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4-Hydroxynonenal induces a DNA-binding protein similar to the heat-shock factor

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By using a gel mobility assay, we have shown that treatment of HeLa cells with 4-hydroxynonenal, a major product of the peroxidation of membrane lipids and an inducer of heat-shock proteins, has the same effect as heat shock in causing the appearance of a protein which binds to the sequence of DNA specific for the induction of heat-shock genes. Lipoperoxidation and heat exposure seem to share a common mechanism of specific gene activation.

INTRODUCTION

Exposure to oxygen after anaerobiosis stimulates the synthesis of heat-shock proteins (hsp) in *Drosophila* (Ropp *et al.*, 1983), CHO cells (Sciandra *et al.*, 1984) and liver (Cairo *et al.*, 1985). The same proteins can be induced by a number of oxidants such as H_2O_2 or quinones, and by depletion of intracellular thiols (Levinson *et al.*, 1980; Lee *et al.*, 1983; Bochner *et al.*, 1984; Courgeon *et al.*, 1988). Recently we have shown that lipoperoxidative damage of isolated hepatocytes or cultivated hepatoma cells by ADP–iron or 4-hydroxynonenal (HNE) induces the synthesis of some proteins which are different under these two conditions, but which are always subsets of the proteins appearing in each type of cells after heat shock (Cajone & Bernelli-Zazzera, 1988). At first we could not demonstrate the synthesis of hsp 70, which is the major and best-conserved of the stress-related proteins, but later we were able to detect both the protein and the related mRNA in HNE-treated cells (Cajone & Bernelli-Zazzera, 1989). These observations suggest that oxidative stress can be, at least in part, responsible for the effects of heat shock. However, the demonstration of a common final effect does not necessarily mean that the same mechanism works under both conditions. We have now performed some experiments to investigate this problem. The activation of heat-shock genes requires a specific DNA sequence called heat-shock element (HSE), and a factor (HSF) that interacts with this sequence and has been identified in yeasts (Sorger & Pelham, 1987), *Drosophila* (Wu *et al.*, 1987) and human cells (Kingston *et al.*, 1987). The HSF is regulated differently in yeast and HeLa cells (Sorger *et al.*, 1987). In the latter, a stress-induced HSE-binding activity, when can be revealed in a gel mobility assay, appears following heat shock (Mosser *et al.*, 1988). By using the same technique we have tried to see if treatment of HeLa cells with HNE, which is one of the main products of the peroxidation of membrane lipids, has the same effect as heat shock in causing the appearance or the translocation of a protein which binds to HSE and thus retards its migration on polyacrylamide gels.

MATERIALS AND METHODS

All cell culture materials were obtained from Flow Laboratories Ltd. (Ayrshire, Scotland, U.K.). 4-Hydroxynonenal, purified by t.l.c., was a gift from Dr. A. Benedetti (Istituto di Patologia Generale, Siena, Italy). [γ-^{32}P]ATP (approx. 3000 Ci/mmol) was obtained from Amersham International (Amersham, Bucks., U.K.). Poly(dI-dC) and T_4-polynucleotide kinase were purchased from Boehringer (Mannheim, Germany). HSE oligonucleotide was synthesized by Dr. G. Saglio (Dipartmento di Scienze Biomediche ed Oncologia Umana, Torino, Italy). All other reagents used were of analytical grade.

Cell culture

HeLa cells were grown in tissue culture flasks (75 cm^2) in Eagle's Minimum Essential Medium supplemented with 10% (v/v) calf serum.

Cell treatment

All experiments were carried out during the exponential phase of growth. Before each experiment, the cell culture medium was removed and replaced by a Hepes/bicarbonate buffer containing 118 mM-NaCl, 5 mM-KCl, 2.5 mM-$CaCl_2$, 1.2 mM-KH_2PO_4, 1.2 mM-$MgSO_4$, 5 mM-$NaHCO_3$ and 20 mM-Hepes, pH 7.4. The standard exposure for heat-shock induction was for 1 h at 43 °C. 4-Hydroxynonenal dissolved in water was added directly to the cell incubation medium at a final concentration of 150 μM, and then the cells were incubated for 1 h at 37 °C.

Preparation of cell extracts

Whole cell extracts were prepared from two flasks according to the procedure described by Mosser *et al.* (1988), and the extracts were frozen in liquid N_2 and stored at −80 °C. Whole cell extracts are a better choice than more purified preparations for comparisons between multiple samples, because variable losses inherent in subcellular fractionation are not incurred (Zimarino & Wu, 1987).

Abbreviations used: hsp, heat-shock protein; HNE, 4-hydroynonenal; HSE, heat-shock element; HSF, heat-shock factor.

