Heat-shock protein 70 attenuates nitric oxide-induced apoptosis in RAW macrophages by preventing cytochrome *c* release

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Heat-shock protein (Hsp) 70 is an inhibitor of apoptosis and has been shown to protect against nitric oxide-mediated toxicity. To gain mechanistic insights into the actions of Hsp70, we stably transfected RAW 264.7 mouse macrophages with the human Hsp70 gene and investigated critical steps in the progression towards cell demise. Incubation of control and Hsp70-transfected macrophages with *S*-nitrosoglutathione induced accumulation of the tumour suppressor p53, expression of p21^{WAF1/CIP1} (where WAF1 corresponds to wild-type p53-activated fragment 1 and CIP1 corresponds to cyclin-dependent kinase-interacting protein 1) and G₁ cell-cycle arrest. However, cytochrome *c* translocation to the cytosol and activation of caspase 9 and caspase 3 were

markedly reduced in Hsp70-overexpressing cells. In addition, changes in nuclear morphology, as determined by Hoechst staining, and the appearance of cells in the sub-G₁ phase were diminished in Hsp70-overexpressing cells compared with controls. We conclude that, in macrophages, Hsp70 interferes with cytochrome *c* release from mitochondria and, thereby, prevents nitric oxide-induced apoptosis, but leaves p53 accumulation and interference in the cell cycle intact.

Key words: caspase 9, cell cycle, mitochondria, p21^{WAF1/CIP1}, p53.

INTRODUCTION

Apoptosis, or programmed cell death, is a highly co-ordinated way of cell disassembly. It is required to control cell homeostasis in multicellular organisms and demands tightly controlled steps of execution (reviewed in [1]). Dysregulation, with too little apoptosis, may favour tumour development, whereas enhanced rates of death may underlie tissue destruction, e.g. as in neurodegeneration. Apoptosis is characterized by morphological changes, such as chromatin condensation, DNA fragmentation and formation of apoptotic bodies. These alterations are brought about by multiple, diverse and pathophysiologically relevant stimuli. Agonists may be grouped as being either membrane receptor-dependent or -independent. Receptor activation by the Fas ligand or tumour necrosis factor α promotes death-inducing signalling complex ('DISC') formation, with subsequent activation of caspase 8 [2]. In contrast, several cytotoxic compounds, such as chemotherapeutic drugs or nitric oxide (NO), induce accumulation of the tumour suppressor p53 [3,4] and/or release of cytochrome *c* from mitochondria [5,6]. This initiates formation of the apoptosome, a large protein complex consisting of cytochrome *c*, apoptotic protease-activating factor 1 (Apaf-1) and caspase 9. Apoptosome assembly in turn processes and activates caspase 9 [7]. Activation of initiator caspases, such as caspase 8 or caspase 9, propagate and amplify the apoptotic signal by processing executioner caspases, which induce the final steps of cell disassembly. Several pro- or anti-apoptotic regulators are known, such as members of the Bcl-2 protein family (reviewed in [8]). Pro-survival proteins, e.g. Bcl-2 or Bcl-x_L, maintain cell integrity, inhibit cytochrome *c* release from mitochondria and attenuate apoptosome assembly. Pro-apoptotic proteins, e.g. Bax, promote cytochrome *c* release and thus initiate cell death.

NO displays both anti- and pro-apoptotic effects. On one hand, NO inhibits processing of caspases, causes S-nitrosation of

active caspases and induces expression of protective heat-shock proteins (Hsps). On the other hand, it evokes accumulation of the tumour suppressor p53, cytochrome *c* translocation, activation of caspases and DNA degradation (reviewed in [9]). In RAW macrophages, accumulation of p53 precedes Bcl-x_L down-regulation and cytochrome *c* release [10]. Transfection of cells with p53 antisense plasmids markedly reduces NO-induced apoptosis. However, p53-negative cells are susceptible to NO-mediated cell death as well [11], pointing to p53-dependent and -independent signalling pathways.

The Hsp70 family of proteins includes constitutively expressed and inducible members (reviewed in [12]), which are up-regulated in some tumours and in response to, for example, heavy metals, NO or peroxynitrite. Hsp70 proteins participate in protein folding and transport, refolding of denatured proteins and cell protection from apoptosis. Hsp70 rescues cells from apoptotic/necrotic death, which normally occurs after heat shock, exposure to tumour necrosis factor α , oxidative stress, ceramide, anti-cancer drugs, radiation or NO [13–16]. Hsp70 was reported to inhibit heat shock-induced apoptosis downstream of cytochrome *c* release but upstream of caspase 3 activation [17], whereas, in H₂O₂-elicited apoptosis, Hsp70 attenuates cytochrome *c* release and mitochondrial depolarization [18]. *In vitro*, Hsp70 associates with Apaf-1 to block apoptosome formation and caspase 9 activation [19,20]. At the cellular level, little is known about the impact of Hsp70 on NO-induced apoptosis, especially with regard to p53 accumulation and cytochrome *c* release.

