Free Rad. Res. Comms., Vol. 7, No. 3–6, pp. 189–194
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THE ACTION OF 4-HYDROXYNONENAL ON HEAT SHOCK GENE EXPRESSION IN CULTURED HEPATOMA CELLS

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Hep G2 cells do not synthesize heat-shock proteins when incubated with ADP-iron, under conditions that can trigger lipoperoxidation. However, exposure of these cells to added 4-hydroxynonenal (HNE), one of the main products of lipoperoxidation, induces the synthesis of hsp70, the most conserved among heat-shock proteins. HNE acts mainly on transcription: in Hep G2 cells the increase in the steady-state level of hsp70 mRNA is detectable by two different hybridization techniques.

KEY WORDS: Lipid peroxidation, heat-shock, heat-shock proteins, heat-shock genes.

INTRODUCTION

Exposure to heat induces a physiological response, which comprises the synthesis of a set of proteins known as heat-shock proteins (hsp).[1–3] The same proteins can be induced by a number of oxidants, such as hydrogen peroxide or quinones, and by the depletion of intracellular thiols caused by diamide, iodoacetamide or cadmium.[4–7]

In a previous paper we have shown that lipoperoxidative damage induces the synthesis of a subset of hsp and we concluded that lipoperoxidation can be implicated, at least in part, in the response of protein snythesis to heat exposure.[8] To obtain a better insight into the mechanism of action of lipoperoxidation we have now studied the induction of one specific protein, hsp70 which is the best known among hsp, and of the related mRNA. Cells were treated with ADP-iron, a condition that trigger lipoperoxidation, or exposed to a major product of lipoperoxidation such as 4-hydroxynonenal. The response to these agencies was compared to the effect of heat shock.

MATERIALS AND METHODS

Cell cultures

Rat hepatoma (MH_1C_1 cell line) and human hepatoma (Hep G2 cell line) were grown in monolayer in Eagle Minimun Essential Medium supplemented with 10% calf serum.

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Cell treatment

All experiments were carried out during the exponential phase of growth. The culture medium was removed and replaced by a Hepes-bicarbonate buffer:NaCl (118 mM), KCl (5 mM), $CaCl_2$ (2.5 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.2 mM), Na-bicarbonate (5 mM), Hepes (pH 7.4, 20 mM) and a methionine-free mixture of 19 L-aminoacids at 5 times the concentration found in the rat serum.[9]

Lipid peroxidation was induced by the adenosine diphosphate-iron complex (2.5 μM-ADP, 100 μM-$FeCl_3$) or 4-hydroxynonenal (150'μM) added directly to the incubation medium, then the cells were incubated for 1 h at 37°C. Heat-shock was carried out by immersion of the culture flask in a water bath for 1 h at 42°C.

After hyperthermia (or lipid peroxidation) Hepes-bicarbonate buffer was renewed and [^{35}S] methionine was added (100 μCi/ml) and labelling of protein was performed at 37°C for the next 2 h. Protein synthesis was stopped by addition of cycloheximide (final concentration 150 μM) and cooling in ice-water.

Gel electrophoresis

Proteins were purified for electrophoretic analysis as described.[8] Samples containing equal amount of radioactivity were loaded onto the gel. One-dimensional (1D) gel electrophoresis was performed using the method of Laemmly.[10] Gels were processed for fluorography and exposed to Kodak XAR5 films at −70°C. Heat shock proteins (hsp; hsps) are identified by their molecular range × 10 calculated from the migration of the following ^{14}C-labelled markers: phosphorylase B (Mr = 100000), bovine serum albumin (Mr = 69000), ovalbumin (Mr = 46000), carbonic anhydrase (Mr = 30000).

Northern blot analysis

Total RNA was extracted with guanidine-HCl 8M.[12] RNA (10 μg) was run in 1.2% agarose gel containing formaldehyde and Northern blotted to nitrocellulose filter.[13]

Slot blot assay

Four different amounts (1–4 μg) of total RNA extracted as described before were transferred to nitrocellulose filters (Schleicher and Schuell BA85—SB) and equilibrated with 20 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M Sodium citrate [pH 7].

