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EFFECT OF STAUROSPORINE ON THE TRANSCRIPTION OF HSP70 HEAT SHOCK GENE IN HT-29 CELLS

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We have investigated the effects of staurosporine (STP), an inhibitor of PKC, on the expression of the HSP70 gene in human colon carcinoma HT-29 cells. Cells were heated at 45°C for 15 min and incubated at 37°C for up to 6 hr with or without STP. When STP (5 μg/ml) was added during 37°C incubation, the accumulation of HSP70 mRNA was suppressed. The suppression of the mRNA accumulation was due to the decrease in initiation and elongation activity of the HSP70 gene. Our study also demonstrated that the effect of STP on the elongation and accumulation of HSP70 mRNA appeared to be selective and the drug did not influence the elongation and accumulation of the house keeping gene, β-actin mRNA. In addition, the drug did not alter the early response of heat shock gene expression, the heat-induced HSF binding activity. These results suggest that STP affects the late response of the transcriptional regulatory system of HSP genes.

The synthesis of heat shock proteins in mammalian cells is a response to stresses such as heat (1), chemical stress (2) or viral infection (3). Synthesis of heat shock proteins has been shown to correlate with the development of thermotolerance (4, 5). Recently we have reported (6) that staurosporine, an antibiotic and a potent protein kinase C (PKC) inhibitor, markedly suppressed thermotolerance development and the expression of HSP70 and HSP28 heat shock genes in colon carcinoma HT-29 cells. In this paper, we further investigated the molecular mechanisms of the effects of STP on the regulation of the expression of the HSP70 gene. We observed that the drug did not alter the early event of heat shock response, i.e. association and dissociation of heat shock factor (HSF) and heat shock element (HSE) complex. However, STP affected the initiation and the elongation of the transcripts of HSP70 gene resulting in a decrease in the level of HSP70 mRNA.

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MATERIALS AND METHODS

Human colon carcinoma HT-29 cells were cultured in McCoy's 5a medium (Cellgro) supplemented with 26 mM $NaHCO_3$ and 10% iron-supplemented calf serum (HyClone). Exponentially growing cultures in T-75 flasks were kept in a 37°C humidified incubator with a mixture of 95% air and 5% CO_2.

The heat treatment was done by total immersion of T-75 flasks in a circulating water bath (Heto) maintained within ±0.05°C of the desired temperature.

Staurosporine (STP, mol. wt. 466.5) was obtained from Sigma Chemical Co. and was dissolved in dimethyl sulfoxide (DMSO). Drug treatment was done by aspirating the medium from the cells and replacing with drug-containing medium. The final concentration of the DMSO added to the medium was 0.1% or less by volume.

The relative level of HSP70 mRNA was determined by the Northern blot analysis. RNA was extracted by the LiCl - urea method (7). Thirty μg of RNA was electrophoresed in a 1% agarose-formaldehyde gel (8), blotted onto nitrocellulose membrane and vacuum baked for 2 h at 80°C. The membrane was prehybridized and hybridized to ^{32}P-labeled HSP70 (pH2.3; 9), or β-actin (10) probes then finally washed as described before (11). Blots were exposed to x-ray film (XR, Fuji Photo Film Co.) in a stainless steel cassette with an intensifying screen. After autoradiography the film was developed with Kodak GBX developer and fixed with Kodak GBX fixer.

The HSF-HSE binding activity was determined by gel mobility-shift assay. Assay conditions, description of the ^{32}P-labeled HSE and the preparation of whole-cell extracts were as published before (12). A double stranded oligonucleotide encoding the HSE portion of the human HSP70 gene (13) was used in the binding reaction with whole-cell extract containing 20 μg protein. The assays were performed at 25°C for 15 min in a final volume of 25 μl. After the incubation, 5 μl of 6X dye solution (0.1% bromophenol blue, 30% glycerol) was added to the reactions and the samples were electrophoresed on a nondenaturing 4.5% polyacrylamide gel for 2.5 hr at 140 V. After electrophoresis, gels were fixed in 7.5% acetic acid for 15 min, rinsed with water for 3 min and dried in a slab gel dryer (Model 483, Bio-Rad) for 1.5 hr at 80°C. The dried gels were autoradiographed on Fuji RX X-ray film for 2-3 days at -70°C.

