Free Rad. Res. Comms., Vol. 3, No. 1–5, pp. 129–139
Photocopying permitted by license only

Printed in Great Britain

OXIDATIVE STRESS AND HEAT SHOCK PROTEIN INDUCTION IN HUMAN CELLS

ROY H. BURDON, VERA M. GILL and CATHERINE RICE-EVANS†

Department of Bioscience and Biotechnology, University of Strathclyde, Glasgow;
† Department of Biochemistry, Royal Free Hospital Medical School, London, UK

(Received July 21st 1986)

Agents which induce heat shock protein synthesis in cultured monolayers of Hela cells such as hyperthermia, ethanol and sodium arsenite can also cause increases in the levels of lipid peroxidation as determined by the formation of TBA-products. The heat induced increases may be diminished by addition to the medium of mannitol or EGTA. These compounds are known to depress heat shock protein synthesis.

Following hyperthermia there is also a decrease in protein synthesis. *In vitro* studies indicate possible damage to ribosomes, and since the heat induced loss of protein synthetic capacity can be increased by superoxide dismutase inhibitors, and prevented by mannitol, such effects may be linked to the increases observed in lipid peroxidation. It is suggested that a connection exists between lipid peroxidation and heat shock protein gene activation.

KEY WORDS: Heat shock proteins, hyperthermia, ethanol, sodium arsenite, oxidative stress, lipid peroxidation.

INTRODUCTION

When human cells are exposed to temperatures a few degrees above their normal growth temperatures, cell killing can result.[1] However when the treatment is sub-lethal, the cells exhibit changes in the patterns of transcription and translation which are known as the 'heat-shock response.'[2] A dramatic feature of this response is the massive increase in synthesis of a small number of 'stress' proteins known as the heat shock proteins.[3,4] Broadly their function appears to be to protect cells from the stressful temperature conditions.[5]

The heat shock protein genes are also activated by various agents other than heat (e.g. ethanol, sodium arsenite) and there are data now accumulating to suggest that the presence within cells of 'abnormal' proteins serves to trigger such gene activity.[6,7] Recently a model has been proposed for the transcriptional regulation of heat shock protein genes based upon competition for degradation between 'abnormal cellular proteins and a labile regulating factor.[7] How heat shock protein gene inducers such as heat, as well as ethanol and arsenite, give rise to the 'abnormal' proteins still remains uncertain. In this report we suggest that such inducers might bring about protein damage through free radical mediated reactions, which may be crucial for the mechanisms of heat shock protein gene activation.

EXPERIMENTAL PROCEDURES

Cell cultures

Hela cells were grown as monolayer cultures in the Glasgow modification of Eagle's minimal essential medium (Gibco Biocult Laboratories, Paisley) supplemented with 10% calf serum.[8]

Incorporation of L-^{35}S-methionine into protein

0.5×10^6 Hela cells were seeded into plastic petri dishes (3.5 cm) and allowed to grow for 24 h at 37°C before being subject to hyperthermia as previously described,[8] or exposed to ethanol (5% v/v) or sodium arsenite (50 μM). Cells were subsequently labelled, as appropriate, with L-^{35}S-methionine and the level of ^{35}S-incorporation into protein determined as previously described.[9] In certain cases the labelled proteins were also analysed for heat shock protein synthesis by dodecyl sulphate-polyacylamide gel electrophoresis, as previously described.[3]

In vitro protein synthetic capacity

Monolayer cultures of Hela cells of 2×10^7 cells were seeded and grown for 2 days in rotating 80 oz Winchester bottles. Where appropriate, hyperthermic treatment was administered as previously described for such cultures.[10] After treatment lysates capable of initiating *in vitro* protein synthesis were prepared in media containing 100 μM haemin by the procedure previously described by Weber *et al.*[11] Protein synthesis was then determined as described by Weber *et al.*,[11] but following the incorporation of L-^{35}S-methionine (634.8 Ci/m mol) into protein in the presence of mixture of 19 unlabelled amino acids. Normally 40 μg of lysate protein were assayed in a reaction volume of 80 μl containing the additions detailed by Weber *et al.*[11] At appropriate time intervals, 10 μl samples were taken for determination of ^{35}S-incorporation into protein. The purified eIF-2 and ribosomal sub-units, 60 S and 40 S (rat liver) were gifts from Dr. D. Leader, Department of Biochemistry, University of Glasgow, Scotland U.K.

