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Oxidants differentially regulate the heat shock response

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Cells, animals, and humans respond to hyperthermia through the synthesis of a family of proteins termed heat shock proteins (HSPs). Because hyperthermic stress may also result in mitochondrial uncoupling and the generation of reactive oxygen species, we wondered whether oxidant stress was sufficient to increase cellular levels of HSP70. HSP70 was detected in cells heated or treated with menadione but not in those treated with hydrogen perioxide or xanthine/xanthine oxidase. We speculate that oxidant stress from menadione exposure is qualitatively different from exposure from hydrogen peroxide or xanthine/xanthine oxidase.

Key words: Heat shock, menadione, oxidants.

1. Introduction

Cells respond to hyperthermic stress by the rapid synthesis of a family of proteins termed heat shock proteins (HSPs). The accumulation of these proteins within cells is associated with the development of tolerance to subsequent, otherwise lethal heat stresses (McAlister and Finkelstein 1980, Li and Werb 1982, Subjeck *et al.* 1982, Landry and Chrétien, 1983) for a review (Moseley 1994). By analogy, the response to oxidant stress is mediated, in part, through the production of a group of anti-oxidant defense proteins including copper-zinc and manganese superoxide dismutase (CuZnSOD, MnSOD), catalase, and the glutathione peroxidases. As is the case with the heat shock proteins, prior elevation of these antioxidant enzymes confers tolerance to subsequent oxidant stresses.

Interestingly, there is a considerable overlap in both the stresses that induce these systems and their abilities to confer tolerance across stresses. For example, hydrogen peroxide exposure as well as heat shock induces resistance to a subsequent hydrogen peroxide exposure (Spitz *et al.* 1987). Cells that have become H_2O_2 resistant are more resistant to a 43 but not a 45°C continuous heat shock (Spitz and Li 1990). In *salmonella*, hydrogen peroxide exposure has been shown to induce proteins that overlap with proteins produced after heat exposure (Morgan *et al.* 1986). Ionizing radiation has also been shown to increase levels of HSP 70 (Sierra-Rivera *et al.* 1993). Freeman *et al.* have shown that the accumulation of the constitutive HSP 70 (HSC 70) is a two-step process, involving first the destabilization of protein structure and then a recognition of the denatured protein (Freeman *et al.* 1995).

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Gluthathione levels have also been shown to be important in a cell's ability to resist heat shock and develop thermotolerance. Glutathione was found to be necessary for protection from intracellular oxidative damage caused by heat shock (Freeman *et al.* 1990). After a reduction in the intracellular levels of reduced gluthatione, rat embryos were less resistant to heat stress even though HSP 70 message levels and synthesis of HSP were not affected (Harris *et al.* 1991). Depletion of glutathione or prevention of glutathione synthesis resulted in inhibition of the development of thermotolerance (Russo *et al.* 1984). Hyperthermia of perfused rat liver results in significant reduction in glutathione levels (Skibba *et al.* 1989). Oxidation of protein thiols after depletion of cellular glutathione levels has been shown to increase levels of HSP 70 (Sierra-Rivera *et al.* 1994). Glutathione has also been shown to increase to 120–200% of control values after exposure to heat shock (Mitchell and Russo 1983).

Endotoxin exposure, which increases HSP production, has also been shown to protect animals from both oxidant-induced acute lung injury (Frank *et al.* 1978) as well as to a subsequent lethal endotoxin exposure (Ryan *et al.* 1992).

Because of the overlap in protective effects from various stresses, we wondered whether oxyradical exposure would result in heat shock protein production within cells and whether different oxyradical stimuli would result in differences in the HSP response, particularly members of the highly heat-inducible 70 kDa HSP family, HSP70. To tes this hypothesis, we used electron paramagnetic resonance to detect free radicals and evaluated cells in culture for HSP70 protein accumulation following various oxidant stresses.

2. Methods

2.1. *Cells*

Human lung carcinoma cells (A549; American Tissue Culture Collection) were the primary cells used for these studies. Cells were grown in minimal essential medium (MEM) plus 10% fetal calf serum (FCS) in a 37° 5% CO_2 incubator and were subcultured at a ratio of 1:5 on every fourth day. Passages 80–110 were used for the studies. In some cases, studies were repeated using COS-1 cells.

