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COMMENTARY

OXIDATIVE INJURY AND THE HEAT SHOCK RESPONSE

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Heat shock response and heat shock proteins

The heat shock response is a conserved, immediate but transient cellular response to elevated temperatures and other types of injury, which consists of the transcriptional activation of a small set of specific genes, with subsequent synthesis of the so-called heat shock, or stress, proteins (HSPs), transcriptional inhibition of normal protein synthesis, and thermotolerance [1, 2]. There is a remarkable conservation in the structure of heat shock genes and heat shock proteins across species, with for example a 50% homology in the 70 kD HSP (HSP70) of *Escherichia coli* and humans. Moreover, the heat shock response has been found in every cell and organism examined heretofore, and this conservation across species and along evolution suggests important functions for the stress proteins [3].

The stress proteins include (i) the HSPs, which are specifically induced by heat; (ii) the glucose regulated proteins (GRPs), for which the inducing signals are, for example, glucose deprivation or calcium ionophores; (iii) ubiquitin, a 76 amino acid polypeptide which plays a role in targeting of proteins for degradation; (iv) metallothioneins; and (v) oxidation-specific stress proteins, such as heme oxygenase (Table 1 and Refs. 1–4).

Functional aspects of heat shock proteins

Thermotolerance, i.e. the ability of cells pre-exposed to non-lethal temperatures to survive subsequent exposure to temperatures lethal under normal conditions, has been the first proposed function for HSPs, and although there has long been controversy about their specific role in thermotolerance, recent experiments indicate that HSPs, and in particular HSP70, indeed play a definitive role in this phenomenon [5, 6]. It also became clear that HSPs are not solely stress proteins, but that they play important functions in unstressed cells as well [7, 8]. The functions of HSPs in unstressed cells appear to relate to "chaperoning," subcellular transport, import-export of other proteins, and prevention of incorrect folding (reviewed in Ref. 9). HSP70 has the ability not only to prevent, but even to reverse protein aggregation by binding to the aggregated proteins and solubilizing them, further releasing itself in an ATP-dependent fashion, thus allowing proteins to fold [1, 9]. Another example for this functional aspect is given by the immunoglobulin heavy chain binding protein, BiP, or GRP78, which in pre-B cells binds heavy chain immunoglobulin molecules while light chains are not yet available, and also can be released from its substrate by ATP [9, 10].

Whereas HSPs represent about 2–3% of total cellular proteins in normal cells, this proportion can reach 20% in cells exposed to heat. In stressed cells, HSPs appear to be essential for survival during and after exposure to cellular injury [5, 6, 11, 12]. An array of events occurring after heat shock which include the heat-induced collapse of intermediate filaments and the disruption of the intranuclear pattern of small ribonuclear proteins, can be prevented by previous synthesis of HSPs [11, 12].

Heat shock proteins and cytokines

The recent discovery that immunodominant antigens from a wide variety of microorganisms also are, by sequence homology, HSPs has brought about an active interest for potential roles of these proteins in infection and immunity [13, 14]. A role for several cytokines, in particular the so-called "endogenous pyrogens" (interleukin 1, tumor necrosis factor α (TNF), interleukin 2, interferons), in the regulation of the stress response has also been suggested, and in this respect it has to be considered that these cytokines, in particular TNF, also regulate the generation of oxygen free radicals by phagocytes [15]. Interleukin 1 induces, in isolated rat islets of Langerhans (in which this cytokine has been suggested to induce toxic radical formation), new proteins suggestive of being stress proteins according to their molecular weight (70 and 32 kD) [16]. On the other hand, TNF induces the synthesis of superoxide dismutase (SOD), and this scavenging enzyme appears essential for cellular protection from subsequent exposure to TNF [17]. In *E. coli*, SOD has been shown to be induced by heat shock itself [18]. We have established that phagocytosis of *Staphylococcus aureus* induces both HSPs and SOD in human monocytes-macrophages (see below; our unpublished data). Taken together, these data suggest multiple links among cytokines, oxidative stress and HSPs.

