A new feature of the stress response: increase in endocytosis mediated by Hsp70

Virginia L. Vega · Wisler Charles · Antonio De Maio

Received: 21 August 2009 / Revised: 19 November 2009 / Accepted: 23 November 2009 / Published online: 31 December 2009
© Cell Stress Society International 2009

Abstract The expression of heat shock proteins (HSP) is a conserved cellular response to a variety of stresses. These proteins have been found to refold denatured polypeptides and stabilize critical cellular processes. In this study, we introduce a new component of the stress response: the increase of receptor-mediated uptake of macromolecules from the external environment. We observed that endocytosis of transferrin, which is involved in the delivery of iron to the cell, was increased after stress induced by heat shock or after incubation with inhibitors of Hsp90 function. In both cases, the increase in endocytosis was reverted by inhibition of transcription, suggesting that gene expression is required. Transfection of cells with *Hsp70* gene or inhibition of its expression by siRNA confirmed the role of this HSP in the increase of endocytosis. The mechanism for the enhancement of transferrin uptake was related to an accelerated internalization of the ligand–receptor complex as well as an increase in receptor recycling. These observations constitute a new paradigm for the cellular protection induced by HSP.

Keywords Heat shock proteins · Transferrin · Geldanamycin · Receptor recycling

Introduction

Cells are constantly adapting to changes in their environment, resulting in a modulation of gene expression to adjust

to the new physiological conditions. Some of these environmental changes may compromise cellular integrity, triggering the stress response. Expression of heat shock proteins (HSP) is a universal response to a variety of physiological, chemical, and physical stresses, which are directed at preserving cell integrity and ensuring survival.

HSP belong to a family of well-conserved proteins, which are classified according to their molecular weights: Hsp100, 90, 70, 60, and small HSP (Lindquist and Craig 1988; De Maio 1999). Some HSP are constitutively present within cells, and they participate in several basic processes, including protein folding, stabilization of substrates, and assembly of macromolecule structures (Bukau et al. 2006). Upon stress, expression of HSP is induced or increased to participate in the folding and degradation of polypeptides that become denatured as a consequence of the insult. Moreover, HSP also stabilize several cellular processes affected by the insult, such as transcription, splicing, translation, and transport. In addition, damaged macromolecules that are beyond repair and other cellular debris are likely to be disposed of by a process requiring HSP (Minowada and Welch 1995; De Maio 1999).

The effect of cellular stressors, such as heat shock (HS), on cellular membranes has gained increasing attention recently. HS changes membrane fluidity, affecting its function, such as the distribution of integral membrane proteins, such as receptors and transporters (Carratù et al. 1996). Several HSP have been shown to associate with membranes, including Hsp70, Hsp60, and small HSP (Multhoff et al. 1995; Vigh et al. 2007; Vega et al. 2008; Schilling et al. 2009). In addition, it has been demonstrated that members of the Hsp70 family, Hsp70 (stress-inducible) and Hsc70 (constitutive), become incor-

V. L. Vega · W. Charles · A. De Maio (✉)
UCSD Department of Surgery,
University of California San Diego,
9500 Gilman Drive, 0739,
La Jolla, CA 92093-0739, USA
e-mail: ademaio@ucsd.edu

porated into artificial lipid bilayers, opening ion conductance pathways (Arispe et al. 2002; Vega et al. 2008). Moreover, Hsp70 has been detected on the surface of transformed and/or stressed cells (Multhoff et al. 1995; Multhoff 2002; Gehrmann et al. 2008; Vega et al. 2008). Although the role of membrane-associated HSP on the stress response is unknown, it could be assumed that this interaction of HSP with membranes may have regulatory effects over different processes, such as cell adhesion, transport, receptors expression, and internalization of macromolecules. The optimization of these processes by membrane-associated HSP may result in faster secretion of toxic metabolites produced during stress as well as an improved internalization of critical nutrients that guarantee cell repair and survival. In the present study, we investigated the effect of HSP expression on clathrin-mediated endocytosis.

