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Antioxidant-mediated inhibition of the heat shock response leads to apoptosis

Adrienne M. Gorman[a,b], Barry Heavey[b], Emma Creagh[c], Thomas G. Cotter[c], Afshin Samali[a,*]

[a] *Institute of Environmental Medicine, Division of Toxicology, Karolinska Institutet, Box 210, S-171 77, Stockholm, Sweden*
[b] *Department of Biochemistry, University College, Galway, Ireland*
[c] *Biochemistry Department, University College Cork, Cork, Ireland*

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Abstract We examined the hypothesis that reactive oxygen species (ROS) contribute to the induction of heat shock proteins (hsps) during stress response. Exposure of HL-60 human myelocytic cells to 42°C induced both hsp72 and hsp27. In the presence of the antioxidant molecules pyrrolidine dithiocarbamate or 1,10-phenanthroline induction of hsp72 and 27 was significantly decreased, while *N*-acetyl-L-cysteine caused a slight reduction. Prevention of hsp induction was associated with heat sensitization and increased caspase activity, indicating that the cells were undergoing apoptosis. These data suggest that ROS contribute to the induction of hsps and furthermore, that hsp induction and apoptosis are mutually exclusive events within the same cell.

Key words: Apoptosis; Heat shock protein; Oxidative stress; Reactive oxygen species; Antioxidant

1. Introduction

Heat shock proteins (hsps) are a set of evolutionary conserved proteins, some of which are constitutively expressed and others whose expression is induced in response to environmental and physiological stresses such as thermal or oxidative stress (for review see [1]). Hsps mediate the refolding or degradation of stress-damaged proteins, thus protecting cells from potential deleterious effects and promoting cell recovery. The accumulation of hsps in cells is associated with an increased resistance to cytotoxic injury by subsequent insults which would otherwise be lethal, a phenomenon known as thermotolerance [2,3].

Apart from the induction of hsps and thermotolerance, heat treatment can also cause a loss of cell viability (for review see [4]). This is particularly obvious when higher temperatures and/or protracted exposure times are used. In general, cell death can follow two distinct pathways, apoptosis or necrosis. Apoptosis is a highly-regulated process involving condensation of nuclear chromatin, cytoplasmic shrinkage, membrane blebbing, nuclear fragmentation and finally formation of apoptotic bodies. One of the early intracellular events during apoptosis is the activation of a family of proteases called caspases. These are cysteine proteases that cleave after aspartic acid residues [5]. Their substrates include fodrin, lamin and poly(ADP-ribose) polymerase (PARP) (reviewed in [6]). Necrosis, on the other hand, is a passive process [7] that occurs when the level of damage to a cell is so great that it cannot execute the apoptotic program. Necrosis is characterized by cell and organelle swelling, followed by lysis of the cell to discharge its contents into the surrounding environment.

The effects of heat on cells (both the induction of thermotolerance and loss of viability) are reported to be associated with oxidative damage to proteins [8]. Although there are reports of increased flux of free radicals in cells subjected to hyperthermia [9,10], it is not known however, whether ROS are involved in the induction of hsps. In this study we decided to examine the effect of a range of antioxidant molecules on the induction of hsp72 and 27 due to heat treatment of HL-60 cells. We furthermore investigated the viability of cells exposed to a combination of antioxidants and elevations in temperature.

2. Materials and methods

2.1. Chemicals

Allopurinol, *N*-acetyl-L-cysteine (NAC), *N*-arginine-methyl ester (NAME), catalase, 4,5-dihydroxy-1,3-benzene-disulphonic acid (TIRON), diphenyliodonium chloride (DPI), 1,10-phenanthroline (Phen), pyrrolidine dithiocarbamate (PDTC) and 2,2,6,6-tetramethyl-1-piperidinyl-1-oxyl (TEMPO) were purchased from Sigma. Ac-Asp-Glu-Val-Asp-α-(4-methyl-coumaryl-7-amide) (DEVD-MCA) was from Peptide Institute Inc. (Minoh-Shi, Osaka 562, Japan). Benzyloxycarbonyl-Val-Ala-Asp (*O*-methyl), fluoromethyl ketone (zVAD-fmk) were purchased from Enzyme Systems Products (Livermore, CA, USA).