Nuclear runon assay procedure of Banerji et al. (14) was adapted and used to investigate the effect of STP on the initiation and elongation of the HSP70 gene transcription. Exponentially growing cells in T-150 flasks were rinsed twice with phosphate buffer solution (PBS) and scraped. Cells were pelleted by centrifugation (300X g, 5 min at 4°C), resuspended with lysis buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$ and 0.5% Nonidet P-40) and incubated on ice for 5 min. The nuclei were pelleted (500 *g* for 5 min) and resuspended in lysis buffer then immediately pelleted again. The nuclei were resuspended in storage buffer (50 mM Tris-HCl [pH 8.3], 5 mM $MgCl_2$, 0.1 mM EDTA [pH 8.0] and 40% glycerol), frozen in liquid nitrogen and stored at -70°C. The elongation of nascent RNA transcripts were carried out in a reaction buffer (25 mM HEPES [pH 7.5], 2.5 mM $MgCl_2$, 2.5 mM dithiothreitol, 5% glycerol, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP and 100 μCi per assay ^{32}P-UTP [700 Ci/mmol, ICN]) containing approximately 200 μg of DNA for 45 min at 26°C. Reaction was stopped by the addition of 5 units RNase-free DNase I (Promega) and incubated further for 15 min at 37°C. The nuclei then were lysed with three volumes of SDS/urea buffer (2% SDS, 7 M urea, 10 mM Tris-HCl [pH 8.0], 0.35 M NaCl, 1mM EDTA, 0.5 mg/ml Proteinase K and 100 μg per assay tRNA) and incubated for 1 hr at 45°C. The RNA was precipitated by the addition of two volumes of cold TCA (20%), after incubation at 4°C for 30 min the precipitate was pelleted by centrifugation at 5,000 *g* for 15 min. The pellet was washed twice with cold ethanol and resuspended in

hybridization solution (50% formamide, 5X Denhardt solution, 6X SSC, 0.2% SDS and 100 μg/ml tRNA).

To investigate the effect of STP on the elongation, the protocol was modified as follows. First, STP (0, 0.1, 0.5, 1.0, and 5.0 μg/ml) was added to the runon reaction and second, the *in vitro* transcription reaction was allowed to run only for 20 min. Our preliminary data indicated that the rate of ^{32}P-UTP incorporation was linear during this time (data not shown).

The hybridization of ^{32}P-labeled RNA to cDNA probes was done as published before (12). Plasmids containing the HSP70 cDNA gene (pH2.3; 9), 28S rRNA gene (15) or β-actin (10) were linearized with restriction enzyme (*PstI*), denatured, dot blotted, and immobilized on nitrocellulose membrane by vacuum baking for 2 h at 80°C. For hybridization, blots were preincubated with hybridization solution for 1 hr at 42°C. The ^{32}P-labeled RNA samples were heated at 60°C for 10 min and added to the prehybridized nitrocellulose membranes. Hybridization was carried out for 72 hr at 42°C. After hybridization blots were washed in 6X SSC containing 0.2% SDS at room temperature and washed twice in 2X SSC with 0.2% SDS for 30 min at 65°C. Blots were air dried and autoradiographed as described above. Blots were quantitated by area integration of the optical density of the film using a computerized laser scanning densitometer (model 300A, Molecular Dynamics, Sunnyvale, CA).

RESULTS

The binding of the HSF to the HSE of the promoter of HSP70 gene is the early event of the heat shock response (Fig. 1, lane H). When cells were heated at 45°C for 15 min and further incubated at 37°C, the HSF-HSE complex dissociated after 4 hr incubation (Fig. 1). Apparently the STP (5 μg/ml) treatment did not change the binding activity of HSF and dissociation of the HSF-HSE complex. The HSF binding activity was highly specific to HSE. The signal was eliminated by the addition of 200 fold molar excess of unlabeled HSE (lane HSE in Fig. 1).