In the present study, we investigate the potential mechanisms of how Hsp70 attenuates NO-mediated apoptosis in macrophages. Although Hsp70 proteins associate with mutated p53 [21], it is unclear whether expression of Hsp70 interferes with wild-type p53 accumulation and/or affects cell-cycle progression to decrease apoptosis. We show that Hsp70 inhibited apoptosis,

Abbreviations used: Ac-DEVD-AMC, *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin; Apaf-1, apoptotic protease-activating factor 1; CIP1, cyclin-dependent kinase-interacting protein 1; DiOC₆, 3,3'-dihexyloxycarbocyanine iodide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSNO, *S*-nitrosoglutathione; Hsp, heat-shock protein; $\Delta\Psi_m$, mitochondrial transmembrane potential; NO, nitric oxide; WAF1, wild-type p53-activated fragment 1.

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but preserved p53 accumulation and the NO-induced cell-cycle arrest.

EXPERIMENTAL

Materials

RPMI 1640 medium and G418 were purchased from PAA Laboratories GmbH (Linz, Austria). Foetal-calf serum was from Gibco BRL (Berlin, Germany). Anti-(Hsp70) antibody was from StressGen Biotechnologies (Victoria, BC, Canada), anti-p53 (clone PAb122) and anti-(cytochrome *c*) antibodies were from BD Pharmingen (Heidelberg, Germany), anti-p21^{WAF1/CIP1} (where WAF1 corresponds to wild-type p53-activated fragment 1 and CIP1 corresponds to cyclin-dependent kinase-interacting protein 1) antibody (C-19) was from Santa Cruz Biotechnology (Heidelberg, Germany), and anti-(mouse caspase 9) antibody was from Cell Signaling Technology (New England Biolabs GmbH, Frankfurt, Germany). Anti- β -tubulin antibody and streptolysin O were obtained from Sigma (Deisenhofen, Germany). Anti-(mouse IgG) was from Amersham Biosciences (Braunschweig, Germany) and anti-(rabbit IgG) was from Promega (Mannheim, Germany). *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin (Ac-DEVD-AMC) fluorogenic substrate was obtained from Biomol (Hamburg, Germany), and propidium iodide and 3,3'-dihexyloxacarbocyanine iodide (DiOC₆) were from Molecular Probes (Leiden, The Netherlands). Streptolysin O was supplied by Sigma.

Cell culture

The mouse monocyte/macrophage cell line RAW 264.7 as well as the Hsp70-expressing clones were maintained in RPMI 1640 medium supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin and 10 % (v/v) heat-inactivated foetal-calf serum. Transfected clones were kept under the selection of G418 (0.2 mg/ml).

Transfection

Plasmids (pZEM-neo and pZEM-hsp70-tag) were obtained from Marja Jäättelä, Department of Tumour Biology, Institute of Cancer Biology, Copenhagen, Denmark [16]. RAW 264.7 macrophages (5×10^4) were seeded into culture flasks 24 h before transfection. Cells were then incubated for 4 h in 6 ml of medium containing 0.1 mM polyethylenimine and 10 μ g of plasmid DNA (pZEM-neo or pZEM-hsp70-tag). After 72 h, the medium was changed, and cells were selected for 4 weeks using 0.2 mg/ml G418. Single cells were seeded into 96-well plates to grow colonies. Expression of Hsp70 in individual colonies was determined by Western-blot analysis.

S-nitrosogluthathione (GSNO) synthesis

S-nitrosogluthathione was synthesized as described previously [22,23].

Cytochrome *c* release

Cells (5×10^6) were washed with PBS and resuspended in 150 μ l of PBS containing 1 unit/ μ l streptolysin O, 1 mM PMSF and 0.01 % BSA. Cells were permeabilized by incubating for 30 min at 37 °C and then centrifuged at 14000 *g* for 30 min at 4 °C [24]. Cytochrome *c* in the supernatant was determined by Western blotting.

Western-blot analysis

Cell lysis was achieved with ice-cold lysis buffer [50 mM Tris/HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5 % Nonidet P40] and sonication for 10 s with a Bandelin Sonoplus HD70 sonifier (Berlin, Germany). Following centrifugation (14000 *g*, 10 min, 4 °C), protein content was measured. Proteins (75 μ g for Hsp70, 50 μ g for p53, 100 μ g for p21, 12.5 μ g for cytochrome *c* and 75 μ g for caspase 9) were subjected to SDS/PAGE using 10 % (w/v) (Hsp70) or 15 % (w/v) (p53, p21, cytochrome *c* and caspase 9) gels, and blotted on to nitrocellulose membranes. After blocking, membranes were incubated with primary antibodies overnight at 4 °C. Horseradish peroxidase-conjugated polyclonal antibodies were used for enhanced chemiluminescence detection. Western blotting of β -tubulin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed to demonstrate equal protein loading.