2.2. Cell culture and treatment of cells

HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere of 5% CO_2 in air at 37°C. The cells were maintained in a logarithmic growth phase by routine passage every 2–3 days.

2.3. DEVD-MCA cleavage activity

Treated cells were collected, centrifuged at 1000 rpm for 5 min and the medium was removed. After washing once with phosphate buffered saline (PBS) at 4°C, the cell pellet was resuspended with 25 μl of PBS. The cells ($2–5\times10^6$ in 25 μl) were rapidly frozen by transferring them directly into a 96-well plate which was floating on liquid nitrogen. Measurement of DEVD-MCA cleavage was as described in [11].

2.4. Western blot

Protein samples (15–20 μg protein per lane) were resolved on 10% SDS-PAGE gels and electrophoretically transferred to nitrocellulose for 2 h at 100 V. Membranes were probed for 1 h with one of the following antibodies: anti-PARP monoclonal antibody (1/2500) (from Biomol, Plymouth Meeting, PA, USA), anti-hsp72 mouse monoclonal (1/1000), anti-hsp27 mouse monoclonal or anti-hsc70 rat monoclonal (1/5000) (all from Stressgen Biotechnologies Corp., Victoria, Canada). This was followed by 1 h incubation with either goat anti-mouse, anti-rabbit or anti-rat IgG-HRP (1:10 000) (from Pierce, Rockford, IL, USA) as appropriate. Bands were then visualized using the Enhanced Chemiluminescence (ECL) Western Blot Detection kit from Amersham Corp. (Buckinghamshire, UK).

*Corresponding author. Fax: +46 (8) 32 90 41.
E-mail: afshin.samali@imm.ki.se

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2.5. Cell viability and morphology

Cell number was assessed using a Neubauer haemocytometer and viability was determined by the ability of cells to exclude trypan blue. Cell morphology was evaluated by staining cytocentrifuge preparations with RapiDiff II (Paramount reagents Ltd., UK). Apoptotic cells were identified as described previously [12].