The finding that STP does not influence the dissociation of the HSF-HSE complex led us to further investigate the effect of STP on RNA synthesis. We have measured ^{32}P-UTP incorporation into nuclear RNA in isolated nuclei under various conditions. Results from runon assays (Figs.2A and 2B) demonstrate that STP significantly delayed and inhibited the initiation of HSP70 gene transcription. The densitometric analysis of dot blots shows that the amount of initiation of the HSP70 gene increased rapidly and reached maximal value 1h after heat shock at 45°C for 15 min. (Fig. 2B) when the drug was not present during 37°C incubation. However, in the presence of 5 μg/ml STP, the initiation of HSP70 gene transcription was delayed. The rate of transcription increased slowly and reached maximal value 3 h after the initial heat shock (Fig. 2B). Figure 2 also shows the effect of heat shock on the initiation of 28S RNA. The transcription level of the 28S RNA initially was suppressed by heat

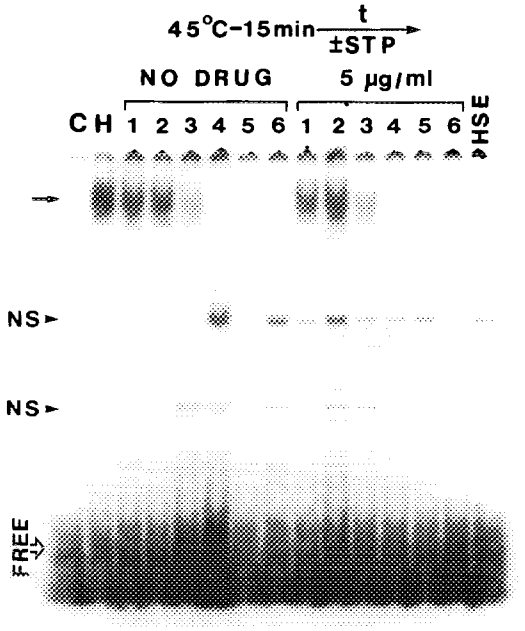

Figure 1.
Effect of STP on the dissociation of heat shock transcription factor-heat shock element complex (HSF-HSE). After heating at 45°C for 15 min cells were incubated at 37°C for intervals marked at the top of each lane without (NO DRUG) or with STP (5 μg/ml) before extraction. Aliquots of cell extracts containing 20 μg protein were used to assay the HSP70 heat shock element (HSE) binding activity in the samples. C = Unheated and untreated control. H = Cells were heated at 45°C for 15 min and extracted immediately after heating. HSE = a 200 fold molar axcess of non-radioactive HSE was added to the reaction before the addition of [^{32}P]HSE. NS = Non specific protein-[^{32}P]HSE complexes. FREE = Unbound [^{32}P]HSE. Arrow indicates the HSE-HSF complex.

shock and recovered after 3 hr incubation at 37°C. To detect the effect of various concentrations of STP on the elongation of HSP70 gene transcripts, nuclear runon assay was performed with or without the drug presence in the reaction mixture (Fig. 3). Data show that the elongation of HSP70 gene transcription was suppressed by 25, 42, 57, and 60% in the presence of 0.1, 0.5, 1.0, and 5.0 μg/ml STP, respectively (Fig. 3). Interestingly, we found little or no effect of STP on the elongation of β-actin mRNA (Figs. 3A and 3B) or on the overall incorporation of ^{32}P-UTP into RNA (data not shown).

To investigate the effect of STP on the level of nuclear and cytoplasmic HSP70 mRNA, we performed northern blot analysis (Fig.4). RNA was isolated from the cytoplasm and from the nucleus of heat treated and control cells in the presence or abscence of STP. Northern blot analysis showed that the level of cytoplasmic HSP70 mRNA increased rapidly over a period of 6 hours. In contrast, the STP (5 μg/ml) markedly suppressed the accumulation of HSP70 mRNA in the cytoplasm (Fig. 4A).

