1990]. Kinase FA/GSK-3α was further identified as a multisubstrate protein kinase. In addition to Mg·ATP-dependent protein phosphatase and glycogen synthase as its substrates, kinase FA/GSK-3α is capable of acting on many substrates including R subunit of cAMP-dependent protein kinase [Hemmings et al., 1982], myelin basic protein [Yang, 1986], nerve growth factor receptor [Taniuchi et al., 1986], G-subunit of phosphatase-1 [Fiol et al., 1988; Dent et al., 1989], neuronal cell adhesion molecule [Mackie et al., 1989], neurofilament [Guan et al., 1991], transcription factors/proto-oncogene products such as c-jun [Boyle et al., 1991; de Groot et al., 1992; Nikolakaki et al., 1993], c-myb and c-myc [Plyte et al., 1992], and CREM [de Groot et al., 1993], synapsin I [Yang et al., 1992a,b], brain microtubule-associated proteins such as MAP-2 [Yang et al., 1991, 1993a] and tau [Yang et al., 1991, 1993b,c, 1994a; Mandelkow et al., 1992; Hanger et al., 1992], brain clathrin-coated vesicles [Yu and Yang, 1993], ATP-citrate lyase [Ramakrishna et al., 1990], acetyl-CoA carboxylase [Hughes et al., 1992], and eukaryotic protein synthesis initiation factor-2B [Welsh and Proud, 1993]. By its dual role as a multisubstrate protein kinase and as a multisubstrate protein phosphatase activator, kinase FA/GSK-3α may modulate phosphorylation and dephosphorylation of many key proteins involved in the regulation of diverse cell and pathophysiological functions

Abbreviations used: kinase FA, protein phosphatase activating factor; GSK-3α, glycogen synthase kinase-3α; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; MBP, myelin basic protein; PKC, protein kinase C; TPA, 12-O-tetradecanoylphorbol-13-acetate; HSP, heat-shock protein.

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[Yang, 1991; Yang et al., 1992c, 1994; Woodgett, 1991; Plyte et al., 1992; Ruel et al., 1993].

In this report, we further identify that the heat stress can induce an increase in protein level and cellular activity of kinase FA/GSK-3α in A431 cells. Most importantly, the heat stress induction process on kinase FA/GSK-3α can be reversibly modulated by okadaic acid (a specific inhibitor for protein Ser/Thr phosphatases 1 and 2A) [Bialojan and Takai, 1988; Cohen et al., 1990] and tumor promoter phorbol ester and sphingosine (the two specific modulators for protein kinase C) [Nishizuka, 1986; Bell et al., 1991], presenting a new mode of signal transduction for the regulation of this multisubstrate/multifunctional protein kinase in cells and a new mode of signaling pathway modulating the heat-induction process.

EXPERIMENTAL PROCEDURES

Materials

[γ-^{32}P]ATP was purchased from Amersham (Buckinghamshire, UK). Human epidermoid carcinoma A431 cells were obtained from American Type Culture Collection ATCC (Rockville, MD). The cell is one of a series of human tumor-derived cell lines. These cells have been used for studies of hormone actions and tumor promoter effects. A431 cells within passages 15–30 were used for all the experiments in this text. Plastic wares for cell culture were from Falcon (Oxnard, CA). Antibiotics, fetal bovine sera, complete and incomplete Freund's adjuvants were from Gibco (Rockville, MD). Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin, gelatin, dimethylsulfoxide (DMSO), sodium orthovanadate, phorbol ester (TPA), sphingosine, Tween 20, genistein, 3-[cyclohexylamino]-1-propane-sulfonic acid (CAPS), and goat antirabbit IgG antibody conjugated with alkaline phosphatase were from Sigma (St. Louis, MO). Alkaline phosphatase conjugate substrate kit were from BioRad. Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate (CSPD), and okadaic acid were from Boehringer Mannheim (Mannheim, Germany). Phenylmethanesulfonyl fluoride (PMSF), benzamidine, aprotinin, Triton X-100, NaF, and glutaraldehyde were from Merck (Darmstadt, Germany). Polyvinylidene fluoride (PVDF) membrane was from Millipore (Bedford, MA). Protein A–Sepharose CL-6B and CNBr-activated Sepharose 4B were from Pharmacia (Uppsala, Sweden).