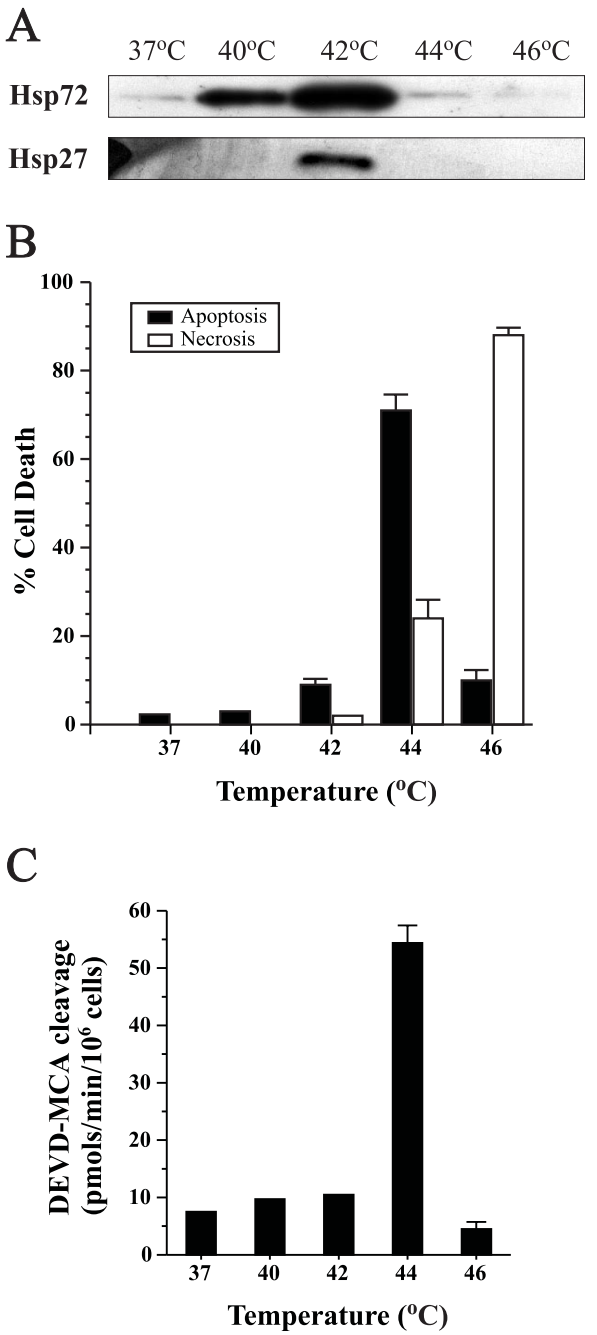

Fig. 1. Effects of heat exposure on hsp induction and cell viability in HL-60 cells. Cells were heat shocked at the indicated temperatures for 1 h followed by 6 h recovery. A: Western blot analysis of hsp72 and hsp27 levels in cells exposed to various temperatures. B: The percentage of cell death by apoptosis or necrosis following exposure to different temperatures was determined based on morphological criteria. C: Caspase-3-like activity (DEVD-MCA cleavage activity) was determined fluorometrically after the 6 h recovery period post-heat shock.

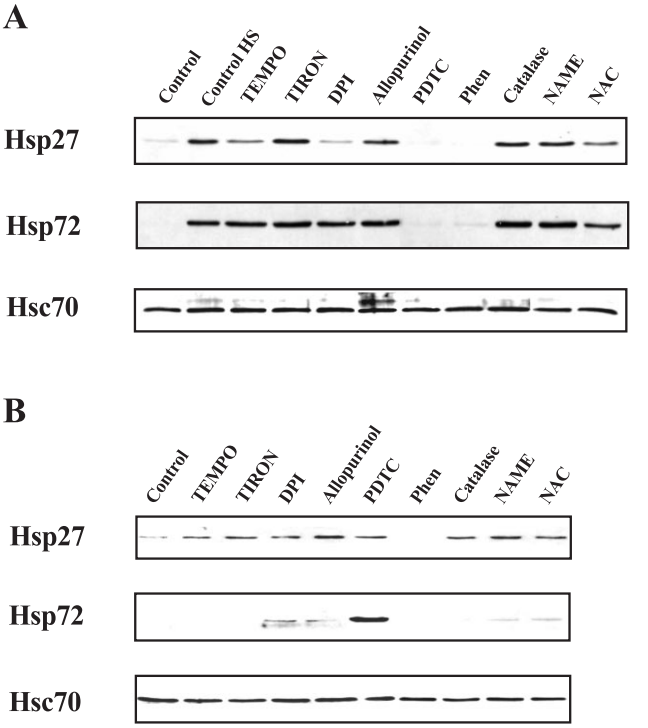

Fig. 2. Effect of antioxidants on hsp induction. A: Cells were heat shocked in the presence of antioxidants at the following concentrations: TEMPO, 1 mM; TIRON, 10 mM; DPI, 1 mM; Allopurinol, 34 μM; PDTC, 100 μM; Phen, 1 mM; Catalase, 500 U/ml; NAME, 1 mM; NAC, 20 mM. After heat shock for 1 h, cells were washed and resuspended in fresh medium in the absence of antioxidants and allowed to recover for 6 h at 37°C. Protein samples were analyzed by Western blotting for the expression of hsp72, hsp27 and hsc72 as indicated. B: Cells were treated as described in A in the absence of heat shock.

3. Results

3.1. Effects of heat exposure on HL-60 cells: hsp induction and cell viability

In order to determine the effect of thermal stress on hsp induction we incubated HL-60 cells at various temperatures (37–46°C) for 1 h and allowed the cells to recover for 6 h prior to Western blot analysis. HL-60 cells do not normally express either hsp72 or 27 (Fig. 1A). However, hsp72 expression was rapidly induced following exposure to 40 or 42°C (Fig. 1A). The expression of hsp27 was also increased at 42°C although not at 40°C (Fig. 1A). Maximal levels of both of these hsps were detectable after heat shock at 42°C.

