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# Oxygen Free Radicals as Inducers of Heat Shock Protein Synthesis in Cultured Human Neuroblastoma Cells: Relevance to Neurodegenerative Disease

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**Summary.** We studied heat shock protein (HSP) synthesis by cultured human neuroblastoma cells in response to either hyperthermia or high levels of superoxide anion (oxygen free radical). Both treatment modalities resulted in induced synthesis of the same major HSP species with an additive effect on the latter and on cell growth inhibition upon combined treatments. Exposure to superoxide anion in the presence of the free radical scavenging enzymes, superoxide dismutase and catalase improved cell survival and prevented HSP induction. These findings suggest a common mechanism by which various forms of injury, such as hyperthermia, cause HSP induction, that is, via oxidative stress or increased production of oxygen free radicals. Increased expression of some HSPs has been detected in association with the pathological lesions that characterize some neurodegenerative diseases such as the neurofibrillary tangles of Alzheimer's disease. This, in turn, suggests that chronic oxidative stress may play a role in the pathogenesis of these disorders.

**Key words:** Free radicals – Heat shock proteins – Neurodegenerative disease

## Introduction

The heat shock protein response is one of the best recognized phenomena in cell biology. Cells of almost all organisms, from bacteria to man, respond to various forms of physical or chemical stress by the induction or preferential synthesis of characteristic polypeptides, termed heat shock proteins (HSPs) (Lindquist and Craig 1988).

Among serveral known HSP families, two with approximate molecular weight ranges of 85–90 kDa (HSP85) and 68–73 kDa (HSP70) are most conserved and best characterized. Attention has also been recently directed to the "smaller" HSPs that can be found within a molecular weight range of 8–35 kDa (Landry et al. 1989). Among these, ubiquitin, a small HSP of about 8.5 kDa, has been the focus of extensive investigation (Rechsteiner 1988). Protein isoforms representative of these families are normally present (constitutive) in the cytoplasm of most cells under physiological conditions, but following stress, the synthesis of similar inducible variants is markedly increased. In stressed cells, HSPs seem to fulfill a "housekeeping" function critical to cell homeostasis, either by ensuring proper protein assembly, folding and transport or by facilitating proteolytic digestion of abnormal or denatured polypeptides (Beckman et al. 1989; Welch et al. 1989).

The role which HSPs may play in the pathogenesis of nerve cell injury is not yet clear. However, accumulating evidence indicates that HSPs may have a critical function in neuronal metabolism, whereby the HSP response is one of the few means by which survival and normal functioning of the individual neurons can be maintained (Hamos et al. 1991). This is particularly true since neurons become postmitotic early in life and regeneration is not an option in the overall process of protecting neural tissue integrity in face of the various forms of stress that the central nervous system may endure. Indeed, many findings characteristic of neuronal degeneration associated with Alzheimer's disease (AD), such as reduction of polysomal mRNA translation (Langstrom et al. 1989), disruption of cytoskeleton with abnormal protein conformations (Muller-Hill and Beyreuther 1989), increased ubiquitin-protein conjugates (Mori et al. 1987; Perry et al. 1987; Pappolla et al. 1989) and the presence of HSP70 in senile plaques and neurofibrillary tangles (Hamos et al. 1991; Perez et al. 1991) are strong indications that brains in neurodegenerative disorders undergo many aspects of the stress (heat shock type) response.

In addition to hyperthermia, HSP inducers include a variety of seemingly unrelated agents or treatments such as reoxygenation after anoxia, hydrogen peroxide, sulfhydryl reagents, oxidizing quinones, glutathione depleters, uncouplers of oxidative phosphorylation and others (Compton and McCarthy 1978; Ashburner and Bonner 1979; Levinson et al. 1980; Omar and Lanks 1984; Scian-