Protein Purification and Analytic Methods

Myelin basic protein (MBP) was purified to homogeneity from porcine brain following the purification procedures as described in previous reports [Yang, 1986; Yu and Yang, 1994c]. Protein concentrations were determined by using BCA protein assay reagent from Pierce (Rockford, IL). Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli [Laemmli, 1970] using 10% gels. Molecular-weight markers used are phosphorylase b (94 kDa), bovine serum albumin (BSA) (68 kDa), glutamate dehydrogenase (55.6 kDa), and glyceraldehyde 3-phosphate dehydrogenase (36 kDa).

Production of Antikinase FA/GSK-3α Antibody

The peptide TETQTGQDWQAPDA, corresponding to the carboxyl terminal regions from amino acids 462–475 of the sequence of kinase FA/GSK-3α [Woodgett, 1990] was synthesized by peptide synthesizer (model 9050, Milligen, Bedford, MA). The cysteine residue was added to the N-terminus in order to facilitate coupling of the peptide to BSA according to the procedure described by Reichlin [Reichlin, 1980] using glutaraldehyde as the cross-linker. The detailed procedure for production and affinity-purification of anti-kinase FA/GSK-3α antibody was as described in previous reports [Yu and Yang, 1994a,b].

Culture of A431 Cells, Heat, and Drugs Treatment and Preparation of Cell Extracts

A431 cells were cultured at 37°C in a 92.5% air/7.5% CO_2 and water-saturated atmosphere in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 25 U/ml penicillin, and 25 μg/ml streptomycin. The exponentially growing A431 cells were freshly plated 2 days before use. The culture medium was changed at night before each experiment. A431 cells were subjected to a sudden and rapid increase in a waterbath at 46°C in a humidified incubator. For drug treatment, aliquots of stock solution of sodium orthovanadate (0.1 M dissolved in H_2O), genistein (100 mM dissolved in DMSO), sphingosine (10 mM dissolved in 50% ethanol), TPA (1 mM dissolved in DMSO), or okadaic acid (100 μM dissolved in DMSO) was added directly into the culture medium to make the required final concentrations. After incubation, A431 cells ($\sim 4 \times 10^6$ cells/100-mm culture dishes) were

washed twice with ice-cold phosphate-buffered saline (PBS), scraped in PBS and collected by centrifugation at 800*g* at 4°C for 10 min. The collected cells were homogenized in 500 μl solution A (10 mM Tris-HCl at pH 7.4, 2 mM EDTA, 1 mM EGTA, 50 mM NaCl, 1% Triton X-100, 1 mM PMSF, 1 mM benzamidine, 0.5 mg/ml aprotinin, 50 mM NaF, and 0.2 mM sodium orthovanadate) on ice by Sonic Dismembrator (model 150, Fisher, Farmingdale, NY) at 40% power output for 3 × 10 sec. The homogenates were then ultracentrifuged at 160,000*g* at 4°C for 30 min, and the supernatants were used as the cell extracts.

Immunoblots

For immunoblotting analysis of kinase FA/GSK-3α from A431 cells, the cell extracts (40 μl) containing ~40 μg of cell protein were subjected to 10% SDS–PAGE, electrotransferred to PVDF membrane, and then immunoblotted with 1 μg/ml of anti-kinase FA/GSK-3α antibody essentially as described in previous reports [Yu and Yang, 1994a,b]. The immunoblot was developed with the CSPD system using 0.25 mM CSPD in 0.1 M Tris–HCl at pH 9.5, 0.1 M NaCl, and 50 mM $MgCl_2$ as alkaline phosphatase substrate at 37°C for 15 min for chemiluminescence detection [Gillespie and Hudspeth, 1991]. The luminescent light emission was recorded on X-ray film and quantified by computing densitometer (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitation and Kinase FA/GSK-3α Activity Assays in the Immunoprecipitates