In contrast, increasing the temperature to either 44 or 46°C did not stimulate the induction of hsp27 or 72 in these cells (Fig. 1A). This was concomitant with the induction of cell death. The mode of death was determined by morphological criteria using cytospin preparation and enzymatically using a caspase assay. Following exposure to 44°C there was a predominance of apoptosis in the cultures while at 46°C the cells were primarily necrotic (Fig. 1B). An increase in apoptosis of cells exposed to 44°C was also shown by an increased level of caspase activity in cells heated to this temperature (Fig. 1C). At 46°C the level of caspase activity in the cells was lower than the background activity in unheated cells, reflecting the extensive necrosis in the cultures treated at this temperature

(Fig. 1C). In contrast, there was no significant alteration in the cell viability of cells heated to 40 or 42°C (Fig. 1B and C).

3.2. Effect of antioxidants on heat shock protein induction

Cells were pre-incubated with various antioxidants prior to heat shock at 42°C for 1 h followed by a 6 h recovery period. Western blot analysis showed that the expression of hsp72 was completely inhibited by pretreatment with PDTC or Phen, while NAC caused a slight decrease in this protein (Fig. 2A). The induction of hsp27 by heat shock was reduced when cells were treated with TEMPO, DPI, PDTC or Phen (Fig. 2A). In contrast, the level of the constitutively expressed member of the hsp70 family, hsc70, was not altered by heat treatment or by antioxidants (Fig. 2A). None of the antioxidants tested, with the exception of PDTC, affected the levels of hsp72 within non-heat-treated cells (Fig. 2B). PDTC treatment alone caused a large elevation in the level of hsp72 (Fig. 2B). However, all of the compounds caused a mild induction in hsp27 (Fig. 2B).

3.3. Apoptosis in heat shocked cells which are pretreated with antioxidants

We decided to examine the status of PARP in these cells. PARP is a caspase substrate whose degradation from 116 kDa to an 85 kDa fragment is indicative of apoptosis. Heat shock alone did not cause a significant increase in PARP cleavage (Fig. 3A). However treatment of the cells with antioxidants prior to heat shock led to extensive cleavage of PARP with some of the antioxidants, namely TEMPO, DPI, Phen and NAC (Fig. 3A). Treatment with Allopurinol, PDTC, catalase or NAME prior to heat shock led to a slight increase in PARP cleavage while TIRON had no effect (Fig. 3A).

In agreement with these observations, treatment of HL-60 cells with either TEMPO, DPI, Phen or NAC prior to heat shock resulted in elevated DEVDase activity compared with heat treatment alone (Fig. 4A). This indicates that there is increased apoptosis in those populations treated with the antioxidants prior to heat treatment. It was observed that treatment of the cells with Phen in the absence of heat treatment led to a large increase in the level of caspase activity while the other antioxidants tested had no effect on caspase activity (Fig. 4B).

Western blot analysis showed that there was almost complete cleavage of PARP in cells treated with Phen alone, while the other antioxidants tested had no effect (Fig. 3B). This demonstrates that Phen treatment induces apoptosis in these cells while the other compounds do not.

Treatment of the cells with the general caspase inhibitor zVAD-FMK did not reverse the reduction in hsp72 or hsp27 levels obtained with PDTC, Phen or NAC (Fig. 5), although zVAD-FMK completely blocked the observed increase in caspase activity as determined by DEVD-MCA cleavage activity (data not shown).

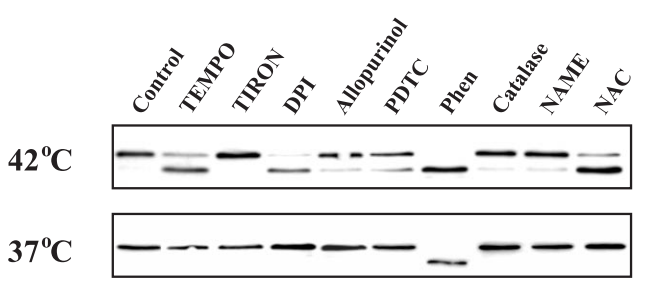

Fig. 3. The effect of heat shock and antioxidants on PARP cleavage. Cells were treated with antioxidants as in Fig. 2. The upper panel shows cells that were also heat shocked, the lower panel shows the effect of antioxidants alone.