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dra et al. 1984). Despite their diversity, many of these inducers seem to act through perturbing respiratory metabolism and subsequently contributing to the accumulation of active oxygen species or free radicals (OFRs). Thus, oxidative stress has been proposed as a key mechanism that medicates HSP induction (Ropp et al. 1983; Keyse and Tyrrell 1987; Omar et al. 1987; Becker et al. 1990). This concept has been supported by several other observations. For instance, membrane lipids and cytoskeletal structures, which are among the main targets of OFRs, are also the primary targets of hyperthermic injury, the "classic" HSP inducer (Glass et al. 1985; Konnings and Ruifrok 1985; Cajone and Bernell-Zazzera 1988). Furthermore, we and others have shown that cellular "defense" response to either oxidative or hyperthermic stress includes, in addition to induced HSP synthesis, an induction or increase in intracellular antioxidant enzyme activities, particularly superoxide dismutase (SOD) (Freeman and Crapo 1982; Loven et al. 1985; Omar et al. 1987; Privale and Fridovich 1987; Hass and Massaro 1988) and exogenously introduced antioxidants can block the cytotoxic and HSP-inducing effects of hyperthermia (Burdon et al. 1987). In our previously reported experiments, peak induction of antioxidant enzyme activities took place several hours following the typically immediate onset of induced HSP synthesis, suggesting a possible regulatory relationship within the overall oxidative (hyperthermic) stress response.

Oxygen free radical reactions are ubiquitious in human cell metabolism and play important roles in inflammation, carcinogenesis, aging and other phenomena. OFRs themselves, however, are short-lived due to their highly reactive nature (Freeman and Crapo 1982). Hence, the availability of a tissue marker that denotes their prior existence, such as inducible HSP species, can be a valuable tool in understanding the pathogenesis of many disease processes. In this regard, the strong expression of some HSPs, particularly ubiquitin and HSP70, within the cytopathological lesions that characterize AD and other neurodegenerative diseases (Lowe et al. 1988; Pappolla et al. 1989; Hamos et al. 1991; Perez et al. 1991), supports the hypothesis that oxidative stress plays a role in the pathogenesis of these disorders (Omar and Pappolla 1990; Pappolla et al. 1990b; Volicer and Crino 1990).

In order to add further support to the proposed OFR-HSP connection and its relevance to nerve cell injury, we undertook to study the HSP response in cells of neuronal origin (cultured human neuroblastoma cells) upon their exposure to various forms of oxidative stress.

## Materials and Methods

### *Cells and Culture Conditions*

The human neuroblastoma cells used in this study were derivatives of the established SK-N-AS cell line (Sugimoto et al. 1984) which we obtained from Dr. L. Helson (New York Medical College, Valhalla, N.Y.). Cells were routinely maintained at 37°C and 5% $CO_2$ in Dublecco's Modified Eagle's Medium (DMEM) supplemented with antibiotics and 15% heat-inactivated fetal bovine serum. Monolayers were seeded in 60 mm tissue culture dishes at a density of $10^4$ cells/cm$^2$ and maintained in the above medium until subconfluence (80–90% of growth surface), at which point they were subjected to the various treatments. Two dishes were set up for each treatment mode, one dish to be used for determining cell survival and the other for studying protein synthesis. Every experiment was repeated at least twice and always included untreated control populations.

### *Hyperthermia and Oxidative Stress*

Exposure to hyperthermia was by incubating cells at 44°C for 1 h in a tissue culture incubator at a similar $CO_2$ concentration and humidity as in the incubator where the cells were routinely maintained. The temperature of this incubator was closely monitored with a precision thermometer and variations were usually within ± 0.2°C.

Oxidative stress was achieved by exposing cultured cells to exogenous superoxide anion ($O_2^-$) or enhancing its intracellular concentration using the drug diethyldithiocarbamate (DDTC). The latter is a copper-chelating agent which inibitis CuZn-SOD, thus leaving its substrate, $O_2^-$, unmetabolized (Frank et al. 1987).

Exposure to exogenous $O_2^-$ was accomplished by incubating cells for 1 h in serum-free culture medium containing 0.10 m*M* xanthine to which xanthine oxidase (0.04 U/ml) was added. $O_2^-$ is generated as a result of this reaction (Flohe and Otting 1984). The incubation was done either at 37°C or 44°C with or without pretreatment with DDTC. The latter was done by incubating cells in culture medium containing 3 m*M* of the drug for 90 min at 37°C followed by 3 × washing with phosphate buffered saline (PBS) before adding the respective treatment medium. Exposure to $O_2^-$ was also done in the presence of either SOD alone (bovine SOD, 50 µg/ml, 2,750 U/mg; Sigma) or in the presence of both SOD and catalase (bovine catalase, 5 µg/ml, 17,600 U/mg; Sigma) in culture medium.