Determination of mitochondrial transmembrane potential ($\Delta\Psi_m$)

Subsequent to incubation with GSNO, cells were washed and resuspended in medium containing DiOC₆ (20 nM) [25]. Cells were incubated for a further 30 min at 37 °C and then analysed by flow cytometry (Coulter-Epics XL; Coulter, Krefeld, Germany). As a control, the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (20 μ M) was added to the cells during equilibration with DiOC₆ in order to ascertain that a decrease in $\Delta\Psi_m$ was reflected by a diminished DiOC₆ fluorescence. Results are expressed as the percentage of cells with depolarized mitochondria.

Measurement of caspase 3 activity

Caspase-3-like activity was determined by the cleavage of the fluorescent substrate Ac-DEVD-AMC. Cell extracts were prepared as for Western-blot analysis. Protein (50 μ g) was incubated in caspase assay buffer [100 mM Hepes (pH 7.5), 10 % (w/v) sucrose, 0.1 % CHAPS, 1 mM EDTA, 10 mM dithiothreitol] in the presence of 26.7 μ M Ac-DEVD-AMC for 1 h at 30 °C. The increase in fluorescence as a result of AMC release was monitored at 465 nm and caspase activity was expressed in nmol of cleaved Ac-DEVD-AMC/h per mg of protein.

Cell-cycle analysis

Cells were collected and washed once with PBS. They were resuspended in buffer containing 10 mM Tris/HCl (pH 7.5), 10 mM NaCl and 1.5 mM MgCl₂, and incubated with 50 μ g/ml RNase for 15 min on ice. Nonidet P40 (1 % final concentration) and 10 μ g/ml propidium iodide were added, and samples were incubated on ice for at least 15 min. Cell-cycle analysis was performed with a Coulter-Epics XL (Coulter).

Hoechst staining

Cells were harvested, washed once and fixed with 3 % paraformaldehyde in PBS. They were spread on to microscope slides, stained with Hoechst dye (8 μ g/ml) for 5 min, washed and examined under a Leica DMR microscope (VisitronSystems, Puchheim, Germany). Cells (500 cells/sample) were counted and the results are expressed as the percentage of apoptotic cells compared with the total number of cells.

Statistics

Statistical analysis was performed using a one-tailed Wilcoxon/Mann–Whitney test, with $P < 0.05$ being considered significant. Results are given as means $\pm$ S.E.M.

RESULTS

Stable expression of Hsp70

To study the role of Hsp70 in attenuating NO-induced apoptosis in RAW264.7 macrophages, we transfected cells with a plasmid encoding the human Hsp70 gene or an empty vector. Following selection with G418, clones were propagated from single cells, and expression of Hsp70 was determined by Western-blot analysis (Figure 1A). Compared with parent RAW macrophages or cells carrying the empty vector (neo2b), Hsp70 expression was enhanced in transfected cells, as shown for clones hsp2a and hsp2b. Quantification of Western blots, with Hsp70 normalized to GAPDH, revealed a 1.9 ± 0.45 -fold ($n = 3$) increased expression of Hsp70 over controls. Enforced expression of Hsp70 was verified following thermal stress. Parent, vector- and Hsp70-transfected macrophages were exposed to 42 °C for 2 h and returned to 37 °C for 3 h before analysing Hsp70 expression. Compared with unstressed cells, expression of Hsp70 was enhanced after thermal stress. This was also apparent in cells constitutively overexpressing Hsp70 (Figure 1B). Despite enforced overexpression of Hsp70, the protein content was subjected to additional regulation by heat stress. A further 1.5 ± 0.12 -fold ($n = 3$) increase in Hsp70 expression was observed following heat stress when Hsp70-transfected cells (hsp2a and hsp2b) were compared with controls (RAW and neo2b). In contrast with the effect of heat stress, exposure of macrophages to 0.3 mM GSNO for 6 h did not alter Hsp70 expression in either control or Hsp70-overexpressing cells (results not shown).

Figure 1 Hsp70 expression in control and heat-treated cells

Hsp70 expression was determined by Western-blot analysis in parent RAW 264.7 macrophages, vector-transfected (neo2b) and stably Hsp70-transfected cells (hsp2a and hsp2b). Hsp70 expression under resting conditions (A) and following heat treatment (2 h at 42 °C followed by a 3 h recovery period at 37 °C) (B). Typical results from four independent experiments are shown. GAPDH served as a protein loading control.