Before immunoprecipitation, protein concentrations of the cell extracts were first diluted to equal amounts with solution A. For immunoprecipitation, 500 μl cell extract (~1.0 mg/ml protein) was incubated with 1 μl affinity-purified anti-kinase FA/GSK-3α antibody (20 mg/ml pure IgG) at 4°C for 1.5 h and then with 100 μl protein A–Sepharose CL-4B (20% v/v in solution A) for another 1.5 h with shaking. The immunoprecipitates were collected by centrifugation, washed three times with 0.5 M NaCl, once with 1 ml solution B (20 mM Tris–HCl at pH 7.0, 0.5 mM dithiothreitol, 1 mM PMSF, 1 mM benzamidine, and 0.5 mg/ml aprotinin), and resuspended in 60 μl solution B. For kinase FA/GSK-3α activity assay in the immunoprecipitate, 10 μl immunoprecipitate at appropriate dilution was incubated with 15 μl mixture containing 20 mM Tris–HCl at pH 7.0, 0.5 mM dithiothreitol, 0.2 mM [γ-^{32}P]ATP (1 pmol ~ 1,000 cpm), 20 mM $MgCl_2$, and 4 mg/ml MBP at 30°C for 10 min. ^{32}P-incorporation into MBP was measured by spotting a 20-μl reaction mixture on phosphocellulose paper (1 × 2 cm), washing 3 times with 75 mM H_3PO_4 and counting in liquid scintillation counter as described in previous reports [Yang, 1986; Yu and Yang, 1994a,b].

RESULTS AND DISCUSSION

When A431 cells were exposed to a rapid and sudden increase from 37°C to 46°C for 30 min, the cellular protein level of kinase FA/GSK-3α could be induced up to ~200% of control level, as demonstrated by immunoblotting analysis of kinase FA/GSK-3α from A431 cell extracts using a potent and immunospecific antikinase FA/GSK-3α antibody followed by chemilumenescence detection and quantification (Fig. 1, lanes 1, 2). More interestingly, when A431 cells were pretreated with 500 nM tumor promoter phorbol ester TPA at 37°C for 30 min and then heat-shocked at 46°C for 30 min, the cellular protein level of kinase FA/GSK-3α could no longer be affected by this heat stress (Fig. 1, lanes 1, 4). Conversely, when A431 cells were pretreated with 1 μM TPA at 37°C for 24 h and then heat-shocked at 46°C for 30 min, the protein level of kinase FA/GSK-3α could be further enhanced up to ~240% of control level (Fig. 1, lanes 1, 6). In sharp contrast, TPA alone caused no change in the immunoblotted protein level of kinase FA/GSK-3α in untreated cells (Fig. 1, lanes 1, 3, 5).

Taken together, the results provided initial evidence that tumor promoter phorbol ester TPA could reversibly modulate the heat induction on kinase FA/GSK-3α. Similarly, when cells were pretreated with 5 μM sphingosine at 37°C for 30 min to inhibit cellular protein kinase C [Bell et al., 1991], the protein level of kinase FA/GSK-3α could be increased up to ~240% of control level (Fig. 2, lanes 1, 6). Conversely, when pretreated with 400 nM okadaic acid at 37°C for 30 min to inhibit protein phosphatases 1 and 2A [Cohen et al., 1990], the heat induction on protein level of kinase FA/GSK-3α could be completely blocked (Fig. 2, lanes 2, 4), whereas okadaic acid or sphingosine alone caused no effect on the basal protein level of kinase FA/GSK-3α in untreated cells (Fig. 2, lanes 1, 3, 5). Okadaic acid and TPA appeared not toxic to A431 cells under the conditions used essentially as described in previous reports [Yu and Yang, 1994b; Yang et al., 1994b].