Hybridization

Nitrocellulose filters were baked for 2 h at 80°C and prehybridized for 12 h at 65°C in a solution containing 3 × SSC, 10 × Denhardt solution (1 × is 0.2% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 0.01% SDS, and 50 μg of sheared salmon sperm carrier DNA per ml. The denatured ^{32}P-labelled probes were added and hybridization was continued for 24 h. The filters were subsequently washed four times for 20 minutes in 0.2 × SSC and 0.1% SDS at 65°C and exposed to Kodak XAR5 films at −70°C.

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Hybridization probes

The probe for hsp70 (human gene) was a 2.3 kb fragment obtained after Hind III-Bam HI digestion from the pH 2.3 sublcone provided by R. Morimoto.[14] The probe for a "housekeeping" mRNA used as control – a gift from J.G. Sutcliffe – was a 0.9 kb fragment obtained from the cDNA clone p1B15 after Pst I digestion.[15]

RESULTS

The human hepatoma cell line Hep G2 responds to 4-hydroxynonenal (HNE) treatment with the synthesis of the most conserved heat inducible hsp, hsp70. On the contrary in these cells the ADP-iron treatment is ineffective (Fig. 1). In order to study the mechanism of induction of hsp70 by HNE, we measured the steady-state levels of

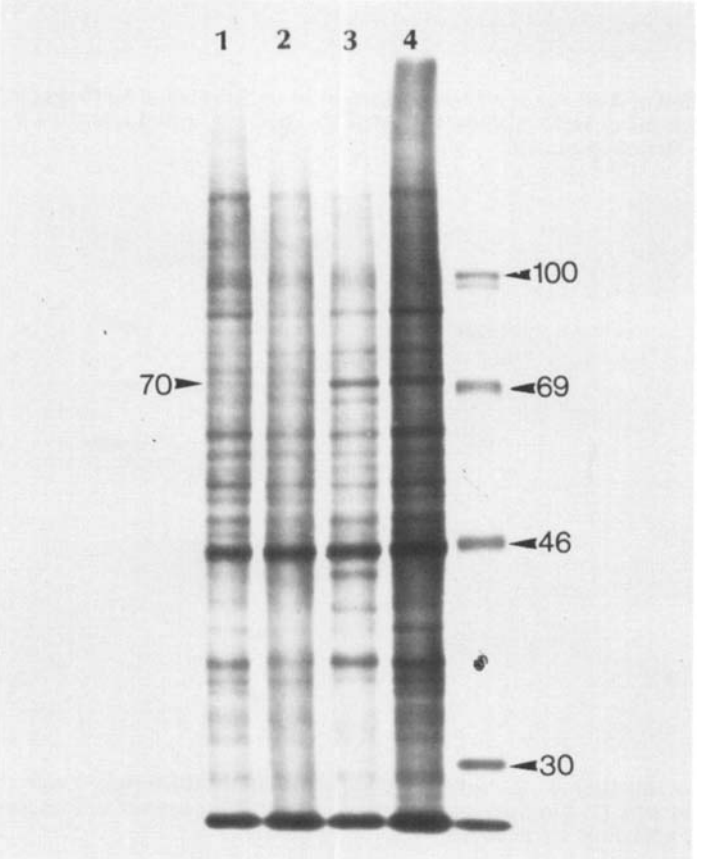

FIGURE 1 Effect of ADP-iron and 4-hydroxynonenal on protein synthesis by Hep G2 cells. Fluorograms of 1D gel electrophoresis of ^{35}S-methionine labelled proteins: (1) Control (2) ADP-iron treated cells. (3) 4-hydroxynonenal treated cells. (4) Heat-shocked cells. Molecular weight markers are shown on the right of the tracing.