The inhibitory effect of DDTC on SOD activity in this cell line was predetermined by exposing separately prepared cell monolayers to various concentrations of the drug (as described above) and measuring their total SOD activity. The latter was done using the cytochrome *c* reduction method as a previously described (Omar et al. 1987).

### *Cell Survival and Protein Synthesis*

For cell survival studies following treatment, cells were removed from tissue culture dishes by trypsinization, counted in a hemocytometer and viability determined by trypan-blue exclusion. Equal number of viable cells were then seeded in 24-well plates at a density $10^3$/well and the plates reincubated under routine conditions. Cells in 3 wells were subsequently counted every other day for 8 days and growth curves constructed. Relative survival at each point was calculated as percent average cell count from a treated population relative to that of the untreated control. This method was favored over the "cloning" efficiency method due to the inherent low clone-forming ability of this cell line. Both methods, by virtue of their long post-treatment follow-up, allow for assessing the "true" survival rate. The latter is different from the trypan-blue-based viability determination since many dye-excluding cells that initially appear viable are in reality "sterilized" and will die in a few days, if maintained in culture, without dividing and contributing to population growth. As a result, when relative growth curves are constructed, one sees an initial decline in relative growth, which may continue over a variable number of days depending on the severity of the treatment, before if levels off and begins to rise again, signaling a positive balance in favor of the truely viable and dividing cells. Thus, the lowest point in the relative growth curve of a population is the true reflection of treatment effect on cell survival.

To study protein synthesis patterns, after treatment cells were immediately washed with PBS, then incubated in methionine-free DMEM containing [$^{35}$S]-methionine (50–100 µCi/ml; 1,200 Ci/

mmol; New England Nuclear), for 2 h at 37°C and 5% $CO_2$. It is assumed that this labeling time is sufficiently short that the rate of [$^{35}$S] methionine incorporation can be considered to represent the rate of protein synthesis rather than the sum of synthesis an degradation. In general, radiolabeling was started within 10–15 min after termination of the treatment. At the end of the incubation period, monolayers were washed again with PBS, scraped off in 1% sodium dodecyl sulfate (SDS)-1% 2-mercaptoethanol and aliquots containing equal counts per minute (cpm) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography as previously described (Omar and Lanks 1984).

Western blot (immunoblot) analysis of electrophoresed proteins was performed as previously described (Pappolla et al. 1987; Omar et al. 1990a), using monoclonal anti-HSP70 (Oncogene Science, Uniondale, N.Y.) and anti-HSP85 (Stress Gen, B.C., Canada) and an immunoalkaline phosphatase detection technique. In the latter, a chemiluminescent, rather than a chromogenic, substrate (adamantyl-1,2-dioxetane phosphate or AMPPD) was used in accordance with the supplier's protocol (Oncogene Science, Uniondale, N.Y.). The immunoreactive protein bands were visualized by exposing the blot to an X-ray film for 1–10 min.

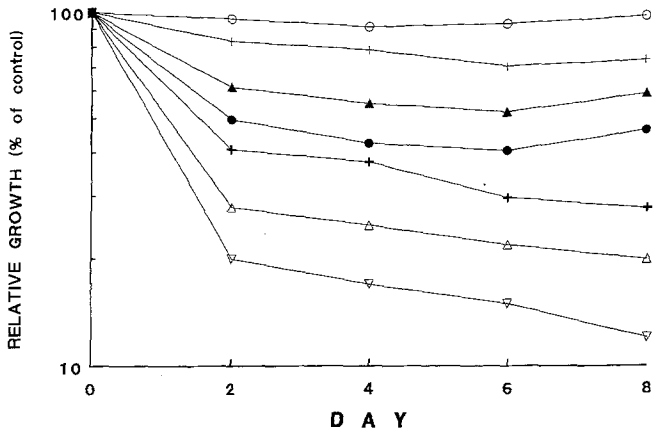


**Fig. 2.** Effect of the various treatments on cell survival. Curves representing the "relative growth" of treated vs untreated (control) cell populations. Each value is the mean of three determinations with a SD ≤ 15% of the mean. —+—, $O_2^-$; —△—, $O_2^-$ + 44°C; —○—, $O_2^-$ + SOD + catalase; —+—, 44°C; —▲—, DDTC; —●—, $O_2^-$ + DDTC; —▽—, DDTC + 44°C

## Results

### *Effect of DDTC on SOD Activity*

The inhibitory effect of various concentrations of DDTC on SOD activity of cultured neuroblastoma cells is shown in Fig. 1. At 3 m*M* concentration, up to 80% of that activity was inhibited.