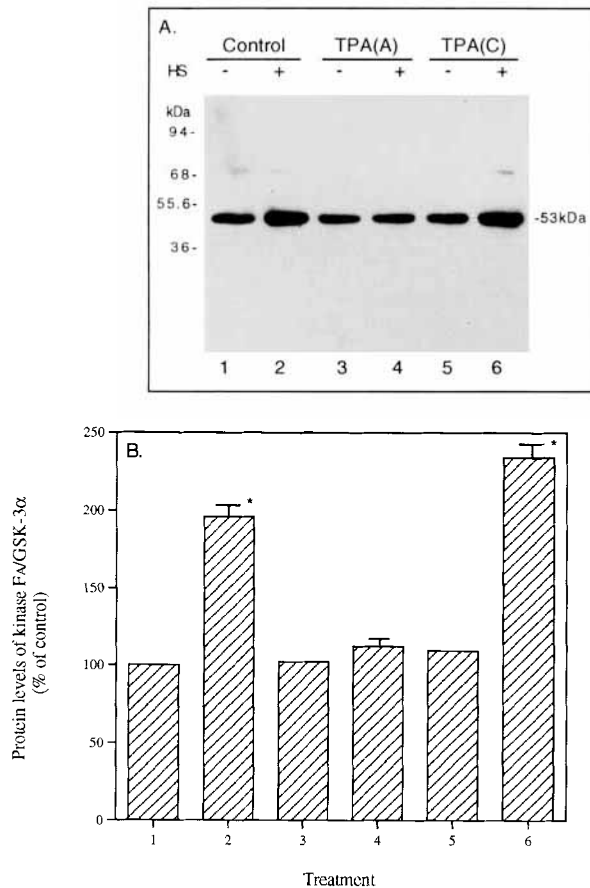

Fig. 1. Effect of TPA on heat-induced protein synthesis of kinase FA/GSK-3α in A431 cells. A431 cells were treated with and without 500 nM TPA at 37°C for 30 min or 1 μM TPA at 37°C for 24 h and then heat-shocked at 46°C for another 30 min. The cell extracts were subjected to immunoblotting analysis (A) with anti-kinase FA/GSK-3α antibody followed by chemilumenescence detection and quantification (B), as described under Experimental Procedures. *Lane 1,* control without any treatment; *lane 2,* heat shock alone; *lane 3,* acutely treated with TPA only; *lane 4,* acutely pretreated with TPA followed by heat shock; *lane 5,* chronically treated with TPA only; *lane 6,* chronically pretreated with TPA followed by heat shock. **A:** Photographs shown are representative results from five independent experiments. **B:** Data expressed as means ±SD. Significance $^*P < 0.05$.