Oxygen free radicals as inducers of the heat shock response: the peculiar case of phagocytes

There are, besides heat, many other inducers of the heat shock response, including oxidative injury (Table 2). Hydrogen peroxide (H_2O_2) induces

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Table 1. Stress proteins and respective inducers

Stress proteins	Main inducers
Heat shock protein families	
HSP110 (110 kD)	cf. Table 2
HSP90 (83–90 kD)	
HSP70 (68–73 kD)	
HSP65 (homologous to GroEL)	
Small HSPs (20–28 kD)	
GRP 78 or BiP (related to HSP70)	Glucose deprivation, calcium ionophores
GRP96 (related to HSP90)	
Ubiquitin (8 kD)	Heat, amino acid analogs, degraded proteins
Other stress proteins	
Metallothioneins	Transition or heavy metals (mostly the dicationic forms: zinc, copper, cadmium, mercury)
Superoxide dismutase (Mn-SOD) (90 kD)	Oxygen reactive species, TNF, and heat in bacteria
Heme oxygenase (32 kD)	Hemin, UV radiations, cadmium, hydrogen peroxide, and, in rodents, heat

Several stress proteins and their main known inducers, are given. Some relatively unrelated proteins, such as HSP70 and heme oxygenase, for example, share one or more of the classical inducers but not others (heat; hemin).

Table 2. Inducers of stress responses, a non-exhaustive list

In vitro	*In vivo*
Heat	Increases in body temperature (heat, D-lysergic acid diethylamide)
Ethanol	
Hydrogen peroxide*	Ischemia/reperfusion injury*
Amino acid analogs	Inflammation*
Erythrophagocytosis*	
Normoxia after anaerobiosis*	
Heavy or transition metals, e.g. cadmium*	
Inhibitors of energy metabolism, e.g. arsenite*	
Drugs used for cancer therapy, e.g. Adriamycin®, bleomycin*	

* Induction may be mediated by oxygen free radicals.

a heat shock response in bacteria [19], and several types of oxidative injuries (UV radiation, sodium arsenite, cadmium) induce stress proteins in human cells. HSPs are also induced in animal models for ischemia and reperfusion injury [20–22]. During reperfusion, oxygen free radicals are generated from molecular oxygen via the metabolism, by xanthine oxidase, of xanthine/hypoxanthine accumulated as a consequence of ATP depletion secondary to ischemia. Synthesis of HSPs in these animal models suggest that oxygen free radicals also have the ability to induce HSPs *in vivo*.

Because phagocytic cells themselves *produce* reactive oxygen species during phagocytosis, through the respiratory burst enzyme NADPH oxidase, we have investigated multiple interactions between oxidative injury and HSPs in human monocytes-macrophages and neutrophils. Phagocyte-derived oxygen metabolites play a definitive role in antimicrobial defense mechanisms but also participate in cellular and tissue injury in particular in inflammation (reviewed in Ref. 23). We first established that in mature peripheral blood monocytes and alveolar macrophages (Fig. 1), but not in the human premonocytic line U937 [24], H_2O_2 induced a heat shock response. As described in bacteria by Christman *et al.* [19], the induction of a heat shock response by H_2O_2 has distinct characteristics as compared to heat. In human monocytes-macrophages, and although inhibition of normal protein synthesis is usually as marked, if not more, when H_2O_2 is used as the inducer of the heat shock response, the amount of stress proteins appears to be less than after heat shock (Fig. 1). Furthermore, exogenous addition of H_2O_2, in contrast to heat