Metabolic stability of cellular proteins

Hela cell cultures of 0.5×10^6 per dish were grown over night and then labelled for 24 h with 2 μC L-^{35}S-methionine (634.8 Ci/m. mole). After this period, the monolayer cultures were washed two times with fresh non-radioactive medium and then returned to 37°C for a further 24 h. At this point cultures were taken for determination of ^{35}S-radioactivity in protein as previously described.[9] These cultures served as zero-time controls. Other cultures were then incubated at 37°C, or 45°C, with, or without, various additions to determine the level of loss of ^{35}S-radioactivity from intracellular protein. In some cases at zero-time, the medium was replaced with fresh medium containing serum, in other cases with fresh medium but minus serum.

Metabolic stability of cellular RNA

The procedure used here was analogous to that used above for assessing protein stability, except that the Hela cell cultures were initially labelled with 2 μC each of

^{3}H-uridine (41 Ci/m mol). To determine the level of ^{3}H-radioactivity in RNA at appropriate times after various treatments, the cultures in question were washed three times with 5% trichloroacetic acid and the cell monolayer dissolved in 880 μl 0.4 M NaOH, neutralised with 20 μl glacial acetic acid and the radioactivity determined.

Lipid peroxidation in Hela cell cultures

Levels of lipid peroxidation were determined using a procedure to determine 2-thiobarbituric acid (TBA) reactive material based on that described by Walls *et al.*[12] and comments of Gutteridge.[13]

Monolayer cultures of Hela cells were established with 10^7 cells in 20 ml medium, in a flat plastic culture vessels of 175 cm^2. The cells were allowed to grow to 2.5 $\times$ 10^7 (3 days) before exposure to the various experimental conditions described in the text.