Experimental procedures

Human hepatoblastoma cells, HepG2, were obtained from American Type Culture Collection. AlexaFluor594 or fluorescein isothiocyanate (FITC)-conjugated transferrin was purchased from Invitrogen (Carlsbad, CA). Antibody against the human Hsp70 cmHsp70.1 was obtained from Multimune GmbH (Munich, Germany). Antibodies against Hsp70 (SPA-810), Hsc70 (SPA-815), Hsp27 (SPA-800), and Hsp90 (SPA-830) were purchased from StressGen Bioreagents (Ann Arbor, MI). Actinomycin D (AcD), geldanamycin (GA) from *Streptomyces hygroscopicus*, β -actin antibody (A5451), and methylthiazole tetrazolium (MTT) were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture conditions and treatments HepG2 cells were grown in MEM medium supplemented with 10% heat-inactivated fetal bovine serum, 10 IU/ml penicillin, and 10 μ /ml streptomycin, L-glutamine, non-essential amino acids, and sodium pyruvate at 37°C with 5% CO₂ in a humidified incubator. Cells were treated with GA (1 μ g/ml) for up to 8 h at 37°C. HS was performed by incubation of the cells for 1.5 h at 43°C followed by a recovery period at 37°C.

Transferrin endocytic assay and visualization of internalized transferrin HepG2 cells (1×10^6 cells) were washed twice with sterile phosphate-buffered saline (PBS) and incubated with serum-free (SF) medium for 0.5 h at 37°C. Endocytosis was evaluated by incubation of cells with FITC- or AlexaFluor594-conjugated transferrin (50 μ g/ml) up to 0.5 h at 37°C. Non-internalized transferrin was removed, and cells were washed twice with PBS pH 5.0. Intensity of the internalized signal was measured using a fluorom-

eter and normalized by the number of live cells in each well, as estimated by MTT assay. Results were expressed as endocytic index (intensity of the signal/MTT). To visualize internalized transferrin, cells (0.5×10^6) on a sterile glass-cover slide were incubated with FITC- or AlexaFluor594-conjugated transferrin (50 μ g/ml) as described above. Then, cells were fixed with 4% paraformaldehyde (PFA) for 10 min at 25°C. Slides were mounted (3.9% DABCO in PermaFluor aqueous mounting medium) and visualized using a fluorescent microscope.

Immunostaining Cells (1×10^6) on a sterile, glass-cover slide were incubated with serum (20%) from the host of secondary antibody and 0.2% Tween-20 in PBS (0.5 h, 25°C) to block nonspecific binding. Then, cells were incubated with primary antibodies (1:200 dilution) for 1 h at 4°C, extensively washed with PBS, and incubated with secondary antibodies (1:1,000 dilution) for 0.5 h at 4°C. When indicated, cells were fixed with 4% PFA (10 min, 25°C) and permeabilized with cold acetone (15 s). Nuclei were stained with 4',6-diamido-2-phenylindole hydrochloride (15 s, 25°C), and cells were visualized using a fluorescent microscope.

Western blotting Cells were washed twice with cold PBS, scraped, and sonicated in 10 mM Tris-HCl pH 7.4 containing 1 mg/ml PMSF and protease inhibitor cocktail from Roche. Protein concentration was determined by the BCA method (Pierce). Total proteins (100–150 μ g) were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (400 mA for 3 h). Membranes were blocked with 5% nonfat milk in Tris-buffered saline (TBS) pH 7.4 for 0.5 h at 25°C. Primary antibodies (1:5,000 dilutions in TBS) were incubated for 16 h at 4°C. Membranes were rinsed twice with washing solution (0.2% Tween-20 in TBS pH 7.4) and incubated with HRP-conjugated secondary antibodies (1:25,000 in TBS) for 3 h at 4°C. Proteins were detected using the SuperSignal Chemiluminescent Detection Kit (Pierce).

Kinetics of transferrin receptor recycling Time of transferrin receptor (TfnR) recycling was estimated in control, heat-shocked, and GA-treated cells as described by Johnson et al. (1994). Cells (1×10^6) in 12-well plates were incubated in SF medium for 16 h at 37°C. Cells were incubated with AlexaFluor594-conjugated transferrin (3 μ g/ml) in the presence (nonspecific signal wells) or absence (signal wells) of 200-fold excess of unlabeled transferrin (20 min at 37°C). Cell-bound transferrin was removed by incubating with 20 mM sodium citrate buffer pH 5.0 (containing 150 mM NaCl; 2 min at 25°C), followed by efflux buffer (0.1 mM desferrioxamine and 3 μ g/ml human diferric unlabeled transferrin in PBS; 1 min, 25°C). Then, cells were incubated with 600 μ l of efflux medium for 2.5, 5, 10, 15, 20, and

30 min at 37°C. Levels of AlexaFluor594-conjugated transferrin were quantified by fluorometry in both supernatants and cell lysates at each time point. Corrected signal in supernatants and cell lysates were calculated by subtracting the nonspecific signal. Percentage of cell-associated signal was calculated at each time point. Time of TfnR recycling was calculated from the plot natural logarithm of cell-associated percentage vs. time.