2.2. *Oxidant and heat stresses*

The A549 cells were plated at a concentration of 4×10^5 cells/dish in 60 mm tissue culture plates in MEM with 10% FCS. Twenty-four hours after plating, the cells were exposed to 44°C for 1 h in MEM, or 37°C in MEM + menadione sodium bisulfate (MEN, 200 μM), hydrogen peroxide (H_2O_2 500μM) or xanthine (100 μM) + xanthine oxidase (X-XO, 10^{-2} Units/ml) or medium alone. Zero percent FCS MEM was used for all treatments and controls. At the end of 1 h, the treatment medium was removed, cells were rinsed 3× with Hank's BSS, 10% MEM was added and the cells were allowed to recover for 8 h. At the end of the recovery period, the cells were rinsed 3× in phosphate buffered saline (PBS; 138 mM NaCl, 2·7 mM KCl, 10 mM Na_2KPO_4, pH 7·4). One ml of 0·25 M TRIS pH 7·8 was added and the cells were scraped with a cell scraper and transferred to an eppendorf tube and spun at 10 000 xg for 5 min at 4°C. The buffer was aspirated and the cell pellet was frozen until the next step.

2.3. *Western blot analysis*

Cellular proteins were quantitated using the Bradford method (Bradford 1976). Following quantitation, 10 μg of each sample were separated on a 12·5% SDS page gel and transferred to nitrocellulose. The membranes were blocked by a 3 h exposure to 10% FCS, 10% Bovine serum albumen (BSA) in TRIS-buffered saline (TBS; 10 mM TRIS, 150 mM NaCl, pH 8·0) at room temperature. They were then rinsed 1× in TBS with 0·05% Tween 20 (Fisher Scientific, Fair Lawn, NJ) and then 2× with TBS. Membranes were then probed for 1 h with monoclonal antibodies specific for either the inducible (72 kDa) or the constitutive and inducible (73 kDa, 72 kDa respectively) HSP (StressGen Biotechnologies Corp., Victoria, BC). The blots were then rinsed 1× in TBS with 0·5% Tween 20, then 3× in TBS. Following the final rinse, they were incubated for 30 min with an alkaline phosphatase linked anti-mouse IgG. The blots were then incubated in 0·45 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma Chemical Co.) and 0·27 mM nitrobluetetrazolium (NBT, Fisher Biotech in AP buffer (100 mM TRIS, 100 mM NaCl, and 5 mM $MgCl_2$, pH 9·5) until the bands developed a blue color.

2.4. *EPR analysis*

To detect directly free radical formation electron paramagnetic resonance (EPR) spectroscopy was employed (Buettner and Oberley 1978, Buettner 1987). EPR spectra were collected using a Bruker ESP300 EPR spectrometer equipped with a TM_{110} cavity and appropriate aqueous flat cell. Free radical formation was monitored using the EPR spin trapping technique; the spin trapping agent 5,5 dimethylpyrroline-1-oxide (DMPO) was purified with activated charcoal and stored as a frozen 1·0 M aqueous solution before use. The spin trapping incubations contained cells in suspension with 100 mM DMPO, and other additions as appropriate. Instrument settings were: sweep rate 80 G/84 s; modulation amplitude (1·0 G; nominal power 40 mW; and time constant 82 min.

3. Results

As expected, A549 cells, under control and all explosure conditions showed appreciable levels of HSC70 (Figure 1A). In addition, there was a second lower band corresponding to the inducible HSP (72 kDa) in cells exposed to heat and menadione. When these same protein samples were probed with an antibody that recognized only the inducible (72 kDa) HSP70, heat and menadione exposure but only xanthine/xanthine oxidase or H_2O_2 exposure resulted in the accumulation of inducible HSP70 at 8 hours post exposure. Studies done at 2,4 and 12 h using concentrations of H_2O_2 from 100 to 500 μM and X-XO concentrations from 10^{-3} to 10^{-1} U/ml, failed to reveal evidence of inducible HSP70. At the highest concentration of H_2O_2 (500 μM) cytotoxicity was $47 \pm 2\%$ at 8 h post exposure. Similar results were seen with COS-1 cells (data not shown), demonstrating that this effect was not cell line specific.