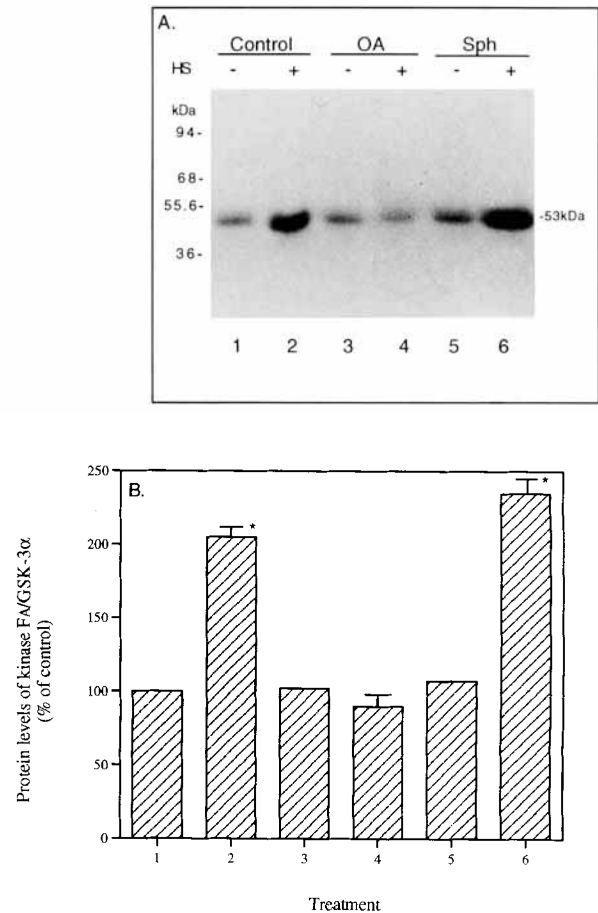

Fig. 2. Effect of okadaic acid and sphingosine on heat-induced protein synthesis of kinase FA/GSK-3α in A431 cells. A431 cells were treated with and without 400 nM okadaic acid or 5 μM sphingosine at 37°C for 30 min and then heat-shocked at 46°C for another 30 min. The cell extracts were subjected to immunoblotting analysis (A) with antikinase FA/GSK-3α antibody, followed by chemilumenescence detection and quantification (B) as described under Experimental Procedures. *Lane 1,* control without any treatment; *lane 2,* heat shock alone; *lane 3,* okadaic acid alone; *lane 4,* okadaic acid + heat shock; *lane 5,* sphingosine alone; *lane 6,* sphingosine + heat shock. **A:** Photographs shown are representative results from five independent experiments; **B:** Data expressed as means ±SD. Significance $^*P < 0.05$.

Moreover, TPA and okadaic acid could reversely potentiate the heat induction of HSP72/73 under identical conditions (data not further illustrated). Therefore, the modulatory effect of TPA and okadaic acid on heat induction of kinase FA/GSK-3α is not due to the cytotoxic effect of these two agents to the cell but can be due to phosphorylation–dephosphorylation mechanisms mediated by TPA and okadaic acid. The protein level and cellular activity of kinase FA/GSK-3α are already manyfold overexpressed in human carcinoma as compared to normal control [Yang et al., 1995]. The heat induction of kinase FA/GSK-3α in the presence of sphingosine (Fig. 2) or TPA (Fig. 1) up to ~240% of this overexpressed protein level in A431 cells might be the maximal level that could ever be reached in cells. We have performed dose–response curve for sphingosine up to 40 μM together with chronic effect of TPA; similar results were obtained (not further illustrated).

The result again points to the fact that ~240% of overexpressed protein level of kinase FA/GSK-3α in human carcinoma A431 cells is in-