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Gel mobility assay

The HSE probe is a double-stranded DNA obtained by annealing two synthetic oligonucleotides containing bases −116 to −87 of the human Hsp 70 promoter. [^{32}P]HSE was prepared by 5′-end-labelling one strand (sense strand) with T_4-polynucleotide kinase and [γ-^{32}P]ATP as described by Maniatis *et al.* (1982). The radiolabelled strand was then purified by polyacrylamide-gel electrophoresis and annealed to the complementary strand. The binding reaction mixture (25 μl) contained 10 mM-Tris (pH 7.8), 5% (v/v) glycerol, 50 mM-NaCl, 1 mM-EDTA, 0.5 mM-dithiothreitol, 0.5 μg of poly(dI-dC) and approx. 0.1 ng of [^{32}P]HSE. Between 5 and 15 μg of whole-cell extracts were used for each binding reaction.

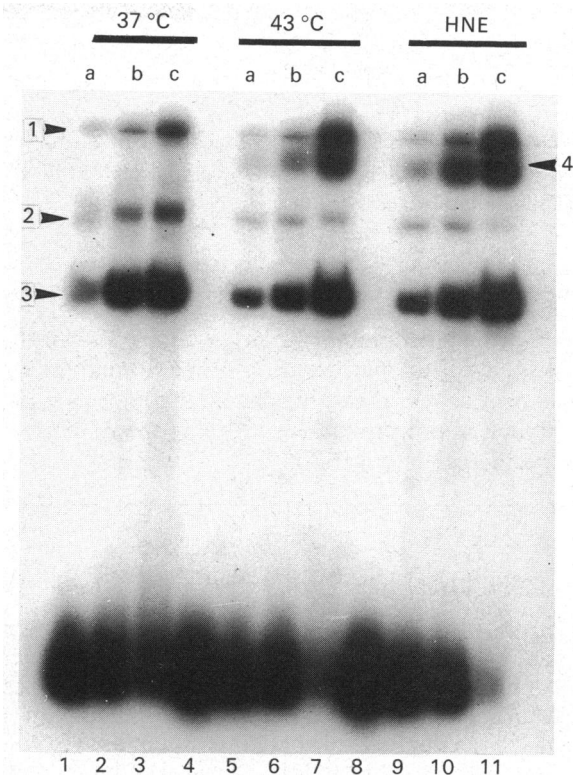

Fig. 1. Gel mobility shift assay performed with a [^{32}P]HSE oligonucleotide and different concentration of cell extracts

Extracts were prepared from HeLa cells maintained at 37 °C (lanes 1, 2 and 3), cells incubated at 43 °C for 1 h (lanes 5, 6 and 7) or incubated at 37 °C in the presence of HNE (lanes 9, 10 and 11). Gel mobility shift assays contained 5 μg (a) 10 μg (b) or 15 μg (c) of proteins. Lanes 4 and 8 lacked extracts. Arrows indicate shifted bands of DNA–protein complexes. Uncomplexed DNA is at the bottom of the lanes.