After treatment, each monolayer culture was washed three times with 0.9% NaCl

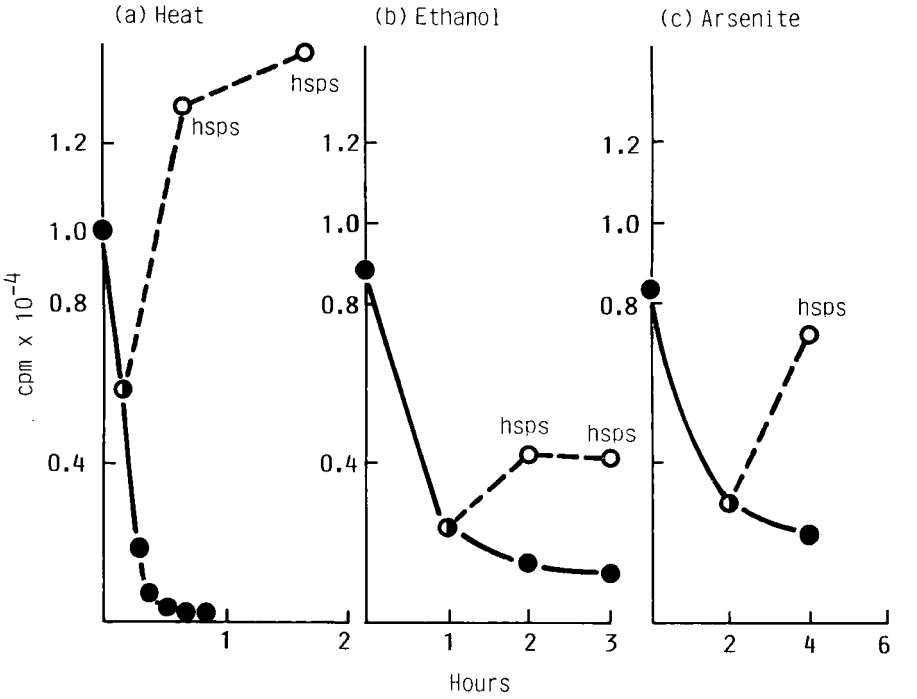

FIGURE 1 Effect of various heat shock protein inducers on incorporation of ^{35}S-methionine into Hela cell protein. (a) Hela cell cultures were held at 45 C for various times and then labelled with ^{35}S-methionine for 30 min at 37 C (●—●). Some cultures were removed after 10 min at 45 C and returned to 37 C for various times before labelling with ^{35}S-methionine for 30 min at 37 C (○- - -○). (b) Hela cell cultures were exposed to 5% ethanol for various times, then labelled with ^{35}S-methionine for 30 min at 37 C (●—●). Some cultures were washed two times with fresh medium, returned to 37 for various times before labelling with ^{35}S-methionine for 30 min at 37 C (○- - -○). (c) Hela cell cultures were exposed to 50 μM sodium arsenite for various times, then labelled with ^{35}S-methionine for 30 min at 37 C. (●—●). Some cultures were washed two times with fresh medium, returned at 37 for various times before, labelling with ^{35}S-methionine for 30 min at 37 C (○- - -○). In (a), (b) and (c) the increased synthesis of heat shock proteins (especially heat shock protein 70) is indicated by 'hsps'.

(w/v) and then the cells scraped off into 2 ml 0.9% NaCl for malonaldehyde measurement. Trichloroacetic acid was added to a final concentration of 10% (w/v) and the suspension centrifuged at 2000 g for 5 min. 1 ml of the supernatant was removed and added to 1 ml 0.75% 2-thiobarbituric acid. Samples were heated at 90°C for 20 min and then centrifuged at 10,000 g for 10 min. The levels of thiobarbituric acid reactive products chromogen in each supernatant fraction were determined by measuring the difference between absorbances at 532 and 580 nm.

RESULTS

A notable effect of heat shock, ethanol exposure or arsenite treatment of Hela cells is an inhibition of protein synthesis (Fig. 1). If the treatments are brief then there is some recovery of protein synthetic ability and a dramatic increase in the synthesis of heat shock proteins, particularly those in the region of 70 kDa.[9] Both the recovery of protein synthesis and heat shock protein induction, appear to require RNA, as they are blocked by low levels of actinomycin D.[9]

If the cultured cells are pretreated with diethyldithiocarbamate, to deplete intracellular levels of superoxide dismutase,[14] then the initial inhibitory effects of heat treatment are more pronounced (Fig. 2a). However if the pretreatment is with 5 mM

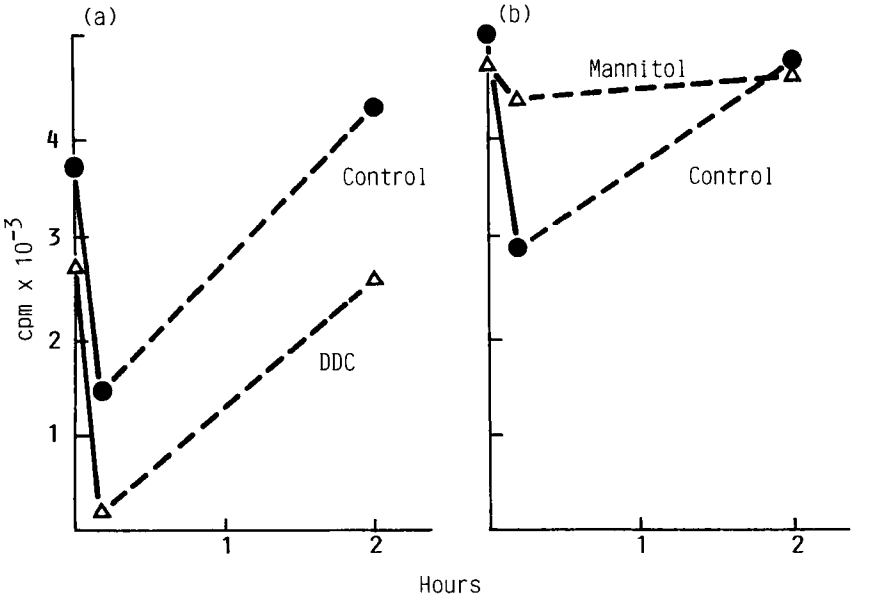

FIGURE 2 Effects of diethydithiocarbamate and mannitol on the heat induced inhibition of ^{35}S-methionine incorporation into protein. (a) Hela cells cultures were pretreated with 1 mM diethyldithiocarbamate (DDC) for 2 h at 37° before subjecting them to 45°C for 10 min (△—△) and returning them at 37°C for 2 h (△- - -△). Untreated control cells at 45°C for 10 min (●—●) and then at 37°C for 2 h (●- - -●). (b) Similar to (a) but cells pretreated with 5 mM mannitol (△) and untreated (●). The incorporation of ^{35}S-methionine into protein after the above treatments is measured over 30 min at 37°C (see Experimental Procedures).