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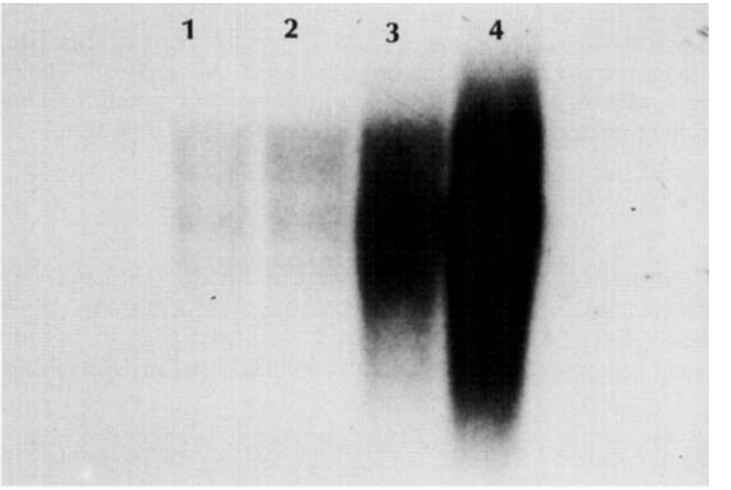

FIGURE 2 Effect of ADP-iron and 4-hydroxynonenal on transcription of hsp70 gene in Hep G2 cells. Northern blot analysis of hsp70 mRNA: (1) Control. (2) ADP-iron treated cells, (3) 4-hydroxynonenal treated cells. (4) Heat-shocked cells.

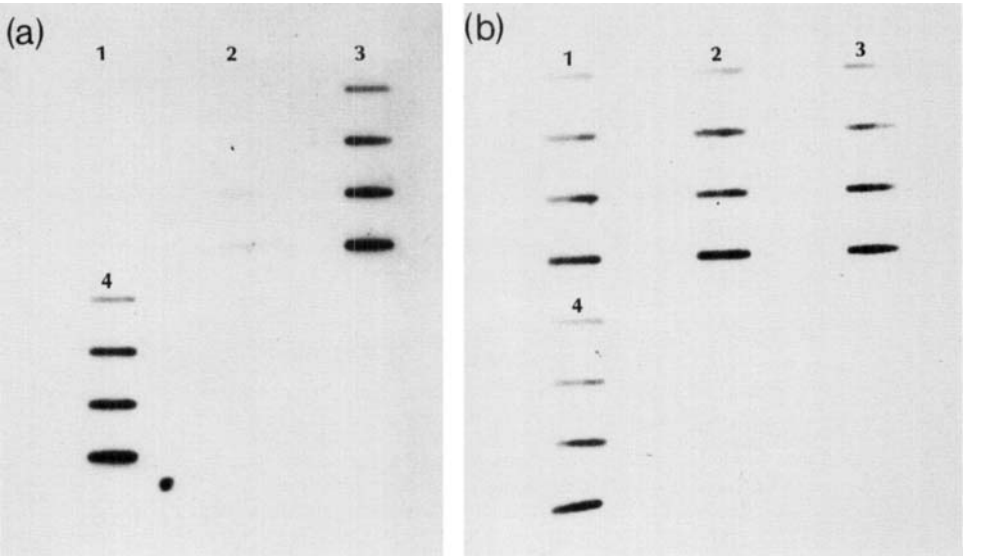

FIGURE 3 Slot blot assay of hsp70 mRNA (A) and plB15 mRNA (B) in Hep G2 cells: (1) Control. (2) ADP-iron treated cells. (3) 4-hydroxynonenal treated cells. (4) Heat-shocked cells. Increasing amounts (1–4 μg) of total RNA from top to bottom.

hsp70 mRNA both after heat-shock and oxidative stress. Results obtained with Hep G2 cells by Northern blot analysis, a technique that minimizes the cross hybridization of the probe with different mRNAs, are shown in Figure 2; hsp70 gene is strongly expressed after heat-shock and well expressed also upon HNE treatment, but