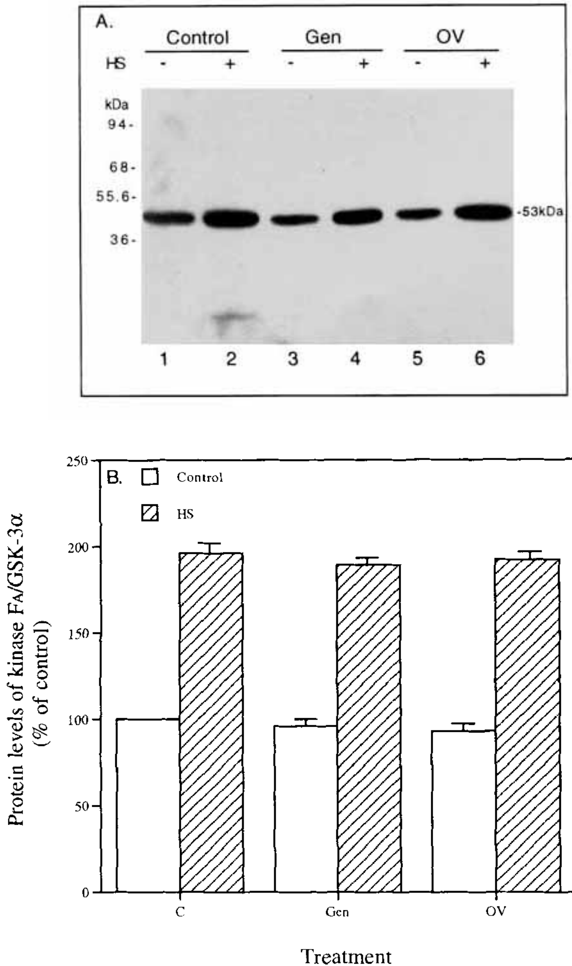

Fig. 3. Effect of genistein and orthovanadate on heat-induced protein synthesis of kinase FA/GSK-3α in A431 cells. A431 cells were treated with and without 400 μM genistein or 500 μM orthovanadate at 37°C for 2 h and then heat-shocked at 46°C for another 30 min. The cell extracts were subjected to immunoblotting analysis (**A**) with antikinase FA/GSK-3α antibody followed by chemilumenescence detection and quantification (**B**) as described under Experimental Procedures. *Lane 1,* control without any treatment; *lane 2,* heat shock alone; *lane 3,* treated with genistein alone; *lane 4,* genistein + heat shock; *lane 5,* treated with orthovanadate alone; *lane 6,* orthovanadate + heat shock. **A:** Photographs shown are representative results from five independent experiments. **B:** Data expressed as means ±SD.

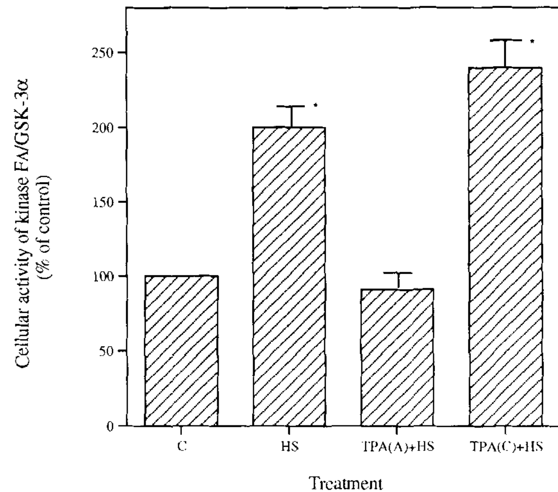

Fig. 4. Effect of TPA on heat-induced cellular activation of kinase FA/GSK-3α in A431 cells. A431 cells were acutely and chronically treated with TPA followed by heat shock under the identical conditions as described in the legend to Fig. 1. The cell extracts were next subjected to immunoprecipitation with anti-kinase FA/GSK-3α antibody followed by kinase activity assay in the immunoprecipitates as described under Experimental Procedures. C, control without any treatment; HS, heat shock alone; TPA(A) + HS, acutely pretreated with TPA followed by heat shock; TPA(C) + HS, chronically pretreated with TPA followed by heat shock. Data obtained from five independent experiments and expressed as means ±SD. Significance $^{*}P < 0.05$.

deed the maximal level that could possibly be reached. This can be the reason why chronic effect of TPA is not so profound as the acute effect of TPA in the heat induction of kinase FA/GSK-3α, as shown in Figures 1 and 2. In sharp contrast, the tyrosine kinase inhibitor genistein [Akiyama et al., 1987] and the tyrosine phosphatase inhibitor orthovanadate [Gordon, 1991] were found to have no effect on the heat-shock induction of kinase FA/GSK-3α under identical conditions (Fig. 3), further indicating that the effect of TPA/sphingosine and okadaic acid on kinase FA/GSK-3α in heat-stressed cells is very specific. To confirm the results further, we also measured the cellular activities of kinase FA/GSK-3α in the immunoprecipitates from the cell extracts using the antikinase FA/GSK-3α antibody as described above.