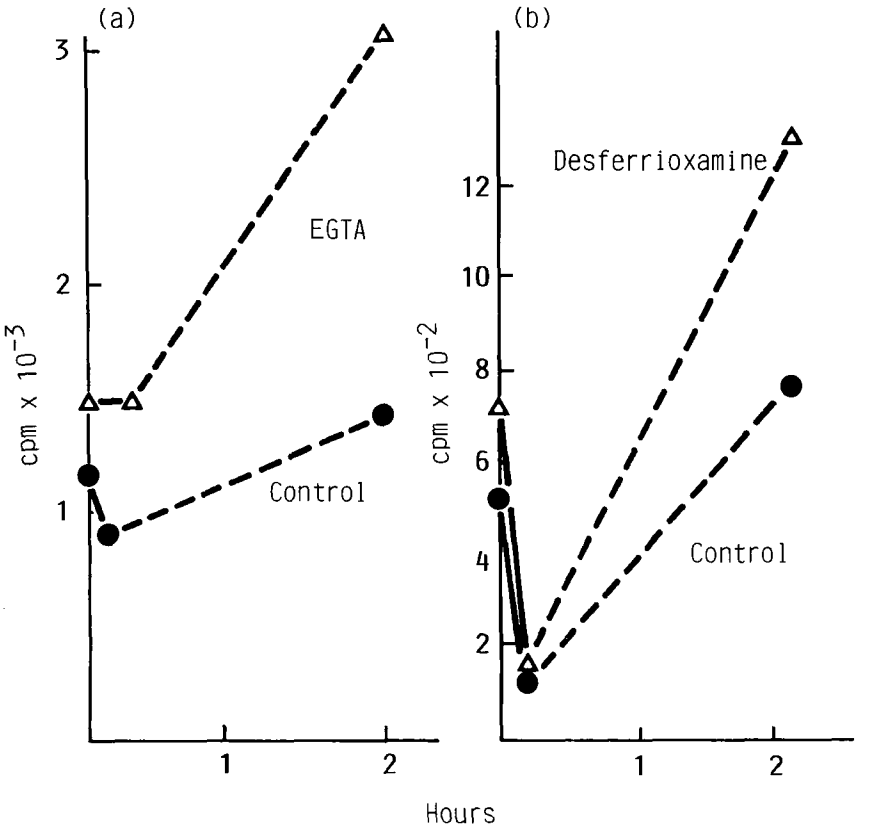

FIGURE 3 Effects of EGTA and desferrioxamine on the heat induced inhibition of ^{35}S-methionine incorporation into protein. The procedure used was similar to that employed in Fig. 2. In (a) cells pretreated with 0.5 mM EGTA for 15 min (△) and untreated control cells (●). In (b) cells pretreated with 0.4 mM desferrioxamine for 15 min (△) and untreated control cells (●).

mannitol (a scavenger of ·OH radicals)[5] then the inhibition is far less marked (Fig. 2b). Moreover there is very little induction of heat shock protein synthesis, suggesting that the initial inhibitory effects on general cellular protein synthesis is somehow linked to the heat shock protein induction process. Another means of reducing the initial inhibitory effect of heat treatment on cellular protein synthesis, is the inclusion in the medium of the calcium-chelator, EGTA (0.5 mM), for 15 min beforehand (Fig. 3a). As was the case with mannitol, induction of heat shock protein synthesis is not observed, again suggesting an important role for the initial heat induced inhibition of protein synthesis in heat shock protein induction. Inclusion of the iron-chelator desferrioxamine,[16] on the other hand does not have any significant effect on the initial inhibition (Fig. 3b).