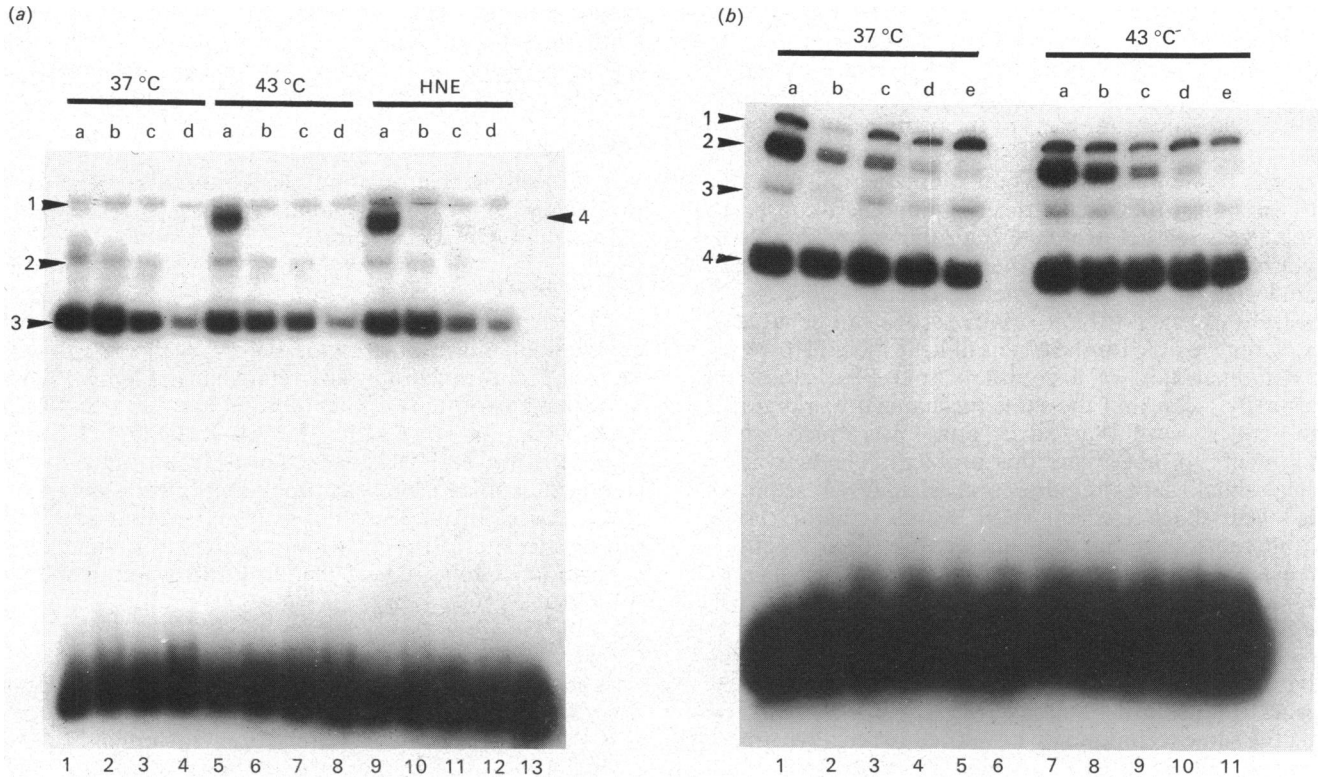

Fig. 2. Gel mobility shift assay performed with a [^{32}P]HSE oligonucleotide and different concentrations of unlabelled HSE

(*a*) Extracts (8 μg of protein) prepared from HeLa cells maintained at 37 °C (lanes 1, 2, 3 and 4), cells incubated at 43 °C for 1 h (lanes 5, 6, 7 and 8) or cells incubated at 37 °C in the presence of HNE (lanes 9, 10, 11 and 12) were used. Lane 13 is without extract. Unlabelled HSE at a 20-fold molar excess (b), 50-fold molar excess (c) or 200-fold molar excess (d) was added where indicated. Lanes a were without competitor. Arrows indicate shifted bands of DNA–protein complexes. Uncomplexed DNA is at the bottom of the lanes. (*b*) As in (*a*), with lower concentrations of competitor ranging from 5–20-fold molar excess. Treatment of the cells as in (*a*): without competitor (a), 5-fold excess (b), 10-fold excess (c), 15-fold excess (d) and 20-fold molar excess (e) of unlabelled HSE. Lane 6 is without extract.

For self-competition experiments, an excess of unlabelled HSE (0.5–20 ng) was added to the binding mixture. The binding reaction was performed at 25 °C for 20 min; the samples were then cooled in ice, 5 μl of dye solution (0.01% Bromophenol Blue/0.05% Xylene Cyanol/5% Ficoll) was added, and samples were loaded on to a 5% polyacrylamide gel in 45 mM-Tris (pH 8.2)/1 mM-EDTA/53.4 mM-boric acid. Gels were run at 5 °C for 3 h at 20 mA and exposed to X-ray films at −70 °C.

RESULTS AND DISCUSSION

Electrophoretic analysis of the mixture containing the HSE-like oligonucleotide and extracts of HeLa cells grown at 37 °C (Fig. 1) shows three retention bands whose intensity is proportional to the amount of protein used in the binding reaction. In addition to these three bands, the extracts of heat-shocked HeLa cells induce the appearance of a new band which is also of an intensity proportional to the amount of extract protein. This new product (band 4) seems to possess a higher affinity for HSE than the band-2 protein of the control sample; the latter, which is proportional to the amount of loaded protein in the cells incubated at 37 °C, decreases in intensity with increasing amounts of protein in the extracts of heat-shocked cells. When the ratio of ligand/probe becomes higher, band-4 protein competes successfully with band-2 protein for the oligonucleotide probe. The results obtained with HNE-exposed cells are superimposable on those of heat-shocked cells.

In the experiments reported in Fig. 2, the amount of protein in the extracts used in the binding reaction was kept constant at a level where the labelled oligonucleotide is never limiting. Under these conditions, one can estimate the competitive effect of a large excess of unlabelled oligonucleotide on the different retention bands. The binding is more specific when the competition is greater. The strongest effect is observed with band-4 protein which appears in heat-shocked and HNE-treated cells; therefore, band-4 protein seems to be the true HSF. In this case also, results with HNE and heat exposure are superimposable.

The demonstration that HNE causes the binding of a specific protein to HSE, in the same way as does exposure to heat, suggests that the stimulation of hsp 70 synthesis induced by this compound occurs by a transcriptional mechanism and confirms our previous observation that hsp 70 mRNA accumulates in HNE-treated as well as in heat-shocked cells (Cajone & Bernelli-Zazzera, 1989). Furthermore, it is clear that a product of lipoperoxidation, rather than events occurring during the process such as the release of active radical species, is an adequate trigger of hsp synthesis. It is still uncertain if HNE plays a direct role in the activation of HSF or if it acts indirectly via denaturation of pre-existing proteins, as it is known that accumulation of abnormal proteins could be implicated in the onset of the stress response (Munro & Pelham, 1985). This dilemma is common to all of the agents that induce the synthesis of hsps, and is further complicated by difficulties in separating the events related to the stress itself and those related to recovery.

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