Figure 2 Accumulation of p53 and p21^{WAF1/CIP1} following incubation with GSNO

Cells were incubated for 6 h with 0.3 mM and 0.5 mM GSNO. p53 (A) and p21^{WAF1/CIP1} (B) were detected by Western-blot analysis. Detection of β -tubulin and GAPDH served as protein loading controls. Typical results from four (p53) or two (p21^{WAF1/CIP1}) independent experiments are shown.

Figure 3 Hsp70 inhibits release of cytochrome *c* into the cytosol

Cells were incubated for 6 h in the absence (—) or presence (+) of 0.3 mM GSNO, harvested and cytosolic fractions were obtained as described in the Experimental section. Cytochrome *c* in the cytosol was determined by Western-blot analysis. Typical results from three independent experiments are shown.

Figure 4 Hsp70 expression prevents caspase 9 activation

Cells were incubated for 6 h in the absence (—) or presence (+) of 0.3 mM GSNO. Processing of caspase 9 from the 49 kDa holozyme to the 39 and 37 kDa fragments was detected by Western-blot analysis. Typical results from four independent experiments are shown.

NO induces p53 accumulation and expression of p21^{WAF1/CIP1}

It is known that NO released from the NO donor GSNO stabilizes the tumour-suppressor protein p53. Incubation of RAW cells with 0.3 or 0.5 mM GSNO for 6 h induced p53 accumulation in parent cells, vector-transfected and Hsp70-overexpressing clones (Figure 2A). Accumulation of p53 in clone hsp2a was slightly impaired but clearly visible.

Table 1 Hsp70 expression reduces caspase 3 activity

Cells were incubated with 0.3 mM GSNO for 6 h. Caspase 3 activity was determined by cleavage of the substrate Ac-DEVD-AMC as described in the Experimental section. Results are means $\pm$ S.E.M. of three experiments. * $P < 0.05$ compared with GSNO-treated neo2b cells.

Cell clone	Activity (nmol/h per mg of protein)	
	Control	GSNO
Neo2b	0.34 $\pm$ 0.19	19.86 $\pm$ 3.12
Hsp2a	0.04 $\pm$ 0.03	2.88 $\pm$ 0.73*
Hsp2b	0.04 $\pm$ 0.04	4.47 $\pm$ 1.92*

Figure 5 Hsp70 expression reduces changes in nuclear morphology

Cells were incubated in the absence or presence of 0.3 mM GSNO for 6 h, fixed and stained with Hoechst dye. **(A)** The percentage of apoptotic nuclei was determined in control cells (open bars) and cells incubated with GSNO (filled bars). * $P < 0.05$ compared with GSNO-treated neo2b cells. **(B)** Representative pictures from four experiments are shown (the arrow indicates a typical apoptotic nucleus). Picture size is 31.9 $\times$ 25.3 μ m.

In order to investigate the response of a classical cell-cycle regulator and established downstream target of p53 [26], we determined the expression of p21^{WAF1/CIP1} upon incubation with GSNO. GSNO caused an up-regulation of p21^{WAF1/CIP1}, but no difference in p21^{WAF1/CIP1} expression was observed between parent

RAW cells, vector- and Hsp70-transfected macrophages (Figure 2B).

Hsp70 inhibits cytochrome *c* release and mitochondrial depolarization

Cytochrome *c* release from the mitochondrial intermembrane space is a central step during apoptosis and can occur through the action of NO or its metabolites on mitochondria or via up-regulation of pro-apoptotic proteins. Incubation of RAW macrophages with 0.3 mM GSNO for 6 h promoted translocation of cytochrome *c* to the cytosol in vector-transfected macrophages, but only to a very minor extent in Hsp70-overexpressing cells (Figure 3).

Reduction of $\Delta\Psi_m$ is a consequence of cytochrome *c* release and commonly observed during apoptosis. However, it has been described that RAW macrophages respond with an increase in $\Delta\Psi_m$ following NO exposure [27]. To determine $\Delta\Psi_m$ under our experimental conditions, we incubated cells with 0.3 mM GSNO for 12 h and used DiOC₆ to assess mitochondrial membrane potential. FACS analysis revealed two populations of cells: intact cells showed increased $\Delta\Psi_m$, whereas dead cells had depolarized mitochondria (results not shown). The percentage of cells with depolarized mitochondria was significantly higher in the neo2b clone (34.1 $\pm$ 4.3 %) compared with the hsp2a (14.8 $\pm$ 3.5 %) or hsp2b (14.6 $\pm$ 0.5 %) clones.