### *Relative Cell Survival (Growth)*

The effect of the different treatments on relative cell growth is presented in Fig. 2. Typically, hyperthermia (1 h at 44°C) had a significant cytocidal effect by itself. This is reflected by the continued decline of relative population growth through the 8th day. Such a population may actually never recover owing to the small number of viable cells left. However, this is a dose-dependent cytocidal effect rather than a qualitative difference

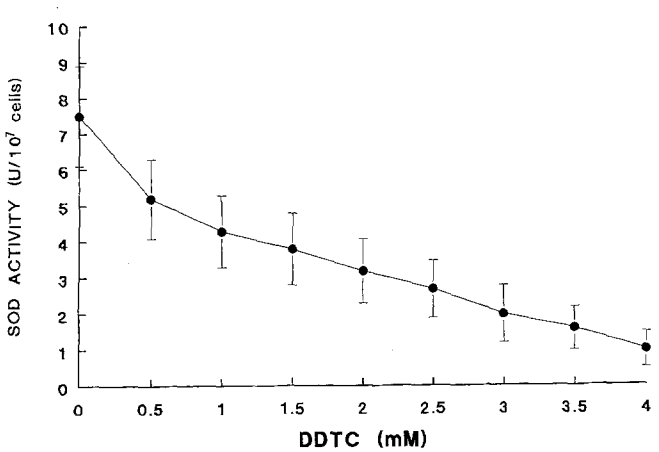


**Fig. 1.** Effect of diethyldithiocarbamate (DDTC) treatment on superoxide dismutase (SOD) activity of SK-N-AS cells. Cells were incubated in culture medium containing 0–4 m*M* DDTC for 90 min at 37°C followed by washing 3 times with phosphate-buffered saline and harvesting cells for enzyme activity assay as previously described. Points show means of six replicates; bars indicate SE

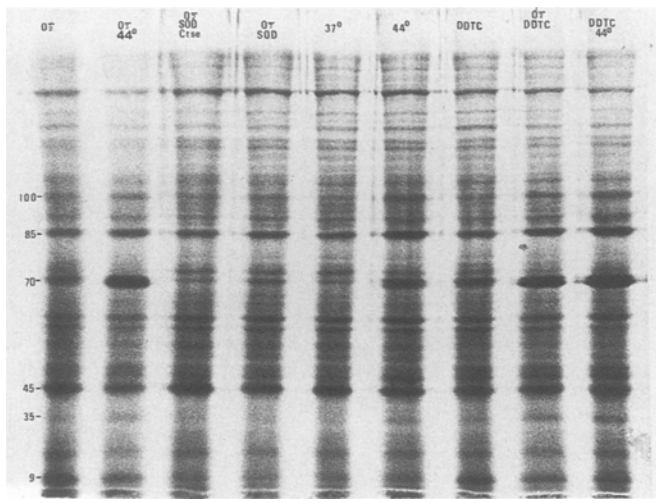


**Fig. 3.** SDS-PAGE of total cell lysates. Autoradiograms of [$^{35}$S]methionine-labeled proteins from cultured SK-N-AS cells which were exposed to various treatments as indicated above individual lanes and as described in Materials and Methods. Molecular weights (× $10^3$) of relevant polypeptides are shown

from the other forms of treatment and a lower temperature could have produced a milder effect with possibly an earlier population recovery. The effect of hyperthermia was potentiated by combining hyperthermia with $O_2^-$ or DDTC treatment. Similarly, the combined effect of $O_2^-$ and DDTC was more significant than the modest cytocidal effect associated with either one of them separately. In case of $O_2^-$ that effect could be substantially reduced or prevented by adding the free radical scavenging enzymes, SOD and catalase.