Whilst these studies were initially aimed at understanding the reasons for the protein synthesis inhibition elicited by heat treatment other experiments indicated that mannitol would also protect the protein synthetic system from the inhibitory effects of arsenite, and to a lesser extent from ethanol exposure.

In order to examine the possible reasons for these various effects on protein synthesis, studies on the overall stability of cellular RNA and protein were initially carried out. From Table I it can be seen that heat, or ethanol, or arsenite treatment, all cause the loss of stable RNA from Hela cells. The effect of heat treatment on RNA is particularly marked in the cells deprived of serum. These losses are however not reduced if mannitol or EGTA is included in the medium. Studies on the stability of total cellular protein also indicated losses from cells exposed to heat, ethanol or arsenite (Table II). As was the case for the RNA losses, these were not prevented by mannitol or EGTA in the medium and thus may not be directly relevant to the early protein synthesis inhibition.

To investigate the reasons for the heat induced early inhibition of protein synthesis, studies were then carried out with cell-free protein synthetic systems prepared from control and heat treated Hela cells. From Fig. 4 it will be seen that the protein synthetic capacity of lysates prepared from briefly heat-treated Hela cells is markedly less than that encountered in lysates from untreated cells. (Lysates from arsenite or ethanol treated cells also exhibited low activities). Because of the composition of the preparation medium and the components normally added to these *in vitro* assays[11] the reduced activity in the lysates from the heated cells is not likely to be due to ATP loss or ion imbalance (ie. K^+, Na^+, Mg^{2+}, Ca^{2+}). Since the addition of glutathione, NADPH or glucose-6-phosphate has no restorative effects, the addition of total Hela cell RNA to the lysate from the heated cells was examined. However, this was without effect. On the other hand when fresh ribosomal subunits (60S and 40S) were added

TABLE I

The effects of heat shock protein inducers on cellular RNA stability

Expt.	Medium	Additions	Duration of 'chase' (min)	Temperature of 'chase'	^{3}H-radioactivity in RNA (cpm × 10^{-3})
1	normal	none	0	37°C	15.49
	normal	none	60	37°C	15.26
	normal	none	60	45°C	14.03
	normal	arsenite (50 μM)	60	37°C	14.14
	normal	ethanol (5%)	60	37°C	9.02
	serum-free	none	60	37°C	14.08
	serum-free	none	60	45°C	8.38
2	normal	none	0	37°C	52.95
	normal	none	60	37°C	56.18
	normal	none	60	45°C	47.81
	normal	mannitol (50 mM)	60	45°C	45.72
	normal	EGTA (0.5 mM)	60	45°C	47.59
	serum-free	none	0	37°C	52.94
	serum-free	none	60	37°C	52.57
	serum-free	none	60	45°C	38.80
	serum-free	mannitol (50 mM)	60	45°C	37.65
	serum-free	EGTA (0.5 mM)	60	45°C	37.21

Monolayer cultures of Hela cells were grown for 24 h, labelled with ^{3}H-uridine for 24 h, then incubated with unlabelled medium for a further 24 h before commencement of the 'chase' as detailed in the Experimental Procedures. The 'chase' was carried out at 37° and 45° with or without the various additions indicated. In some cases the medium was replaced for the period of the 'chase' but lacking the normal serum component.

TABLE II

The effect of heat shock protein inducers on cellular protein stability

Medium	Duration of chase (min)	Temperature	^{35}S-radioactivity in protein (cpm)
normal	0	37°C	345
normal	180	37°C	320
normal	60	45°C	308
plus arsenite (50 μM)	180	37°C	171
plus ethanol (5%)	180	37°C	266

Monolayer cultures of Hela cells were grown for 24 h, labelled with L-^{35}S-methionine for 24 h, then incubated with unlabelled medium for a further 24 h before commencement of the 'chase' as detailed in the Experimental Procedures. The 'chase' was carried out at 37° and 45° with and without the various additions indicated.

to lysates from the heat-treated cells a partial restorative effect was observed (Fig. 4). Such an approach suggests the possibility that damaged, or inactivated, ribosomes, rather than RNA loss *per se* may contribute to the reduced protein synthetic capacity. It is of course also possible that other components of the system become inactivated during the heat treatment. For instance experiments in which purified eIF-2 was added to the lysates, also indicated a restorative effect, although this was not sustained (Fig. 5), possibly due to the presence of inactivated ribosomes in the lysates from the heated cells.