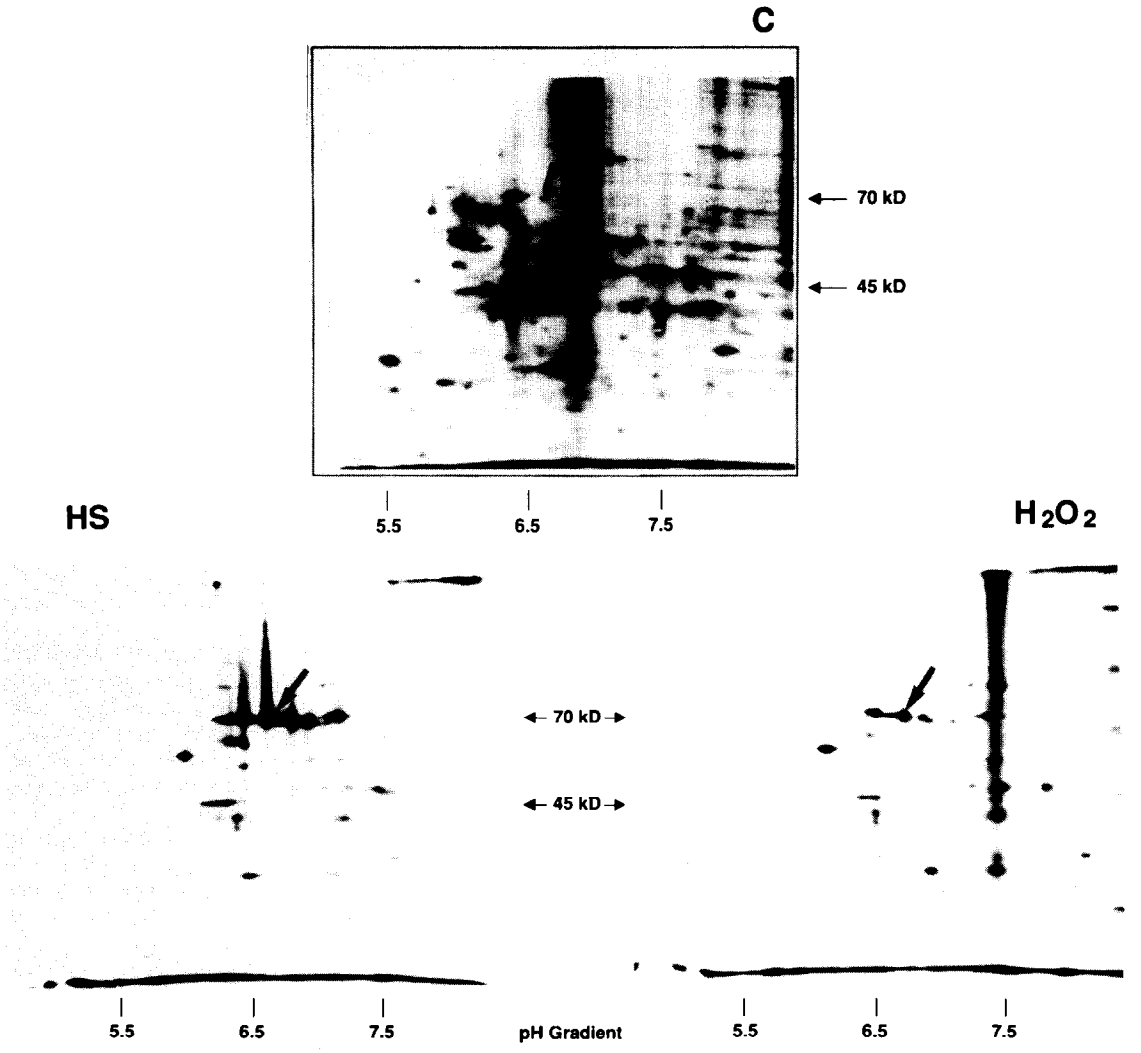

Fig. 1. Induction of HSP70 by heat shock or H_2O_2 in human alveolar macrophages. Alveolar macrophages were obtained by *ex vivo* bronchoalveolar lavage of lungs excised for carcinoma, washed, purified by adherence, and cultured in RPMI without methionine (Gibco, Paisley, U.K.), with 5% fetal calf serum (Gibco), exposed to 45° for 20 min (HS), or to H_2O_2 (5×10^{-3} M) for 1 hr, or maintained at 37°, and then labeled with 10 μCi/mL [^{35}S]methionine (Amersham, Buckinghamshire, U.K.) for 1 hr. Cell lysates corresponding to equal cell number were then analyzed by two-dimensional gel electrophoresis as previously described [24]. Arrows indicate HSP70.

shock, does not induce in all cells examined detectable amounts of stress proteins [24].

We then investigated whether HSPs were also synthesized by phagocytes during the stimulation of endogenous oxygen free radical production associated with phagocytosis. As a model system, we used sheep erythrocytes (SRBCs) as phagocytic stimulus, and the U937 cells, which in their undifferentiated state are non-phagocytic and unable to produce superoxide, but in which phagocytosis and a functional NADPH oxidase can be induced by incubation with the steroid hormone 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] [25]. In this model, we have shown that, indeed, generation of oxygen free radicals during erythrophagocytosis is associated in 1,25-$(OH)_2D_3$-differentiated U937 cells with the synthesis of the classical HSPs and of the 32 kD oxidation specific stress protein [26–28]. Stress protein synthesis was also observed after incubation of SRBCs with normal human monocytes or alveolar macrophages, but not with non-phagocytic cells. Identity of the 32 kD protein to heme oxygenase has been established recently by Keyse and Tyrrell [4]. Interestingly, heme oxygenase which had been known for a long time to be induced during erythrophagocytosis, has definitive antioxidant potential [29, 30]. Because neither other stimuli of the respiratory burst nor hemoglobin-depleted red cell ghosts induced stress protein synthesis, we hypothesize that hydroxyl radicals, generated through the metal-catalyzed Haber–Weiss reaction in the presence of hemoglobin-derived iron, are involved in the mechanisms leading to stress protein synthesis. The synthesis of both the classical HSPs and of heme oxygenase

appears in this case to be mediated by oxidative injury, because both were prevented by the free thiol *N*-(2-mercaptoethyl)-1,3-propanediamine, WR 1065 (dephosphorylated from the radioprotective agent WR 2721), which prevents H_2O_2-induced cell death in U937 cells [31].