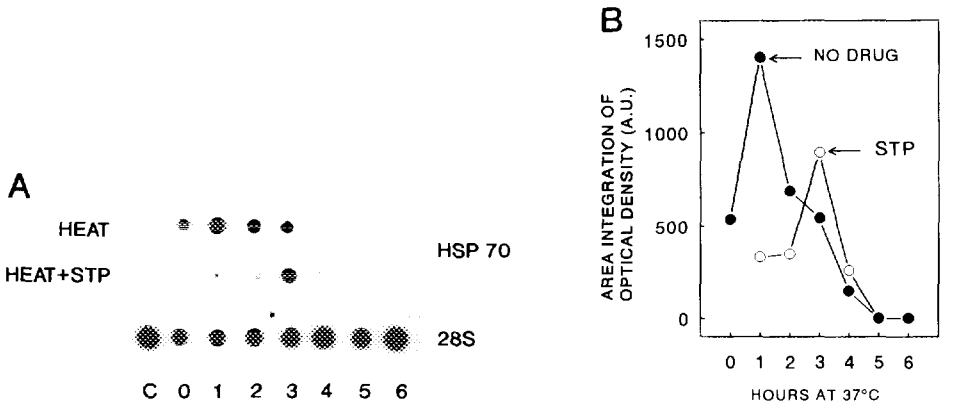

Figure 2.

Effect of STP on the initiation of HSP70 gene transcription. Cells were heat treated at 45°C for 15 min and incubated at 37°C for 0-6 hr as indicated at the bottom of the panel without the drug (HEAT) or with drug (5 μg/ml). Nuclei were isolated and labeled with ^{32}P-α-UTP. The purified RNA transcripts were hybridized to HSP70 or 28S gene probes spoted onto nitrocellulose membrane and (**A**) autoradiographed. C = Nuclei from untreated cells. (**B**) The intensity of the dots was analyzed by densitometry and plotted as a function of hours at 37°C without (●) or with (o)drug .

There was no extraordinary accumulation of HSP70 mRNA in the nucleus during STP treatment. These results suggest that the transport of the HSP70 mRNA into the cytoplasm was not effected by the STP treatment (Fig. 4C). Northern blots have been developed for β-actin mRNA as control. Heat and STP treatment did not influenced the cytoplasmic β-actin mRNA level.

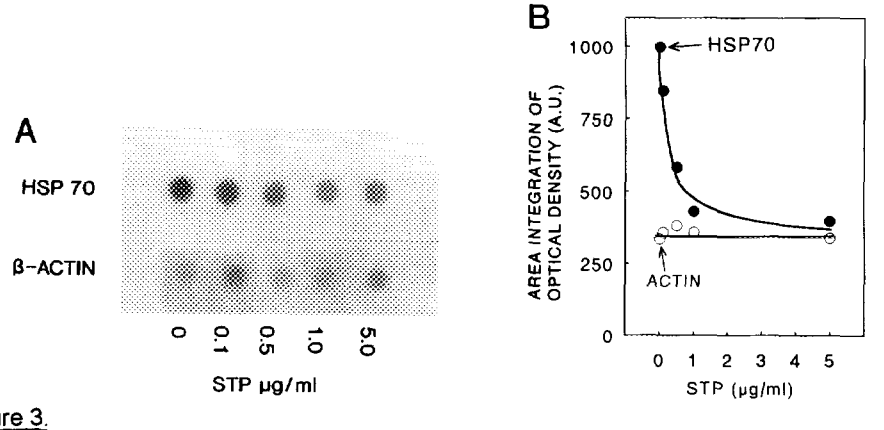

Figure 3.

The effect of STP on the elongation of HSP70 and β-actin mRNA. Cells were heated at 45°C for 15 min and further incubated at 37°C for 1 hr. Nuclei were isolated from the cells and labeled with ^{32}P -α-UTP for 20 min in the presence of different concentrations of STP (0, 0.1, 0.5, 1.0 and 5.0 μg/ml). The *in vitro* transcribed ^{32}P -labeled RNA from the nuclei was isolated and hybridized to HSP70 (●) and β-actin (o) gene probes, autoradiographed (**A**) and analyzed by densitometer (**B**).