Cell transfection HepG2 cells (30% confluency) were transfected using FuGENE with pSG5–12c, a WT Hsp70 plasmid. After transfection (48 h), transferrin internalization was evaluated as described above. Hsp70 expression was measured by Western blot or by immunostaining.

Results

Endocytosis of transferrin was enhanced upon treatment of HepG2 cells with GA GA is a specific inhibitor of

Hsp90 function that combines with the adenosine triphosphate (ATP)-binding site of the molecule (Necker and Ivy 2003). In addition, treatment with GA induces the expression of HSP by disrupting the interaction of Hsp90 with HSF-1 (Zou et al. 1998). Thus, the effect of GA on cells could be due to the inhibition of Hsp90 function, induction of the HS response, or both. To investigate the effect of GA on clathrin-dependent endocytosis, HepG2 cells (human hepatoblastoma) were treated with GA (1 µg/ml) for 0 to 5 h and incubated with FITC-conjugated transferrin for 0.5 h at 37°C. Then, cells were washed extensively with acidic PBS (pH 5.0) to remove the non-endocytosed ligand, and the internalized transferrin signal was measured using a fluorometer or visualized by fluorescent microscopy. GA treatment resulted in a significant increase in endocytosis of transferrin proportionally to the incubation time with the drug in comparison with untreated cells (Fig. 1a). This observation was confirmed by fluorescent microscopy (Fig. 1b). The increase in transferrin uptake induced

Fig. 1 Treatment with GA resulted in increased transferrin endocytosis. **a** HepG2 cells were incubated with GA (1 µg/ml) from 1 to 5 h in the presence or absence of AcD (10 µg/ml) at 37°C. Then, cells were incubated with SF medium (30 min) followed by the addition of FITC-conjugated transferrin (50 µg/ml) in SF medium for 0.5 h at 37°C. Extracellular transferrin was removed by washing with acidic PBS (pH 5.0), and the non-internalized signal was quenched by trypan blue. Intensity of the signal was measured using a fluorometer and normalized by the number of cells in the well detected by the MTT method. The results were compared with cells treated under identical conditions, but in the absence of GA or AcD ($t=0$). Results are expressed as arbitrary units (intensity signal/MTT). **b** The internalization of FITC-conjugated transferrin (50 µg/ml) under the same

conditions described above was visualized by fluorescent microscopy (3 h GA or AcD treatment). **c** Cells were incubated in the presence or not of GA for 5 h followed by incubation with different concentrations of FITC-conjugated transferrin (from 15 to 100 µg/ml) SF medium for 0.5 h at 37°C. The internalization of transferrin was determined as described above. **d** Cells were treated with GA, AcD, or a combination of both, for 5 h at 37°C and incubated with FITC-conjugated transferrin (50 µg/ml) for 0, 15, 30, and 60 min at 37°C. The internalization of transferrin was determined as described above. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by Newman–Keuls test. * $p<0.05$ vs. untreated cells

by GA was abolished by pre-incubation of the cells with the transcription inhibitor AcD (Fig. 1a, b), suggesting that gene expression is required for the increase in endocytosis. Incubation of HepG2 cells with AcD alone did not have any effect on transferrin endocytosis in comparison with untreated cells (Fig. 1b). The effect of GA treatment on endocytosis was observed at different concentrations of FITC-conjugated transferrin (Fig. 1c) or at different incubation times with a fixed amount of FITC-conjugated transferrin (Fig. 1d). These results suggest that GA treatment induced an increase in transferrin endocytosis, which requires new gene expression, rather than inhibition of Hsp90 function.

The effect of GA treatment on endocytosis was independent of oxidative stress Previous reports have suggested that the effect of GA on cells could be due to oxidative stress generated during the metabolism of the quinone group within the drug by cytochrome P450 (Billecke et al. 2002; Dikalov et al. 2002; Dey and Cederbaum 2006). To address whether or not the effect of GA on transferrin endocytosis is related to oxidative stress, cells were incubated with radicicol (RD), another inhibitor of the Hsp90 family, which lacks the quinone group and, therefore, does not generate an oxidative stress (Schulte et al. 1999; Billecke et

al. 2002; Sreedhar et al. 2003). Cells were incubated with RD or GA (1 $\mu\text{g/ml}$) for 5 h, and the uptake of FITC-conjugated transferrin was monitored up to 30 min at 37°C. Both RD and GA treatments resulted in a similar increase in transferrin internalization with respect to untreated cells (Fig. 2a, b). In addition, cells that were incubated with GA in the presence or absence of *N*-acetylcysteine, a potent antioxidant, displayed no significant differences in GA-enhanced endocytosis (data not shown), supporting the evidence that oxidative stress is not involved in the GA-associated increase in transferrin endocytosis.