Having indicated possible inactivation of protein components of the protein synthetic apparatus following treatment with heat, the nature and causes of such inactivation remain to be understood. A pointer however comes from consideration of another cellular stress which results in heat shock protein induction. This is the reoxygenation of mammalian cells following a period of anoxia.[17,18] This has led to speculation that oxygen derived free radical activity in this particular instance may be an element in the induction mechanism. This raises the possibility that other inducers such as heat, ethanol and arsenite, might also elicit free radical activity, which might be an element in a trigger mechanisms involving macromolecular damage.

The ability of heat, ethanol or arsenite to elicit cellular lipid peroxidation was then examined by measuring the formation of thiobarbituric acid reactive products as shown in Table III. Both ethanol and arsenite treatment of the monolayer cultures resulted in notable increases in lipid peroxidation. A significant response to heat treatment however was more difficult to detect with this technique, at the cell densities that were employed. Nevertheless a significant increase in intracellular lipid peroxidation was observed if the serum component of the medium was removed prior to the heat treatment. Under such conditions the response could also be diminished if mannitol or EGTA were included in the medium.

DISCUSSION

The observation that there are increases in lipid peroxidation following treatment of Hela cells with heat, ethanol or arsenite, does not necessarily imply a role in the induction of heat shock proteins. What may be more relevant however is the observation that the increases in lipid peroxidation brought about by heat can nevertheless be modulated by calcium levels in the medium, as well as by free radical scavengers

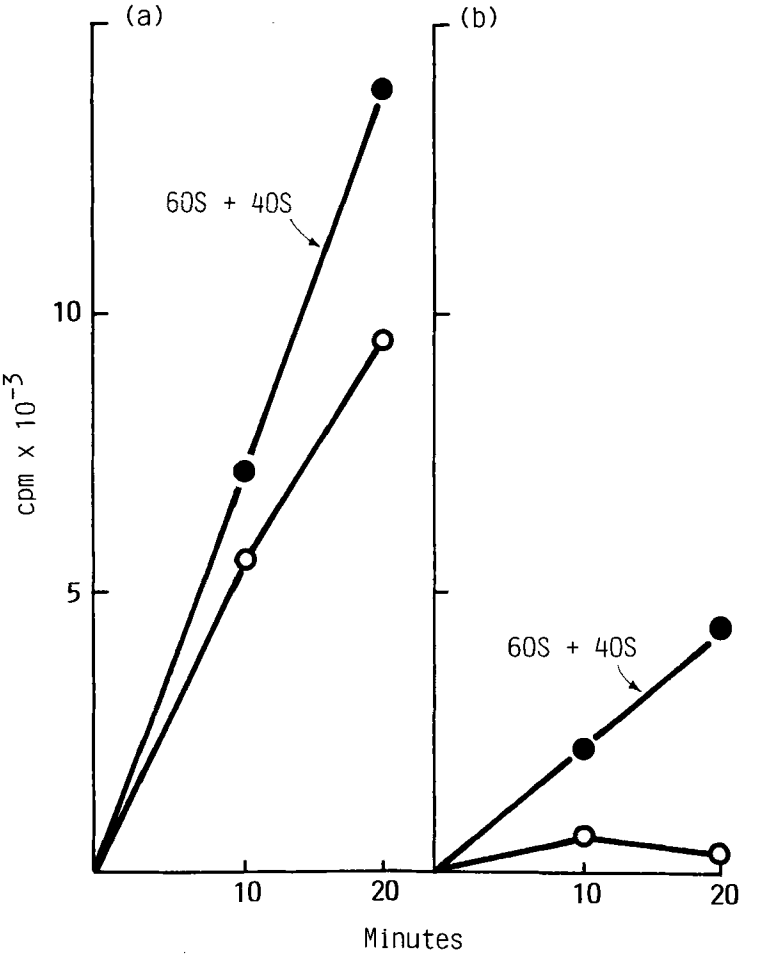

FIGURE 4 Effects of purified ribosomal sub-units on Hela cell *in vitro* protein synthesis. Lysates capable of *in vitro* protein synthesis were prepared from (a) untreated Hela cell monolayer cultures (b) from monolayer cultures heated at 45°C for 10 min (see Experimental Procedures). Protein synthesis was determined by ^{35}S-methionine incorporation into protein in the presence (●) and absence (○) of added purified rat liver ribosomal sub-units (an equal mixture of 60S and 40S units equivalent to 54 μg RNA in reaction mixtures of total volume 80 μl containing cell lysate equivalent to 40 μg protein, and the other components as described by Weber *et al.*[11]