### *HSP Protein Synthesis*

Patterns of protein synthesis in neuroblastoma cells exposed to various treatments are presented in Fig. 3. The

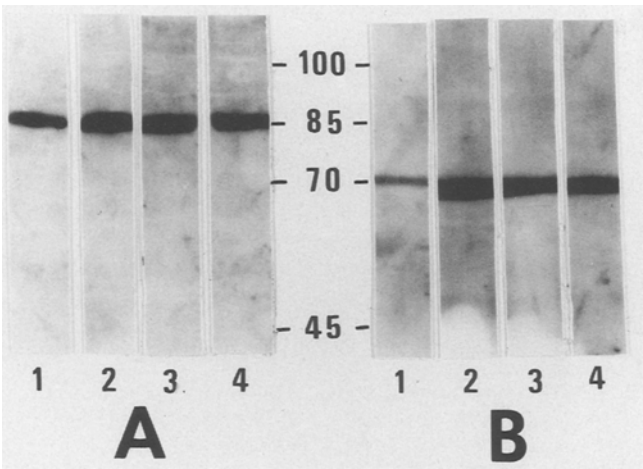


**Fig. 4A, B.** Western blot (immunoblot) analysis of electrophoresed proteins from cells grown at 37°C (lanes A1 and B1), cells exposed to 44°C (lanes A2 and B2), $O_2^-$ (lanes A3 and B3) or DDTC (lanes A4 and B4). Following electrophoresis, proteins were transfered to nitrocellulose sheets and reacted with either anti-HSP85 (**A**) or anti-HSP70 (**B**). Single immunoreactive bands corresponding to HSP85 and HSP70 are seen. Positions of relevant molecular weights ($\times 10^3$) are indicated

basic pattern produced at 37°C is seen, whereby relatively faint bands corresponding to the major HSP species can be recognized. Characteristically, exposure to 44°C resulted in the enhanced synthesis of several HSP species, the most prominent of which were HSP 85 and HSP 70. The identity of these HSPs was confirmed by Western blot analysis (Fig. 4). Less prominent bands corresponding to HSP100 and some small HSPs (apparent molecular weights between 8 and 35 kDa) can also be seen. Exposure to either $O_2^-$ or DDTC alone induced the synthesis of the same HSPs, although to a lesser extent, which was potentiated by added exposure to hyperthermia or by combined exposure to both $O_2^-$ and DDTC. Exposure to $O_2^-$ in the presence of SOD and catalase almost totally inhibited HSP induction while SOD alone permitted a slight degree of synthesis enhancement.

## Discussion

We have shown that increased concentration of superoxide anion in the environment of cells from neuronal origin is associated with enhanced HSP synthesis and reduced cell survival, as is the case with hyperthermia. Both treatment modalities seem also to have an additive or synergistic effect on these parameters, suggesting a similar mode of injury, i.e., via OFR reactions.

Among the induced HSP species, the HSP of approximate molecular weight of 32–35 kDa has been previously shown by other investigators to be the most responsive to oxidative stress such as in reoxygenation following extended anoxia (Ropp et al. 1983) or upon treatment with hydrogen peroxide (Keyse and Tyrell 1987). However, induction of other HSPs, particularly HSP 70, under conditions of oxidative stress has also been reported (Salo et al. 1991). In our experiments, although HSP 85 and HSP 70 were the most strongly induced, a mild induction of a 32–35 kDa protein band could also be seen.