To determine whether the oxy-radical generating systems above were indeed producing radicals, EPR analysis was done using the spin trap DMPO (Figure 2). When A549 cells were exposed to menadione in the presence of the spin trap DMPO, DMPO/$^{\bullet}$OH ($a^N = a^H = 14{\cdot}9$ G) was observed. As expected the xanthine/xanthine oxidase sytem produced both DMPO/$^{\bullet}$OOH ($a^N = 11{\cdot}3$ G, $a^H = 14{\cdot}36$, $a^H = 1{\cdot}2$ G) and DMPO/$^{\bullet}$OH ($a^N = a^H = 14{\cdot}9$ G) , consistent with the formation of

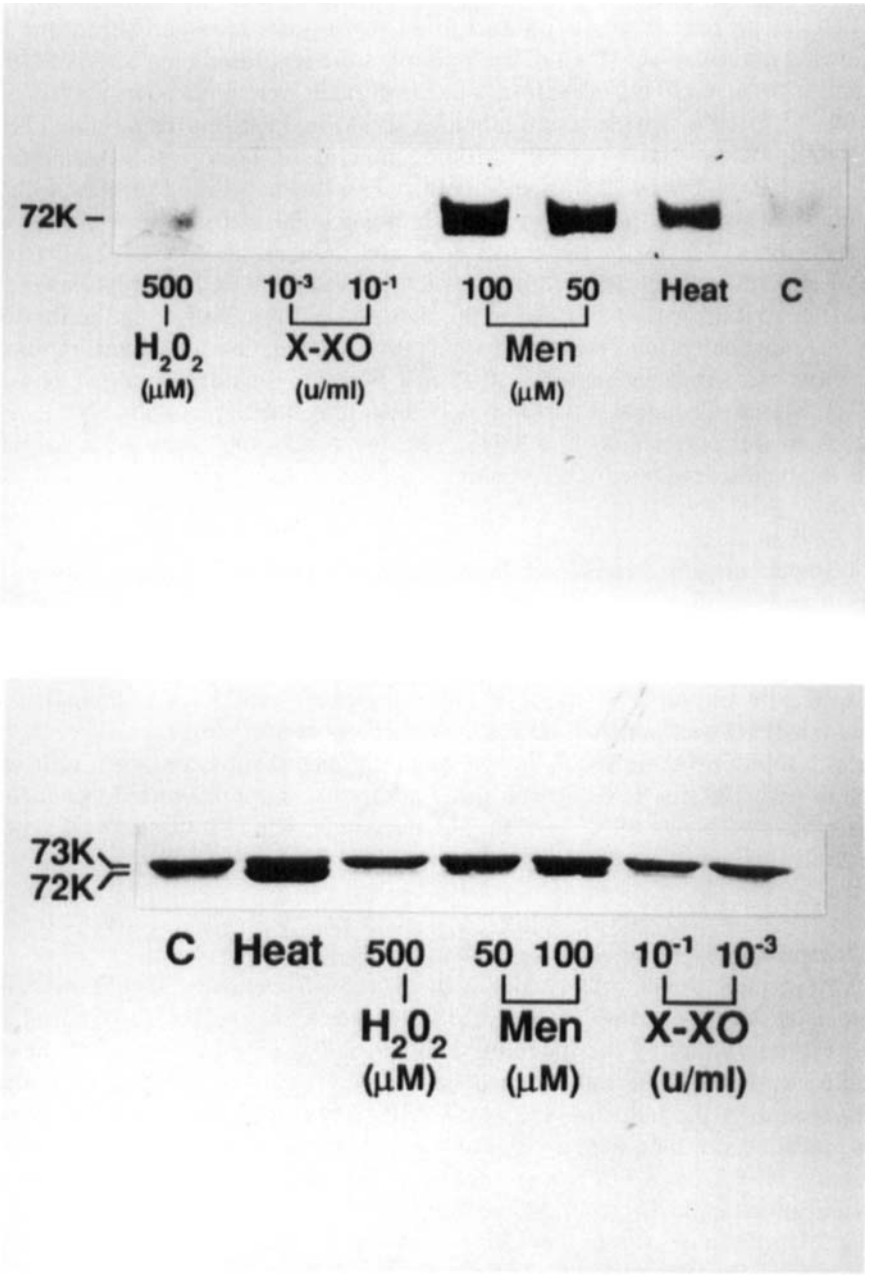

Figure 1. Western Blot of A549 cells exposed to heat (44°C ambient temperature), hydrogen peroxide (H_2O_2; 500 μM), menadione (MEN; 50 and 100 μM), or xanthine/xanthine oxidase (X-XO; 10^{-3} or 10^{-1} U/ml). Proteins were isolated by gel electrophoresis, transferred and probed with an antibody specific for both the constitutive (HSC70) and inducible (72kDa) forms of HSP70 (1A) or an antibody that recognizes only the inducible (72kDa) form of HSP70 (1B).

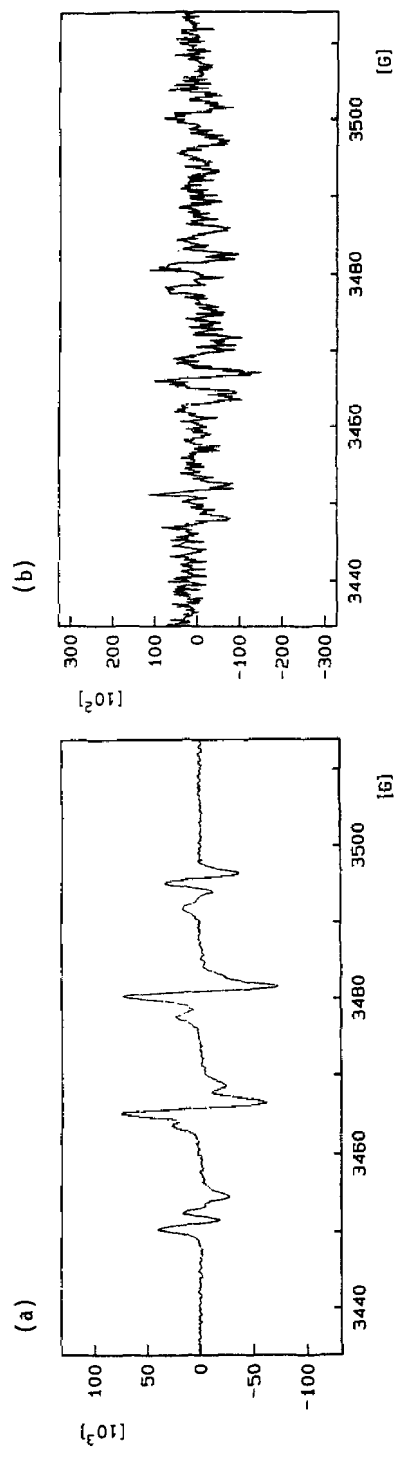

Figure 2. EPR spin trapping reveals radical production by COS cells exposed to menadione: (a) xanthine/xanthine oxidase (10^{-2} U/ml) in PBS; (b) cells suspended in PBS; (c) menadione (100 μM) in PBS; (d) cells suspended in PBS and exposed to menadione (100 μM). All spectra were obtained using the spin trap DMPO. Cells were gently scraped from tissue culture plates, suspended in buffer, additions made as appropriate then immediately placed in the EPR flat cell and scans were initiated. The spectra were collected in the time frame of 3–10 min after all additions were made.

superoxide and hydroxyl radicals. Neither menadione alone nor cells alone generated oxyradicals.

4. Discussion

These studies reveal that the cell-associated oxyradical generator menadione, like heat, is a potent inducer of the HSP 70 response while hydrogen peroxide exposure and xathine/xanthine oxidase exposure (as an $O2^{\bullet -}$ generator) do not cause inducible HSP 70 accumulation. The lack of HSP 70 accumulation is not due to impaired radical generation since $O2^{\bullet -}$ production by xanthine/xanthine oxidase was verified by EPR.