Activation of caspase 9 and caspase 3 is attenuated by Hsp70

Cytosolic cytochrome *c* induces oligomerization of Apaf-1 and caspase 9 in an ATP/dATP-dependent manner. In turn, caspase 9 becomes processed and subsequently activates the downstream effector caspase 3. Incubation of neo2b macrophages with 0.3 mM GSNO for 6 h promoted cleavage of the caspase 9 holoenzyme (Figure 4), whereas processing of caspase 9 was blocked in cells overexpressing Hsp70. As expected, caspase 3 activity increased in response to incubation with GSNO in vector-transfected cells, but was largely attenuated in Hsp70-transfected macrophages, as determined by cleavage of Ac-DEVD-AMC (Table 1).

Hsp70 reduces DNA degradation but does not prevent G₁ cell-cycle arrest

In order to assess degradation of DNA, which is considered a late marker of apoptosis and cell-cycle arrest, nuclei were stained with Hoechst or propidium iodide. Figure 5(A) shows the percentage of apoptotic nuclei after incubation of cells with 0.3 mM GSNO for 6 h. Hsp70-expressing cells revealed significantly less apoptotic nuclei than control cells, and examples are shown in Figure 5(B). Macrophages overexpressing Hsp70 revealed markedly less condensed chromatin compared with neo2b clones.

In the next set of experiments, cells were exposed to 0.3 mM GSNO and stained with propidium iodide. After 16 h, cell-cycle distribution was analysed (Table 2). Examples of original traces are given in Figure 6. In control cells, GSNO induced some reduction in the number of cells in the G₂/M and S phase, with significant amounts of cells exhibiting a sub-G₁ peak. Furthermore, the percentage of cells in G₁ phase was increased. Expression of Hsp70 did not prevent a significant decrease in the number of cells in G₂/M phase. However, the amount of apoptotic cells showing a sub-G₁ peak was drastically reduced in Hsp70-overexpressing macrophages (Table 3).

Table 2 Changes in cell-cycle distribution following the addition of GSNO

Cells were incubated in the absence or presence of 0.3 mM GSNO for 16 h, harvested, stained with propidium iodide and analysed by flow cytometry. Results are means $\pm$ S.E.M. from four independent experiments. * $P < 0.05$ between control and GSNO-treated cells.

Cell clone	G ₁ phase (%)		S phase (%)		G ₂ /M phase (%)	
	Control	GSNO	Control	GSNO	Control	GSNO
Neo2b	54.2 $\pm$ 1.2	62.6 $\pm$ 2.2*	32.2 $\pm$ 1.2	29.0 $\pm$ 2.5	13.7 $\pm$ 1.4	8.4 $\pm$ 1.8
Hsp2a	63.3 $\pm$ 1.9	69.5 $\pm$ 3.7	30.4 $\pm$ 1.4	28.4 $\pm$ 3.7	6.1 $\pm$ 1.0	2.1 $\pm$ 0.7*
Hsp2b	58.4 $\pm$ 1.8	60.8 $\pm$ 1.2	37.0 $\pm$ 1.3	38.6 $\pm$ 1.7	4.6 $\pm$ 1.3	0.7 $\pm$ 0.7*

Figure 6 Cell-cycle distribution following incubation with GSNO

Cells were incubated in the absence or presence of 0.3 mM GSNO for 16 h, harvested, stained with propidium iodide and analysed by flow cytometry. Cell-cycle distribution was determined as described in the Experimental section. Typical examples of original data are shown.

Table 3 DNA degradation evoked by the exposure to GSNO

Cells were incubated in the absence or presence of 0.3 mM GSNO for 16 h, harvested, stained with propidium iodide, analysed by flow cytometry, and the percentage of cells in sub-G₁ phase was determined. Results are means $\pm$ S.E.M. from five independent experiments. * $P < 0.05$ compared with GSNO-treated neo2b cells.

Cell clone	DNA degradation (%)	
	Control	GSNO
Neo 2b	2.7 $\pm$ 0.5	24.4 $\pm$ 3.3
Hsp2a	0.7 $\pm$ 0.1	12.5 $\pm$ 1.9*
Hsp2b	1.1 $\pm$ 0.1	17.1 $\pm$ 0.9*

DISCUSSION

In the present study, we provide evidence that Hsp70 diminished NO-induced apoptosis in RAW 264.7 macrophages, whereas the accumulation of the tumour suppressor p53 and cell-cycle arrest remained effective. We also observed reduced cytochrome *c* release from mitochondria and thus no processing/activation of caspase 9 and caspase 3.