GA treatment did not alter transferrin binding to its receptor, but resulted in faster ligand–receptor internalization The effect of GA on transferrin endocytosis could be due to an increase in the binding of transferrin to its specific cell surface receptor, TfnR. To test this possibility, cells were incubated in presence or absence of a combination of GA and AcD for 1 to 5 h at 37°C. Then, cells were placed in prechilled SF medium (30 min, 4°C) and incubated with FITC-conjugated transferrin (30 min at 4°C). Unbound transferrin was removed by extensive washing at 4°C, and the intensity of the bound signal was measured using a fluorometer or visualized by fluorescent microscopy. No significant differences in transferrin bind-

Fig. 2 Effect of GA on endocytosis of transferrin was not due to oxidative stress. **a** HepG2 cells were treated or not with GA (1 $\mu\text{g/ml}$) or RD (1 $\mu\text{g/ml}$) for 5 h at 37°C, followed by incubation with FITC-conjugated transferrin (50 $\mu\text{g/ml}$) in SF medium for 5, 15, or 30 min. Extracellular transferrin was removed by washing with PBS and the non-internalized signal was quenched by trypan blue. Intensity of the

signal was measured using a fluorometer and normalized by the number of cells in the well detected by the MTT method. Results are expressed as arbitrary units (intensity signal/MTT). **b** The uptake of transferrin was visualized by fluorescent microscopy (GA or RD for 5 h and incubation with FITC-conjugated transferrin for 30 min)

ing were observed between the four experimental groups (Fig. 3a). In other words, transferrin binding was similar between cells treated or not with the combination of GA and AcD. In addition, treatment with GA did not alter the cell surface level of TfnR as visualized by immunostaining of nonpermeabilized cells (Fig. 3b). Moreover, total cellular content of TfnR was not different between cells treated or not with GA as detected by Western blotting (Fig. 3c). These results suggest that the effect of GA on transferrin endocytosis was not due to an increase in the expression of TfnR. If the receptor levels are not altered after GA treatment, the increase in endocytosis could be due to accelerated internalization of the ligand–receptor complex. To

evaluate this possibility, HepG2 cells were incubated with FITC-conjugated transferrin (30 min at 4°C), and the unbound ligand was removed by washing with PBS at 4°C. These conditions allowed transferrin binding but not internalization into the cells. A similar transferrin binding pattern was observed in cells treated or not with GA (Fig. 4). Then, cells were warmed up at 37°C for 5, 10, and 20 min. The non-internalized signal was quenched by incubation with trypan blue, and internalized FITC–transferrin was visualized by fluorescent microscopy. A more rapid internalization of FITC-conjugated transferrin was observed in cells treated with GA in comparison with untreated cells (Fig. 4). This observation suggests that accelerated transferrin–TfnR

Fig. 3 Binding of transferrin was not affected by GA treatment. **a** HepG2 cells were incubated with GA (1 µg/ml), AcD (10 µg/ml), a combination of both, or in the absence of both drugs for 1 to 5 h at 37°C. Then, cells were incubated in prechilled SF medium (30 min at 4°C) and incubation with FITC-conjugated transferrin (50 µg/ml) was performed for 30 min at 4°C. Unbound transferrin was removed by washing with PBS, and the intensity of the transferrin signal was measured using a fluorometer and normalized by the number of cells in the well detected by the MTT method. **b** Alternatively, binding of

FITC-conjugated transferrin to the cell surface was visualized by fluorescent microscopy (incubation with GA or GA + AcD for 3 h). **c** The cell surface levels of TfnR were visualized in nonpermeabilized HepG2 cells after 5 h of treatment with GA, GA + AcD, or in the absence of both drugs by fluorescent microscopy using antibody specific for the receptor followed by a FITC-conjugated secondary antibody. **d** Total cellular levels of TfnR were measured by Western blotting in lysates of cells treated or not with GA for 5 h

Fig. 4 Internalization of transferrin was accelerated by pre-treatment with GA. HepG2 cells were incubated with GA (1 $\mu\text{g}/\text{ml}$) for 5 h at 37°C, followed by incubation in pre-chilled SF medium (30 min, 4°C) and further incubated with FITC-conjugated transferrin (50 $\mu\text{g}/\text{ml}$) for 30 min at 4°C. Unbound transferrin was removed by washing with PBS at 4°C. Then, cells were incubated in SF medium without transferrin for 5, 10, and 20 min at 37°C. The bound (4°C) or internalized (quenched with trypan blue) signal was visualized using fluorescent microscopy

complex internalization is responsible for the high level of endocytosis observed after GA treatment.