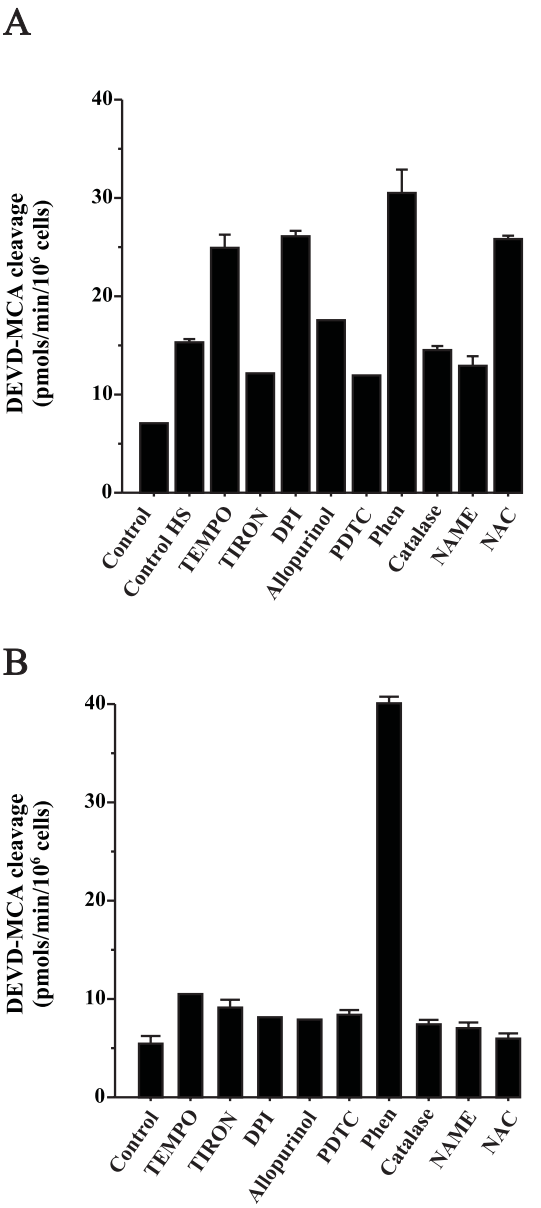

Fig. 4. The effect of heat shock and antioxidants on caspase activity. Cells were treated with antioxidants as described in Fig. 2 either in the presence (A) or absence (B) of heat shock. DEVD-MCA cleavage activity was measured and expressed per 10^6 cells.

4. Discussion

In the present study we show that cells respond to elevations in temperature in different ways depending on the temperature. Exposure to mild increases in temperature (i.e. 42°C) induces hsp72 and 27. This was associated with thermotolerance (data not shown). Indeed, we have previously demonstrated that expression of hsp72 and 27 increases resistance of U937 cells to apoptosis [13]. In contrast, further elevations in temperature induced apoptosis or necrosis, with the mode of

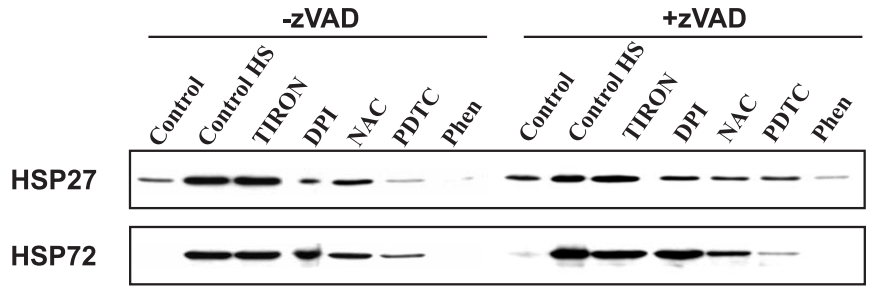

Fig. 5. Lack of effect of zVAD-fmk on the induction of hsps by heat. Cells were treated as in Fig. 2 in the presence or absence of zVAD-FMK (200 μM) as indicated. ZVAD-FMK was included in the medium for 30 min prior to heat shock and was maintained in the medium during heat shock and the 6 h recovery period. Protein samples were analyzed by Western blotting for the expression of hsp72, hsp27 and hsc72 as indicated.

cell death being dependent on the actual temperature (see Fig. 1). It was noted that at lethal temperatures (44 and 46°C) neither hsp72 nor 27 were induced, suggesting that the induction of hsps and of cell death may be mutually exclusive processes.