Our understanding of Hsp70 is no longer restricted to its function as a molecular chaperone that facilitates protein folding, since the anti-apoptotic actions of Hsp70 have become apparent [17,18]. Our present results are consistent with an earlier observation reporting that attenuated necrotic cell death was a result of Hsp70 overexpression [16]. We have extended this finding by delineating the central apoptotic steps in macrophages in order to clarify the interference of Hsp70 in this pathway. In RAW 264.7 macrophages, accumulation of p53 in response to NO represents an early and critical step in propagating a pro-apoptotic signal. This was confirmed by eliminating p53 using antisense technology and, thereby, largely attenuating apoptosis [11]. Although Hsp70 effectively inhibited apoptosis, accumulation of p53 remained unaffected. This implies that at present unidentified pathways, which induce the stabilization of p53 under the influence of NO, remain intact. Candidate targets that may be affected by NO to stabilize p53 are the proteasomal degradation pathway and/or alterations in p53 distribution. Experiments are under way to clarify this issue. However, in thymocytes, irradiation-induced p53 accumulation was attenuated by heat shock pretreatment [15]. An explanation for these variations is that different signalling pathways are used by irradiation and NO to promote p53 accumulation. Current information does not imply that nuclear damage occurs as a result of NO treatment, which is in contrast with concepts of irradiation-induced damage.

Along with the unaltered accumulation of p53 in macrophages, we observed the up-regulation of p21^{WAF1/CIP1} during exposure to GSNO. Considering that p21^{WAF1/CIP1} expression in RAW 264.7 macrophages requires p53 stabilization [26], it can be concluded that p53 transcriptional activity is unaltered, which allows downstream signalling to halt cell-cycle progression. This is highlighted by cell-cycle analysis showing that Hsp70 overexpression does not interfere with NO-mediated G₁ cell-cycle arrest and/or alterations seen in G₂/M.

There is a degree of similarity between the protection afforded by overexpression of Hsp70 and Bcl-2. Bcl-2 has been shown to interrupt the apoptotic cascade at several steps by inhibiting the release of cytochrome *c* from mitochondria [28] and nuclear import of p53 [29]. Moreover, Bcl-2 reduces p21 expression [30] and protects Apaf-1^{-/-} cells from apoptosis [31]. Taking into account that Hsp70 has been reported to stabilize mitochondria [32], an action shared by Bcl-2, it can be speculated that Hsp70

confers resistance to mitochondria in macrophages by not allowing propagation of the apoptotic signal at this site. Unfortunately, previous studies using heat treatment to up-regulate Hsp70 also induced Bcl-2 at the same time, making it difficult to draw conclusions about the action of Hsp70 on mitochondria [32]. Release of cytochrome *c* from mitochondria is central for propagating apoptotic signals and, therefore, signalling effects of Hsp70 at this level have been investigated in previous studies [17,18,24,33]. However, these studies have produced contradictory conclusions, and have not investigated the effects of NO intoxication.

Cytochrome *c* translocation in H₂O₂-exposed Jurkat T-cells was suppressed in clones expressing Hsp70 [18], and heat pretreated RAW macrophages did not release cytochrome *c* after addition of NO [33]. Furthermore, expression of full-length Hsp70, but not Hsp70 without ATPase activity, blocked cytochrome *c* release after heat treatment [24]. In contrast, in the macrophage-type cell line U937, expression of Hsp70 still allowed cytochrome *c* release after heat treatment, but abrogated caspase 3 activation, suggesting that signalling between cytochrome *c* and caspase 3 was interrupted [17]. This concept has now been confirmed by two *in vitro* studies showing that Hsp70 interferes with apoptosome assembly [19,20]. We have studied the activation of caspase 3 *in vitro* by adding cytochrome *c* and dATP to cytosol from control and Hsp70-overexpressing cells (hsp2a and hsp2b clones), and found only 10–20% lower caspase 3 activity in cytosol from hsp2a and hsp2b clones (S. D. Klein and B. Brüne, unpublished work). Further examination is needed to address the question of whether the level of Hsp70 expression defines its action. It may turn out that higher Hsp70 concentrations are needed for blocking apoptosome assembly, whereas lower expression uses different pathways to attenuate cell death. In our present study, expression of Hsp70 was moderate, since heat treatment still allowed a further increase. No decrease in the growth rate was evident in our cells, although this has been reported for cells that overexpress Hsp70 [24]. Multiple actions of Hsp70, besides blocking apoptosome assembly, are also apparent when Hsp70 protects Apaf-1^{-/-} cells against death induced by serum withdrawal [34]. In addition, it has been reported that Hsp70 prevents changes in nuclear morphology even after caspase 3 has been activated [35]. A recent study provided evidence for the synergistic effects of chaperone pairs, namely Hsp70/dj1 or Hsp70/dj2, in attenuating NO-induced apoptosis and caspase 3 activity, whereas transfection of Hsp70 alone was inactive [33]. In contrast with those experiments performed by transient transfection, we have used stably transfected macrophages to avoid any variation on the basis of transfection efficiency. Our present results indicate that Hsp70 expression alone is sufficient in blocking apoptosis, although an even stronger apoptosis-interfering effect of Hsp70 may be possible upon co-transfection with dj1 or dj2. However, the question still remains as to how these divergent effects can be combined to obtain one unifying hypothesis for Hsp action. Depending on the type of apoptotic stress, distinct pathways are engaged to transmit death signals and these signalling cascades may have different susceptibilities towards inhibition by Hsp70. Our present study shows that Hsp70 expression in RAW macrophages renders them resistant to NO-induced apoptosis by preventing cytochrome *c* release from mitochondria. On the basis of *in vitro* experiments, it appears likely that later steps in the apoptotic cascade are affected by Hsp70 as well [19,20], but these findings need confirmation in cells that express gradually increasing levels of Hsps in order to dissect primary versus secondary targets. Hsp70 expression does not affect p53 accumulation, allowing cells to eventually recover from a period of