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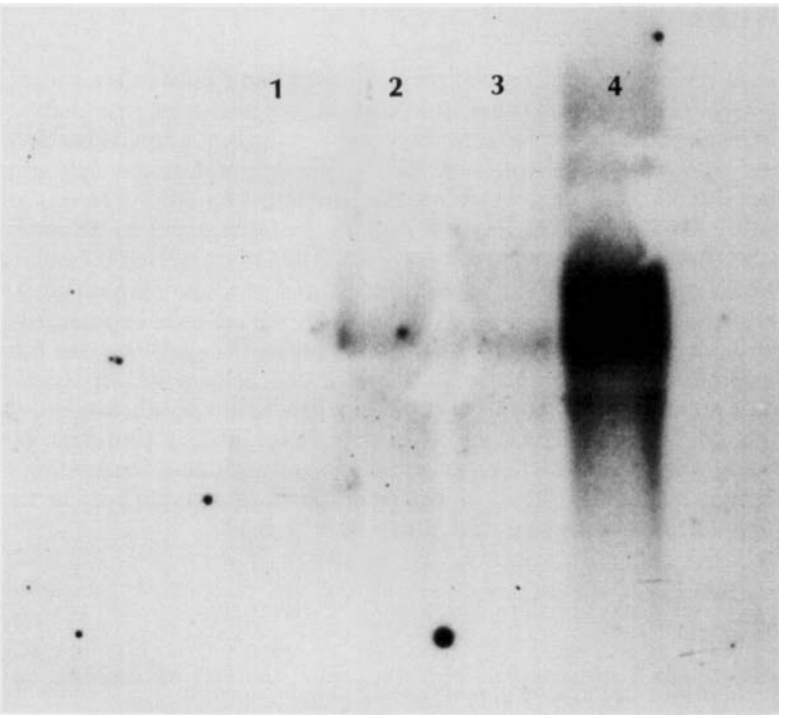

FIGURE 4 Northern blot analysis of hsp70 mRNA in MH_1C_1 cells: (1) Control. (2) ADP-iron treated cells. (3) 4-hydroxynonehal treated cells. (4) Heat-shocked cells.

exposure of Hep G2 cells to ADP-iron does not raise the steady-state level of hsp70 mRNA. Figure 3 shows a Slot blot assay of mRNA. This technique allows a better calibration of the amount of total RNA used for hybridization. The expression of hsp70 gene is shown in Figure 3a: the expression of this gene is easily seen and, under these conditions, at approximately the same level in both heat-exposed and HNE-treated cells, but again ADP-iron treatment has no effect. When the same filter is hybridized with a probe for a "housekeeping" mRNA, the levels of expression of the latter gene are the same in all samples, thus confirming the specific nature of the changes in expression of hsp70 gene. The induction of synthesis of hsp70 and the increase in the steady-state level of related mRNA seem to occur in Hep G2 cells only after treatment with HNE, in addition to the expected effect after heat challenge. In a previous paper,[8] we have demonstrated that in another cell line, MH_1C_1 hepatoma, the synthesis of heat-shock proteins was induced both by ADP-iron and HNE, though the main product was a peptide of molecular range higher than hsp70 for which there is no probe available. Therefore we have now tried to measure the level of hsp70 mRNA in MH_1C_1 cells exposed to ADP-iron and HNE. The results, shown in Figure 4 show that both these agents increase the levels of hsp70 mRNA, but the effect is much lower than the one obtained by heat-shock.

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DISCUSSION

The present research completes our previous work[8] and shows that a transcriptional mechanism, which does not exclude the possibility of post-trancriptional modulation, is mainly responsible for the induction of hsp70 – the best known heat shock protein – in cells exposed to ADP–iron and HNE. This interpretation is further supported by the fact that Hep G2 cells, which are not stimulated by ADP-iron but are induced to synthesize hsp70 by HNE, respond only to the latter agent by an increase in the steady-state level of the corresponding mRNA. The failure of Hep G2 cells to respond to ADP-iron can tentatively be interpreted as due to a low amount of P-450 or of enzymes of the peroxidative pathways; but when the cells are exposed to a product of lipoperoxidation there is synthesis of hsp. This fact suggest that the formation of lipoperoxidation products can be part of the mechanism of hsp synthesis upon heat-shock; alternatively heat shock and lipoperoxidation products could stimulate a final common trigger. We expect to gain a better insight into this problem by investigating if lipoperoxidation as a process, or products therefrom, can cause changes in the heat-shock factor – which is known to activate heat-shock genes – comparable to those occurring during exposure to heat.

Acknowledgements

We than Mr. Angelo Rossi for skillful technical assistance. This work was supported by CNR grants 87.01182.44 P.F. Oncologia and CT 87.01312.04.115.19891.

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Accepted by Prof. T.F. Slater

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