We further showed that the induction of hsps by heat could be modulated by certain antioxidant molecules. In particular, PDTC and Phen inhibited while NAC reduced the induction of hsp72. PDTC and Phen also inhibited hsp27 induction while TEMPO, DPI, Allopurinol and NAC caused a reduction in its synthesis. This suggests an involvement of oxidative stress or reactive oxygen species in the cellular response to elevations in temperature. However, the inhibition profiles for the two hsps were quite distinct (although overlapping in some cases) suggesting that hsp27 induction is more sensitive to modulation of redox status. There is much evidence to support a role for reactive oxygen species in the cellular response to heat, irrespective of whether the outcome is thermotolerance or cell death [14–16]. However, while a number of the antioxidant compounds tested in the present study caused a reduction or complete inhibition of hsp induction, this was concomitant with an increase in caspase activity, indicative of apoptosis. There are at least two possible explanations for these results.

One is that the antioxidants prevented hsp induction and thus sensitized the cells to the effects of mild elevations in temperature which would ordinarily be counteracted by the rapid induction of protective stress proteins. This hypothesis implies that the induction of heat shock proteins is dependent on ROS or on oxidative stress. In fact, an increased flux of free radicals has been reported in cells subjected to hyperthermia [10] and we have observed that there is a rapid (within 20 min) 5-fold increase in superoxide anion levels in HL-60 cells incubated at 42°C (data not shown). The appearance of hsps following generation of superoxide may suggest a role for this radical in induction of hsp72 and 27. These observations are further suggested by the report that hsp72 gene expression is dependent on superoxide generation in pig liver ischemia [14].

The second possible explanation for the death of cells treated with antioxidants and exposed to 42°C is that the combination of stress due to elevated temperature and incubation with antioxidant compounds is too great for the cells' protective machinery to cope with. Therefore the apoptotic program is initiated before hsps can be induced. If this is the case then treatment of the cells with the cell-permeable caspase inhibitor, zVAD-fmk, should prevent apoptosis and allow hsp induction. However, zVAD-fmk did not alter the hsp status of cells exposed to heat shock and antioxidants. This suggests that the former explanation, i.e. that the induction of death is due to the lack of hsps, is the more plausible. In agreement with this it has been reported that blocking the endogenous increase in hsp72 by antisense converts a mild hypoxic injury to a pattern of cell injury seen with severe injury [17].

Although a number of the antioxidant molecules that we have used in this study have been demonstrated to have confer protection against the induction of apoptosis in different model systems, some have been shown to also have a pro-apoptotic action in certain conditions. For example, while the spin trapping compound TEMPO has been shown to inhibit apoptosis by both etoposide and the glucocorticoid methylprednisolone in thymocytes [18] it has also been reported to have a cytotoxic action on endothelial cells [19]. Dithiocarbamates, including PDTC, can induce apoptosis in thymocytes by raising the intracellular level of redox-active copper [18,20] but on the other hand, PDTC has also been used to successfully decrease apoptosis in PC12 cells [21]. In the short term, however, PDTC exerts mainly antioxidant effects and inhibits apoptosis. In fact, dithiocarbamates can directly inhibit activation of the caspase-3 proenzyme [22]. Over the duration of the experiments presented here, there was no induction of caspase activity in cells which were treated with PDTC alone (Figs. 2B and 4B) although there was a marked elevation in hsp72 levels (Fig. 2B). In fact none of the antioxidant molecules employed in the present study caused an increase in caspase activity when used alone, with one notable exception being Phen which caused a massive increase in caspase activity and in PARP cleavage after 6 h. This is in accordance with a previous report showing that Phen induces apoptosis in rat liver cells by promoting the redox activity of endogenous copper ions [23].

In conclusion, these data show that cell death, either by apoptosis or necrosis, and hsp induction are mutually exclusive events, particularly with regard to hsp27. Certain antioxidant compounds prevent hsp induction and the combination of antioxidant treatment with exposure to heat stress led to death of the cells. This effect is in agreement with the hypothesis of a reciprocal relationship between cell death and hsp induction.

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