such as mannitol. These 'modulators' also modify the ability of heat to inhibit overall cellular protein synthesis. Whilst the reasons for the initial inhibition of protein synthesis is not clear, they could in part be an outcome of the general intracellular loss of cellular RNA or protein brought about by heat and other heat shock protein inducers. However the observation that neither mannitol nor EGTA prevented RNA or protein loss suggests that this is not of primary significance. Whilst various covalent modifications of initiation factors e.g. eIF2[19], or ribosomal proteins (e.g. S6

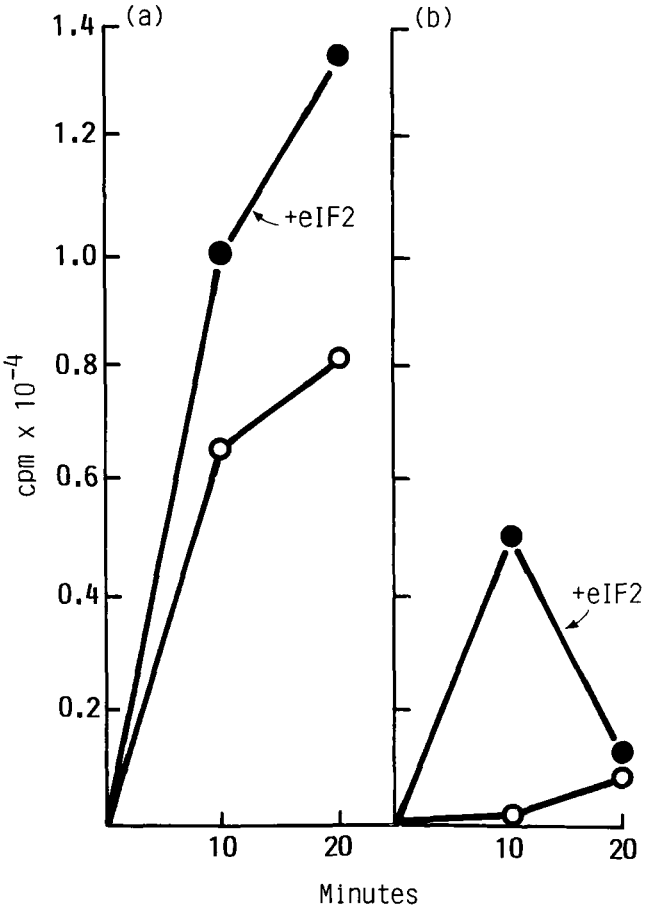

FIGURE 5 Effects of purified initiation factor eIF-2 on Hela cell *in vitro* protein synthesis. The procedure was exactly that used in Fig. 4 except that 6 μg of purified rat liver eIF-2 was added per reaction mixture instead of the ribosomal subunits. ○, no added initiation factor; ●, plus initiation factor.

and L14)[20] may also play a role, possible effects brought about by free radicals should not be discounted. For instance radicals generated in lipid peroxidation reactions might cause alterations or damage to ribosomes and other cell proteins, which may serve as the 'abnormal' molecules proposed as essential for the triggering of heat shock protein gene induction. Particularly relevant therefore the observation of ours, and others,[21] that calcium depletion from the medium prevents the heat induction of heat shock proteins and also prevents heat induced lipid peroxidation and protein synthesis inhibition. Another treatment which produced similar effects is addition of mannitol to the medium. Thus from work with Hela cells at least there is reason to suspect a possible connection between lipid peroxidation and heat shock protein induction which may be of relevance to cellular homeostatic mechanisms.