In close correlation with the results as described in Figure 1, the same heat stress could also induce an increase in the cellular activity of kinase FA/GSK-3α up to ~200% of control level, which could be blocked by acute treatment with TPA but could be further activated by chronic treatment with TPA (Fig. 4). Similarly, the heat stress-induced cellular activation of kinase FA/GSK-3α could also be blocked by okadaic acid but could be further potentiated by sphingosine up to ~250% of control level (Fig. 5), in good correlation with the results as described in Figure 2, demonstrating that kinase FA/GSK-3α is a newly described heat stress-inducible protein kinase subjected to cellular activation in heat-shocked cells and may not represent a constitutively active/mitogen-inactivated protein kinase as previously conceived [Woodgett, 1991; Hughes et al., 1993]. Taken together, the results provide

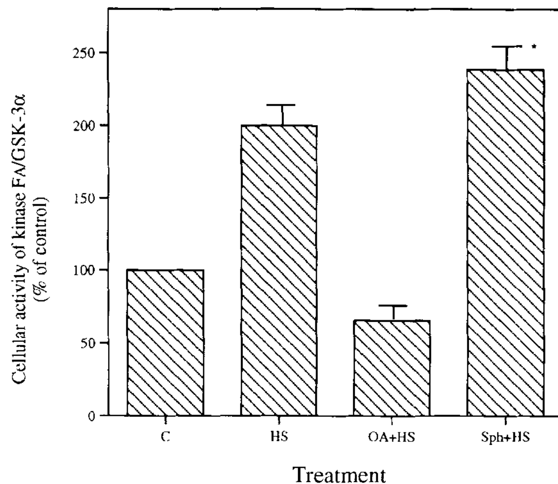

Fig. 5. Effect of okadaic acid and sphingosine on heat-induced cellular activation of kinase FA/GSK-3α in A431 cells. A431 cells were treated with okadaic acid or sphingosine followed by heat shock under the identical conditions as described in the legend to Fig. 2. The cell extracts were next subjected to immunoprecipitation with antikinase FA/GSK-3α antibody followed by kinase activity assay in the immunoprecipitates as described under Experimental Procedures. C, control without any treatment; HS, heat shock alone; OA + HS, pretreated with okadaic acid followed by heat shock; Sph + HS, pretreated with sphingosine followed by heat shock. Data obtained from five independent experiments and expressed as means ±SD. Significance $*P < 0.05$.

initial evidence that the heat induction on kinase FA/GSK-3α can be reversibly modulated by TPA/sphingosine and okadaic acid, suggesting that phosphorylation/dephosphorylation mechanisms are involved in the regulation of the heat-shock induction of kinase FA/GSK-3α in cells.

In comparison with the well-established heat-induction process on heat-shock protein 70 kDa (HSP 70), the results presented here provide a new mode of signal transduction pathway modulating the heat induction process [Sorger and Pelham, 1988; Schlesinger, 1990; Sorger, 1991; Welch 1992; Morimoto, 1993; Sarge et al., 1993]. For instance, protein serine/threonine phosphorylation has been proposed as a key step to activate the heat-shock transcription factors to enhance the expression of heat-shock genes to increase synthesis of HSP70 and okadaic acid has been reported to markedly potentiate the heat-induced HSP70 promoter activity [Chang et al., 1993], leading to an increase in protein level of HSP70 after heat shock [Mivechi et al., 1994]. In sharp contrast, as presented in this study, okadaic acid, possibly via inhibition of protein phosphatases to enhance cellular protein phosphorylation, appeared to induce a decrease in protein level of kinase FA/GSK-3α (Fig. 2, lanes 1–4) (Figs. 1, 2) in heat-stressed A431 cells, presenting an opposite control mechanism for modulating the heat-induction process.

The putative heat-shock transcription factor of kinase FA/GSK-3α possibly subjected to phosphorylation/inactivation by TPA-activated protein kinase C and to dephosphorylation/activation by okadaic acid-sensitive protein phosphatases obviously presents an intriguing issue deserving further investigation. Nevertheless, the present study clearly demonstrates that kinase FA/GSK-3α is a newly described heat stress-inducible protein kinase subjected to cellular activation in heat-stressed cells and the specific protein serine/threonine phosphatases inhibitor okadaic acid and the specific protein kinase C modulators TPA and sphingosine can reversibly modulate the heat-stress induction process on kinase FA/GSK-3α in A431 cells, representing a new mode of signal transduction for the regulation of this multisubstrate/multifunctional protein kinase and a new mode of signaling pathway modulating the heat-induction process.

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