An overlap of the response to heat and oxidant stress has also been found following exposure to hydrogen peroxide. Accumulation of a heat inducible 70 kDa protein has been reported following hydrogen peroxide exposure (Spitz *et al.* 1987, Courgeon *et al.* 1988). These studies demonstrated the presence of the inducible 70 kDa protein using radio-labeled proteins on a two-dimensional gel. They did not probe the gels using any available anti-HSP antibodies. Their studies suggest that there may be a 70 kDa protein, induced by hydrogen peroxide exposure, which has not yet been characterized.

The ability of menadione but not xanthine/xanthine oxidase or H_2O_2 to cause heat shock protein accumulation is intriguing. Since both H_2O_2 and menadione generate hydroxyl radicals, one possibility is that hydroxyl radical is not responsible for the accumulation of HSP 70 seen. Alternatively, the cellular substrate affected by these agents may be important in the HSP 70 response. Menadione is a vitamin K analog with an electrophilic carbon center. When taken up by cells, menadione may be redox cycled, generating oxy-radicals such as the hydroxyl radical (Trad and Butterfield 1994). In studies with isolated hepatocytes, exposure to menadione did not cause lipid peroxidation (Orrenhius *et al.* 1985). In studies of isolated erythrocyte membranes, menadione caused no significant effects on membrane fluidity, but significantly increased alterations in cytoskeletal proteins. This alteration in protein appeared to be related to the oxidation of specific protein components (Trad and Butterfield 1994). It may also react directly with molecules such as glutathione and thereby impair the cell's ability to resist oxidant stress (Di Monte *et al.* 1984). Because of this ability to react directly with protein thiols as well as creating oxidant species, menadione exposure may be qualitatively different than other types of oxidant exposures.

Menadione also induces DNA strand breakage in Chinese hamster fibroblasts, associated with oxy-radical production. In a study by Calderaro *et al.* (1993) the reaction was potentiated in part by menadione associated increases in free intracellular Fe and Cu, suggesting that menadione metabolism increases the production of $O2^{\bullet -}$ and H_2O_2 and the levels of Fe and Cu available for catalysis of DNA oxidation (Calderaro *et al.* 1993). Menadione can also react directly with glutathione to produce a menadione-glutathione conjugate, leaving cells less able to recover from an oxidative challenge (Di Monte *et al.* 1984). In fact, the cell's ability to withstand low level heat stress and oxidant stresses may both be somewhat related to the levels of glutathione in the cell. H_2O_2 resistant Chinese hamster fibroblasts, characterized as having greater ability to withstand oxidative challenge, as shown by increased levels of glutathione, glutathione peroxidase, superoxide dismutase and catalase activity (Spitz *et al.* 1988, 1989), were significantly more resistant to a 43°C heat shock than control cells although the protection disappeared if the cells were heated to 45°C.

The cells were also less well protected than thermotolerant cells (Spitz and Li 1990). Glutathione depletion was found to inhibit the ability of Chinese hamster V79 cells to develop thermotolerance and led to a decrease in levels of total protein as well in levels of heat shock proteins. Sierra-Rivera *et al.* (1993, 1994) found increases in the constitutive HSP 70 (HSC 70) following exposure to menadione. The authors suggest that the cell's inability to maintain glutathione in a reduced form during menadione redox cycling results in protein thiol accumulation which in turn results in an accumulation of HSC 70 mRNA and protein (Sierra-Rivera *et al.* 1994). They did not find an increase in the levels of inducible HSP 70 protein, but did not probe specifically for it. Freeman *et al.*, found that heat shock promotes oxidative damage and that glutathione is required for protection (Freeman *et al.* 1990).

In summary, these data reveal that the intracellular oxyradical generator menadione, like heat, is capable of inducing cellular accumulation of inducible HSP70 protein. In contrast, H_2O_2 and X-XO, which may have important effects on the cell membrane, cause no HSP70 protein accumulation in spite of their ability to generate oxygen radicals and to activate the HSP70 promoter.

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