TABLE III

Effect of heat shock protein inducers on the generation of 2-thiobarbituric acid reactive material in cultured Hela cell monolayers

Expt.	Treatment	n mol TBA reactive (product per 10^{10} cells)
1	37°C 1 h	470.4 ± 40.9
	ethanol (5%) 37°C, 1 h	1147.2 ± 40.9
	arsenite (50 μM) 37°C, 1 h	1290.4 ± 81.9
2	fresh medium 37°C, 3 h	327.6 ± 40.9
	fresh medium 37°C, 3 h then 45°C, 1 h	450.5 ± 204.8
	fresh medium minus serum 37°C, 3 h	368.7 ± 81.9
	fresh medium minus serum 37°C 2 h, then 45°C, 1 h	2027.5 ± 245.7
3	fresh medium minus serum at 37°C, 3 h	266.2 ± 20.5
	fresh medium minus serum 37°C, 2 h then 45°C, 1 h	409.6 ± 20.5
	fresh medium minus serum 37°C 2 h then 45°C, 1 h with mannitol at 50 mM	40.9 ± 61.4
	fresh medium minus serum 37°C, 2 h then 45°C, 1 h with EGTA at 0.5 mM	286.7 ± 40.9

Acknowledgements

This work is partly supported by Grants from Scottish Home and Health Department and Cancer Research Campaign.

References

1. Henle, K.J. and Dethlefsen, L.A. Annals of the New York Academy of Sciences, **335**, 234, (1980).
2. Craig, E.A. *Crit. Rev. in Biochem.*, **18**, 329, (1985).
3. Slater, A., Cato, A.C.B., Sillar, G.M., Kioussis, J. and Burdon, R.H. *Eur. J. Biochem.*, **117**, 341, (1981).
4. Burdon, R.H. in Hyperthermic Oncology, ed. J. Overgaard (Taylor & Francis, London, 1984) Vol. 2 p 223.
5. Li, G.C. and Hahn, G.M. in Hyperthermic Oncology, ed. J. Overgaard (Taylor & Francis, London 1984) Vol. 2 p 231.
6. Finley, D., Ciechanover, A. and Varshavsky, A., *Cell*, **37**, 43, (1984).
7. Ananthan, J., Goldberg, A.C. and Voellmy, R. *Science*, **232**, 522, (1986).
8. Burdon, R.H., Kerr, S.M., Cutmore, C.M., Munro, J. and Gill, V. *Br. J. Cancer*, **49**, 437, (1984).
9. Burdon, R.H., Slater, A., McMahon, M. and Cato, A.C.B. *Br. J. Cancer*, **45**, 953, (1982).
10. Burdon, R.H. and Cutmore, C.M.M. *FEBS Letts.*, **140**, 45, (1982).
11. Weber, L.A., Feman, E. and Baglioni, C. *Biochemistry*, **14**, 5315, (1975).
12. Walls, R., Kumar, K.S. and Hochstein, P. *Archives Biochem. Biophys.*, **174**, 463, (1976).
13. Gutteridge, J.M.C. *Free Radical Research Commun.*, **1**, 173, (1986).
14. Evans, R.G., Nielsen, J., Engel, C. and Wheatley, C. *Radiation Res.*, **93**, 319, (1983).
15. Halliwell, B. and Gutteridge, J.M.C. *Biochem J.*, **219**, 1, (1984).
16. Keberle, H. *Ann. New York Acad. Sci.*, **119**, 758, (1964).
17. Li, G.C. and Shrieve, D.C. *Expt. Cell Res.*, **142**, 464, (1982).

18. Sciandra, J.J., Subjeck, J.R. and Hughes, C.S. *Proc. Natl. Acad. Sci. USA*, **81**, 4843, (1984).
19. De Benedetti A. and Baglioni, C. *Proc. Natl. Acad. Sci. USA*, **261**, 338, (1986).
20. Kennedy, I.M., Burdon, R.H. and Leader, D.P. *FEBS Letts.*, **169**, 267, (1984).
21. Lamarche, S., Chretien, P. and Landry, J. *Biochem. Biophys. Res. Commun.*, **131**, 868, (1985).

Accepted by Prof. H. Sies and Dr. J.V. Bannister