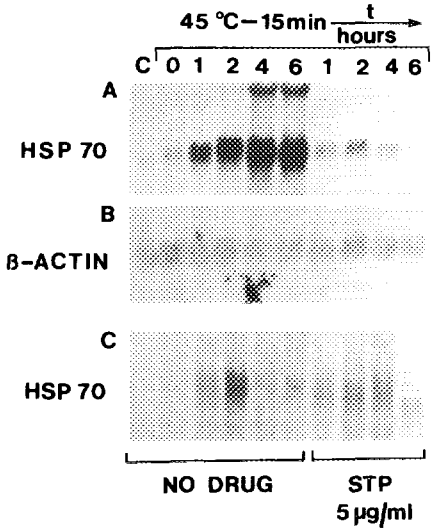

Figure 4.
Northern blots of HSP70 and β-actin mRNA. Cells were heated at 45°C for 15 min and incubated at 37°C for 0, 1, 2, 4, and 6 hours as indicated at the top of each lane in the presence or absence of STP (5 μg/ml). Autoradiographs of (**A**) HSP70 and (**B**) β-actin mRNA from cytoplasm and (**C**) HSP70 mRNA from the nucleus.

DISCUSSION

Our data demonstrate that STP, a PKC inhibitor, considerably suppressed the expression of the HSP70 gene. The STP treatment resulted in a decrease in the level of HSP70 mRNA accumulation (Fig. 4) that was due to suppressed initiation and elongation activity of the gene (Figs. 2 and 3). However, STP did not effect the attenuation of HSF-HSE binding activity (Fig. 1). Interestingly, STP selectively inhibited the transcription of HSP70 mRNA but did not alter the level of a house keeping gene, β-actin mRNA (Figs. 3 and 4). Transcription of the HSP genes requires the proper engagement of the RNA polymerase II in the promoter of the gene and binding of the activated HSF to the HSE (16, 17). The activation of HSF might require the process of a trimerization step and postranslational modification such as phosphorylation (18). Kinases, specificaly cAMP dependent protein kinase (PKA) (19) and as well PKC (6) have been implicated in the signal transduction pathway of heat shock . Recently Lee et al. (20) have reported that PKC rather than PKA is involved in this process probably through the phosphorylation of the HSF or other transcription factor(s) of the HSP70 gene. Heat shock activation of PKC could occur through the activation of phosphatidyl inositol-4,5-bisphosphate phosphodiesterase that is involved in production of IP3 and diacylglycerol (21), activators of PKC. The activated PKC may

translocate into the nucleus and activates the expression of the HSP70 gene by phosphorylation of the HSF and/or by phosphorylation of other transcription factor required for HSP70 gene transcription. Initially STP has been characterized as a specific PKC inhibitor (22) but it also has been shown to have antiproliferative effect by inhibiting the activation of receptor tyrosine kinases (23, 24). In addition, STP inhibits asialoglycoprotein internalization with an action independent from the inhibition of PKC (25) and can sensitize tumor cells to TNF through a kinase independent action (26). Therefore, it should be considered that the action of STP on heat shock genes may not be the result from the inhibition of PKC alone but also from the effects of the drug on the initiation/elongation complex of the heat shock genes independently from PKC. Usheva et al. (27) reported that the phosphorylation of the carboxy-terminal domain of RNA polymerase II is necessary for the transition from an initiation complex to an elongation-competent complex. It has been speculated (18) that HSF may promote the phosphorylation of the carboxy-terminal domain of the largest subunit of RNA polymerase II and results in the elongation of HSP70 gene products. Full transcriptional activation of the HSP70 gene involves at least two phosphorylation steps. The first step is the phosphorylation of HSF, followed by a second one, the phosphorylation of the carboxy-terminal domain of RNA polymerase II. Our results (Fig. 3) indicate that STP specifically interferes with the elongation-competent complex during elongation and effectively decreased the transcription level of the HSP70 gene. It is commonly accepted that the elongation of mRNA is controlled by the general transcriptional apparatus. However, our data suggest that the elongation process of HSP70 and β-actin mRNA is somewhat different. We hypothesize that STP may interfere during early elongation with the recruitment of specific factors necessary to overcome the block in transcription. Alternatively the drug might displace this heat shock gene specific factor(s) causing selective stalling of the RNA polymerase II on the HSP70 gene. At the present time, we have shown that the drug interacts with the transcriptional apparatus of the HSP70 gene at multiple points. But to understand the mechanism which by STP affects the transcriptional regulation of the HSP genes and results in a suppression of the gene expression needs further studies at the molecular level.

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