NO-intoxication. Macrophages may need such protective mechanisms, as the production of NO is used to fight invading pathogens without causing autotoxicity.

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REFERENCES

- Vaux, D. L. and Strasser, A. (1996) The molecular biology of apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 2239–2244
- Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S. and Dixit, V. M. (1998) An induced proximity model for caspase-8 activation. *J. Biol. Chem.* **273**, 2926–2930
- Fritsche, M., Haessler, C. and Brandner, G. (1993) Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. *Oncogene* **8**, 307–318
- Messmer, U.K., Ankarcrone, M., Nicotera, P. and Brüne, B. (1994) p53 expression in nitric oxide-induced apoptosis. *FEBS Lett.* **355**, 23–26
- Di Matola, T., D'Ascoli, F., Fenzi, G., Rossi, G., Martino, E., Bogazzi, F. and Vitale, M. (2000) Amiodarone induces cytochrome *c* release and apoptosis through an iodine-independent mechanism. *J. Clin. Endocrinol. Metab.* **85**, 4323–4330
- Fulda, S. and Debatin, K. M. (2000) Betulinic acid induces apoptosis through a direct effect on mitochondria in neuroectodermal tumors. *Med. Pediatr. Oncol.* **35**, 616–618
- Zou, H., Li, Y., Liu, X. and Wang, X. (1999) An APAF-1 cytochrome *c* multimeric complex is a functional apoptosome that activates procaspase-9. *J. Biol. Chem.* **274**, 11549–11556
- Adams, J. M. and Cory, S. (1998) The bcl-2 protein family: arbiters of cell survival. *Science (Washington, D.C.)* **281**, 1322–1326
- Brüne, B., von Knethen, A. and Sandau, K. B. (1998) Nitric oxide and its role in apoptosis. *Eur. J. Pharmacol.* **351**, 261–272
- Brockhaus, F. and Brüne, B. (1999) p53 accumulation in apoptotic macrophages is an energy demanding process that precedes cytochrome *c* release in response to nitric oxide. *Oncogene* **18**, 6403–6410
- Messmer, U. K. and Brüne, B. (1996) Nitric oxide-induced apoptosis: p53-dependent and p53-independent signalling pathways. *Biochem. J.* **319**, 299–305
- Kiang, J. G. and Tsokos, G. C. (1998) Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. *Pharmacol. Ther.* **80**, 183–201
- Jäättelä, M., Wissing, D., Bauer, P. A. and Li, G. C. (1992) Major heat shock protein hsp70 protects tumor cells from tumor necrosis factor cytotoxicity. *EMBO J.* **11**, 3507–3512
- Mosser, D. D., Caron, A. W., Bourget, L., Denis-Larose, C. and Massie, B. (1997) Role of the human heat shock protein hsp70 in protection against stress-induced apoptosis. *Mol. Cell. Biol.* **17**, 5317–5327
- Gordon, S. A., Hoffman, R. A., Simmons, R. L. and Ford, H. R. (1997) Induction of heat shock protein 70 protects thymocytes against radiation-induced apoptosis. *Arch. Surg. (Chicago)* **132**, 1277–1282
- Bellmann, K., Jäättelä, M., Wissing, D., Burkart, V. and Kolb, H. (1996) Heat shock protein hsp70 overexpression confers resistance against nitric oxide. *FEBS Lett.* **391**, 185–188
- Li, C.-Y., Lee, J.-S., Ko, Y.-G., Kim, J.-I. and Seo, J.-S. (2000) Heat shock protein 70 inhibits apoptosis downstream of cytochrome *c* release and upstream of caspase-3 activation. *J. Biol. Chem.* **275**, 25665–25671
- Creagh, E. M., Carmody, R. J. and Cotter, T. G. (2000) Heat shock protein 70 inhibits caspase-dependent and -independent apoptosis in Jurkat T cells. *Exp. Cell Res.* **257**, 58–66
- Beere, H. M., Wolf, B. B., Cain, K., Mosser, D. D., Mahboubi, A., Kuwana, T., Tailor, P., Morimoto, R. I., Cohen, G. M. and Green, D. R. (2000) Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. *Nat. Cell Biol.* **2**, 469–475
- Salah, A., Srinivasula, S. M., Balkir, L., Robbins, P. D. and Alnemri, E. S. (2000) Negative regulation of the Apaf-1 apoptosome by Hsp70. *Nat. Cell Biol.* **2**, 476–483
- Fourie, A. M., Hupp, T. R., Lane, D. P., Sang, B.-C., Barbosa, M. S., Sambrook, J. F. and Gething, M.-J. H. (1997) HSP70 binding sites in the tumor suppressor protein p53. *J. Biol. Chem.* **272**, 19471–19479
- Hart, T. W. (1985) Some observations concerning the S-nitroso and S-phenylsulphonyl derivatives of L-cysteine and glutathione. *Tetrahedron Lett.* **26**, 2013–2016
- Brockhaus, F. and Brüne, B. (1999) Overexpression of CuZn superoxide dismutase protects RAW 264.7 macrophages against nitric oxide cytotoxicity. *Biochem. J.* **338**, 295–303