Are HSPs and oxidation-specific stress proteins identical or distinct?

The simultaneous induction of HSPs and oxidation-specific stress proteins suggests that they are part of the same system. Such a possibility would require the existence of a common pathway(s) for their induction.

Denatured, unfolded or "abnormal" proteins have been suggested to represent the signal sensed by the cell for transcriptional activation of heat shock genes [9, 32]. Proteins altered by heat, oxidation, or any other metabolic stresses will bind HSP70 or ubiquitin. Whether the presence of the unfolded proteins, the absence of HSP70, or deubiquitination of the heat shock transcription factor plays the key role in triggering the heat shock response remains to be established [9]. The possibility that heat shock induces HSPs (as well as SOD) via generation of oxygen free radicals and lipid peroxidation is supported by several observations: (i) heat shock has been shown to increase lipid peroxidation as evidenced by the rise in 2-thiobarbituric reactive material [33]; (ii) modulation of the levels of lipid peroxides by the use of free radical scavengers prevents lipid peroxidation as well as induction of HSPs by heat [33]; and (iii) both heat shock and oxidative stress also lead to accumulation of adenylylated nucleotides or "alarmones" [34, 35].

This hypothesis, however, remains controversial. We found no generation of superoxide during exposure of human neutrophils to temperatures ranging from 37° to 45° [36]; rather heat shock was found to inhibit superoxide generation by neutrophils [36]. Thus, the possibility that induction of HSPs and induction of oxidation-specific stress proteins are regulated by separate pathways has to be considered. In particular, the potential common mechanisms for induction of stress proteins by heat or oxidative injury mentioned above do not account for the specific synthesis of heme oxygenase under certain conditions of oxidative injury. Indeed, heme oxygenase (the 32 kD oxidation-specific stress protein) has been shown to be induced in human cells specifically by oxidant stresses such as H_2O_2 or UV radiation but not by heat [27], although in rodent cells it is a classical HSP [28]. It therefore appears that in the human system, the proteins induced by oxidative injury *include* the classical HSPs, all of the latter being induced by oxidative injury, but not all oxidation-specific stress proteins being induced by heat shock, whereas in bacteria, there are common and distinct proteins induced by both types of stresses.

Another argument that supports distinct mechanisms for thermal and oxidative injury is the role played by calcium in the latter but not in the former. When U937 cells are exposed to H_2O_2, calcium enters the cells and participates to cell death, inasmuch as removal of extracellular calcium partially prevents H_2O_2-induced cell death [37]. On the other hand, we have established in the same cellular system that calcium plays no role in induction of HSPs by heat shock, nor in thermal killing [38]. Furthermore, whereas 1,25-$(OH)_2D_3$ increases the synthesis of HSPs upon exposure to heat and protects cells from thermal injury, the hormone in contrast does *potentiate* oxidative injury [37, 39]. We have established that this potentiation of H_2O_2-induced cell death relates to a 1,25-$(OH)_2D_3$-mediated increase in cellular calcium [37, 39].

Table 3. Protection of 1,25-$(OH)_2D_3$-treated U937 cells from subsequent exposure to H_2O_2 by preexposure to heat shock (HS)

Treatment	Cell viability (%)		P
Preexposure to HS	−	+	
Exposure to H_2O_2 (mM)			
0.0	98 ± 1	99 ± 1	NS
0.1	90 ± 2	92 ± 1	NS
0.5	80 ± 2	88 ± 1	<0.01
1.0	68 ± 3	80 ± 2	<0.01
5.0	47 ± 5	69 ± 2	<0.01
10.0	36 ± 2	54 ± 1	<0.001

U937 cells were exposed to heat shock (45°, 20 min), allowed to recover at 37° for 4 hr, and then exposed to increasing concentrations of H_2O_2 for 30 min. Cell viability was evaluated by trypan blue exclusion as previously described [37]. Values are means ± SE of quadruplicate determinations. NS = not significant.