Our findings support the hypothesis that HSP response is mediated by increased production of OFRs. The mechanism by which OFRs may induce HSP synthesis is not known. Low levels of OFRs, particularly $O_2^-$, are routinely produced as byproducts of normal metabolism, but are quickly neutralized or "scavenged" by a variety of low molecular weight reductants and specialized antioxidant enzymes. Typically, $O_2^-$ is metabolized by SOD into hydrogen peroxide which is, in turn, metabolized into water and molecular oxygen by catalase and glutathione peroxidase (Freeman and Crapo 1982). OFRs in excess of cellular antioxidant defenses can enter in destructive chain reactions with various biomolecules such as nucleic acids, membrane lipids and cytoskeletal components. Accumulation of thus-generated aberrant or denatured molecules has been suggested as a key triggering mechanism for the activation of HSP genes (Ananthan et al. 1986). This could be simulated in vitro by inducing synthesis of abnormal proteins with amino acid analogues (Kelley and Schlesinger 1978). In vivo, several human diseases are characterized by intracytoplasmic accumulation of abnormal cytoskeletal proteins in the form of "inclusion bodies." Examples of such are "Mallory bodies" in alcoholic liver disease, "Lewy bodies" in Parkinson's disease, "neurofibrillary tangles" in AD and "Pick's bodies" in dementia of Pick's type. In all these diseases, the presence of ubiquitinilated conjugates has been demonstrated (Lowe et al. 1988) and occasionally, increased expression of some other HSPs, particularly HSP 70, has also been found (Omar et al. 1990a, b; Hamos et al. 1991; Perez et al. 1991). Furthermore, we have recently shown (Pappolla et al. 1992) increased immunoreactivity for SOD and catalase within tangle-bearing neurons of AD. These data strongly suggest that chronic oxidative stress plays an important role in the pathogenesis of AD and other cytodegenerative disorders. Support for this possibility can be found in numerous reports describing typical OFR types of injury in association with aging and AD. These include increased protein and lipid peroxidation (Oliver and Ahn 1987; Subbarao et al. 1990), changes in membrane composition and permeability (Schroeder 1984; Keyser et al. 1990), cytoskeletal derangements (Lowe et al. 1988; Pappolla et al. 1990a) and antioxidant enzyme abnormalities (Zelman et al. 1989; Somerville et al. 1991).

The function of many HSPs remains unclear. However, it is assumed that their increased levels are associated with enhanced ability of some cells to survive subsequent injury. It has been shown that ubiquitin is covalently conjugated to denatured or aberrant cell proteins that are generated under physiological or stressful conditions, thus making them more susceptible to either lysosomal or non-lysosomal proteolytic systems aimed at ridding the cells of such metabolic byproducts (Goff et al. 1988; Mayer et al. 1991). The other major HSPs may help protect critical cellular molecules by "coating" or forming "loose" aggregates with them during cellular

stress or by facilitating the process of ubiquitinilation and protein degradation through their own proteolytic or ATPase activity (Schlesinger 1986; Lindquist and Craig 1988). They probably also participate in the reparative process by "chaperoning" the proper assembly, folding and transport of nascent polypeptide species (Beckman et al. 1989; Welch et al. 1989). It is also possible that some HSPs may have an antioxidant potential, either as free-radical scavengers or as inducers of antioxidant enzymes (Omar et al. 1987; Polla 1988; Becker et al. 1990). Overwhelming of these defenses leads to the accumulation of abnormal metabolites, complexed with HSPs, in the form of the already mentioned inclusion bodies. Conversely, failed clearance of damaged proteins in some diseases may be due to inhibited or abnormal HSP response, in which case inadequate amounts of these "protective" polypepetides are made. For instance, it has been shown that in response to stress, aged human fibroblasts synthesize less HSP 85 and HSP 70 than young cells (Lu et al. 1989), which leaves open the possibility that some cases of AD may actually show decreased neuronal expression of some HSPs. This, however, seems to be the exception rather than the rule, since an increase in HSP expression has been found in the majority of AD brains thus far studied (Hamos et al. 1991; Perez et al. 1991).

The relevance of the OFR-HSP connection, as demonstrated in our data, to neurodegenerative disease lies not only in improving our understanding of disease pathogenesis, but also in suggesting new diagnostic-prognostic and therapeutic avenues. For the former, the amount of inducible HSPs in brain tissue may serve as a measure of the oxidative (metabolic) stress sustained by nerve cells. For the latter, "preventive" antioxidant therapy as well as "preventive" induction of cross-tolerance to oxidative stress (by raising HSP levels via prior treatment with a low, HSP-inducing but cell-sparing, dose of a relatively harmless, HSP inducer, such as mild hyperthermia) may be considered.

Obviously, these and other possible aspects of oxidative stress and HSP connection to CNS pathology remain in need of further investigation.

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