- 24 Mosser, D. D., Caron, A. W., Bourget, L., Meriin, A. B., Sherman, M. Y., Morimoto, R. I. and Massie, B. (2000) The chaperone function of hsp70 is required for protection against stress-induced apoptosis. *Mol. Cell. Biol.* **20**, 7146–7159
- 25 Métivier, D., Dallaporta, B., Zamzami, N., Larochette, N., Susin, S. A., Marzo, I. and Kroemer, G. (1998) Cytofluorometric detection of mitochondrial alterations in early CD95/Fas/APO-1-triggered apoptosis of Jurkat T lymphoma cells. Comparison of seven mitochondrion-specific fluorochromes. *Immunol. Lett.* **61**, 157–163
- 26 Yang, F., von Knethen, A. and Brüne, B. (2000) Modulation of nitric oxide-evoked apoptosis by the p53-downstream target p21^{WAF1/CIP1}. *J. Leukocyte Biol.* **68**, 916–922
- 27 Hortelano, S., Alvarez, A. M. and Boscá, L. (1999) Nitric oxide induces tyrosine nitration and release of cytochrome *c* preceding an increase of mitochondrial transmembrane potential in macrophages. *FASEB J.* **13**, 2311–2317
- 28 Shimizu, S., Narita, M. and Tsujimoto, Y. (1999) Bcl-2 family proteins regulate the release of apoptogenic cytochrome *c* by the mitochondrial channel VDAC. *Nature (London)* **399**, 483–487
- 29 Beham, A., Marin, M. C., Fernandez, A., Herrmann, J., Brisbay, S., Tari, A. M., Lopez-Berestein, G., Lozano, G., Sarkiss, M. and McDonnell, T. J. (1997) Bcl-2 inhibits p53 nuclear import following DNA damage. *Oncogene* **15**, 2767–2772
- 30 Bukholm, I. K., Nesland, J. M., Karesen, R., Jacobsen, U. and Borresen-Dale, A. L. (1997) Interactions between bcl-2 and p21 (WAF1/CIP1) in breast carcinomas with wild-type p53. *Int. J. Cancer* **73**, 38–41
- 31 Haraguchi, M., Torii, S., Matsuzawa, S., Xie, Z., Kitada, S., Krajewski, S., Yoshida, H., Mak, T. W. and Reed, J. C. (2000) Apoptotic protease activating factor 1 (Apaf-1)-independent cell death suppression by Bcl-2. *J. Exp. Med.* **191**, 1709–1720
- 32 Polla, B. S., Kantengwa, S., François, D., Salvio, S., Franceschi, C., Marsac, C. and Cossarizza, A. (1996) Mitochondria are selective targets for the protective effects of heat shock against oxidative injury. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 6458–6463
- 33 Gotoh, T., Terada, K. and Mori, M. (2001) hsp70-DnaJ chaperone pairs prevent nitric oxide-mediated apoptosis in RAW 264.7 macrophages. *Cell Death Differ.* **8**, 357–366
- 34 Ravagnan, L., Gurbuxani, S., Susin, S. A., Maise, C., Daugas, E., Zamzami, N., Mak, T., Jäättelä, M., Penninger, J. M., Garrido, C. and Kroemer, G. (2001) Heat-shock protein 70 antagonizes apoptosis-inducing factor. *Nat. Cell Biol.* **3**, 839–843
- 35 Jäättelä, M., Wissing, D., Kokholm, K., Kallunki, T. and Egeblad, M. (1998) Hsp70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. *EMBO J.* **17**, 6124–6134

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