Heat shock proteins and protection from oxidative injury

Another aspect of the relationships between heat shock and oxidative injury that we are investigating is the possible cross-protection between heat shock and oxidative injury. In bacteria, heat shock induces thermotolerance but no resistance to H_2O_2 [19]. In Chinese hamster fibroblasts, Spitz *et al.* [40] reported resistance to H_2O_2 after exposure to heat, but no resistance to heat after exposure to H_2O_2. We hypothesized that HSPs may protect human monocytic cells not only from thermal, but from oxidative injury as well. The coordinate upregulation, during phagocytosis, of enzymes with known antioxidant potential (heme oxygenase, SOD) and of the classical HSPs supports the hypothesis that in our system HSPs may be induced as part of cellular mechanisms for protection from oxidative injury. Indeed, preexposure of 1,25-$(OH)_2D_3$-treated U937 cells to temperatures leading to the synthesis of HSPs induces partial but significant protection from subsequent exposure to H_2O_2 ([37]; Table 3). We had shown previously that 1,25-$(OH)_2D_3$ does increase the synthesis of HSPs and protects monocytes from thermal injury [24, 41]. The fact that protection by heat shock was observed solely in the cells synthesizing more HSPs provides one more argument for a specific role of these proteins in protection, although the possibility that 1,25-$(OH)_2D_3$ protects phagocytes by an increase in SOD—as it increases phagocytosis

and generation of toxic oxygen metabolites by these cells—has to be considered as well [25, 42].

Preliminary experiments also indicate that heat shock protects cells from subsequent exposure to bleomycin, an antitumor agent which exerts its toxic effects through the intracellular generation of reactive oxygen species and DNA damage, and in parallel primes monocytes-macrophages for extracellular superoxide production, both *in vitro* and *in vivo* ([43] and our unpublished data). Interestingly, the toxic effects of bleomycin appear to be mediated at least in part by TNF [44], and heat shock has been suggested to provide protection from TNF-induced cell lysis as well [45].

Further experiments will be required to establish whether these protective effects of heat shock may have any pharmacological application. First, it has to be shown whether protection would occur *in vivo*, and second, the mechanism(s) for these protective effects has to be unraveled. The fact that HSPs are induced in *in vivo* situations of oxygen free radical generation (such as reperfusion injury) does support the first possibility. Moreover, heat has been used as therapy for centuries, initially, and still today, in tumors, then in infections and inflammatory lesions (reviewed in Ref. 46). Oxygen free radicals have been suggested to play a role in these various pathologies [47, 48]. The elevated temperatures observed in inflamed joints could also serve a protective purpose through induction of HSPs [49]. The beneficial potential of fever, first suggested by Hippocrates, then established by Kluger and coworkers in the 1970s [50, 51] could actually well be mediated by induction of HSPs *in vivo*. Finally, with respect to allergy, it has been reported that an increase in local temperature prevents in the nose the symptoms associated with allergic rhinitis [52], another situation in which oxygen free radicals could be involved [53].

Potential mechanisms for protection from oxygen free radicals by HSPs include prevention from protein degradation, membrane lipid peroxidation or calcium intrusion from the extracellular milieu, maintenance of ATP levels, induction of classical scavengers such as SOD or glutathione (which itself plays a role in induction of HSPs [54, 55]), inhibition of any of the multiple steps involved in oxidative injury-mediated cell death, such as uncoupling of oxidative phosphorylation, decrease in calcium-ATPase, activation of phospholipase A_2, or maintenance of a normal cellular (ultra)structure. Whereas prevention from protein aggregation is a recognized function of HSPs, we found no effect of specific modifications in membrane fatty acid composition on U937 cell thermotolerance or HSP synthesis [56]. Furthermore, we have established that although preexposure to heat shock partially protects U937 cells from H_2O_2-induced cell death, the rise in cytosolic free calcium concentrations induced in these cells upon exposure to H_2O_2 is not affected in heated cells [37]. Protection from oxidative injury by heat shock appears, therefore, to be distal to calcium intrusion. In contrast, pharmacological protection of oxidative injury by WR 1065 is associated with prevention from calcium intrusion in U937 cells. Although heat shock also protects the glycolytic-deficient CHO mutants DS7 [57] from injury associated with inhibition of oxidative phosphorylation by oligomycin, the decrease in ATP levels upon exposure to this drug is similar in heated and control cells [58].

All these negative results with respect to potential mechanisms for protection of oxidative injury by heat shock leave open many directions for further research; some of those we are pursuing are mentioned above. Despite the exciting possibilities for therapeutic applications of heat or modulation of the heat shock response in human diseases associated with oxidative injury, such applications must await understanding of the mechanisms involved in protection.

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