GA treatment resulted in a reduction of receptor recycling time In addition to accelerated vesicle internalization, it is possible that receptor recycling could be enhanced after GA treatment. TfnR recycling kinetics was estimated as previously described (Johnson et al. 1994). Cells were incubated (20 min, 37°C) with AlexaFluor594-conjugated transferrin in the presence or absence of 200-fold excess of unlabeled transferrin. Unbound and cell surface (non-internalized) transferrin were removed by sequential washes with 20 mM sodium citrate buffer at pH 5.0. Then, cells were incubated with efflux buffer (0.1 mM desferrioxamine and 3 $\mu\text{g}/\text{ml}$ human diferric unlabeled transferrin in PBS) for 2.5, 5, 10, 15, 20, and 30 min at 37°C. This process allowed the recycling of transferrin–TfnR complex to the cell surface and the subsequent release of fluorescent transferrin into the extracellular medium. The extracellular medium was collected, and cells were lysed. The content of AlexaFluor594-conjugated transferrin in each fraction at the different times of incubation was quantified using a fluorometer. A representative experiment is presented in Fig. 5. The percentage of cell-associated signal was calculated at each time point, and the time of transferrin recycling was calculated from the plot Ln of the percentage of cell-associated transferrin vs. time of incubation with efflux buffer (Fig. 5). A significant increase in TfnR recycling was observed in cells after GA treatment in comparison with control cells (Table 1), suggesting a faster recycling of TfnR to the cell surface.

Transferrin endocytosis was enhanced after HS The preceding results suggest that the effect of GA on transferrin

endocytosis requires gene expression. Since GA treatment has been shown to induce the HS expression, we hypothesize that expression of HSP, rather than Hsp90 inhibition, is responsible for the increase in transferrin endocytosis after incubation with GA. If this hypothesis is correct, then other inducers of the stress response, such as HS, should result in a similar increase in transferrin endocytosis. To test this possibility, HepG2 cells were heat-stressed (43°C for 1.5 h) followed by recovery at 37°C for 3, 8, and 24 h. Increased expression of Hsp70 and Hsp27 was clearly observed after HS by Western blotting. However, no increase in Hsp90, Hsc70, or Grp94 was observed (Fig. 6a). The pattern of gene expression after HS was compared with that observed after GA treatment. An increase in the expression of Hsp70 and Hsp90 was observed after GA treatment. An increase of Hsp27 could only be detected after 16 h of GA treatment (not shown). In contrast, Hsc70 or Grp94 expression was not induced by GA incubation (Fig. 6a). Transferrin endocytosis was evaluated in cells that were heat-shocked as described above and recovered at 37°C for 0, 3, 6, and 24 h. A significant increase in FITC-conjugated transferrin internalization was observed in cells recovering from heat stress in comparison with non-HS cells (Fig. 6b). In addition, the effect of HS on transferrin endocytosis was blocked by incubation with AcD during HS and the recovery period (Fig. 6b). We noticed an early spike in endocytosis within 3 h after HS. This initial spike could be a contribution of the effect of hyperthermia in the cell physiology, perhaps due to changes in membrane fluidity (Balogh et al. 2005). This early spike in endocytosis has been previously observed during phagocytosis (Vega and De Maio 2005). The increase of transferrin endocytosis after HS/recovery was confirmed by fluorescent microscopy (Fig. 6c). To

Fig. 5 GA treatment and HS resulted in faster TfR recycling. The time of TfR recycling was evaluated (as described in the [Experimental procedures](#) section) after treatment with GA (5 h) at 37°C, or after HS (43°C, 1.5 h), and recovery for 24 h at 37°C. A typical result obtained in control or GA-treated cells is presented

further compare the contribution of HS and GA on transferrin endocytosis, cells were stressed at 43°C for 1.5 h and recovered at 37°C for 24 h. Then, cells were incubated with GA (1 µg/ml) for 5 h at 37°C. Transferrin endocytosis was evaluated as described above and compared with cells that were heat-shocked without GA incubation or cells that were treated with GA in the absence of HS. There were no significant differences between the combination of HS and GA incubation compared with the individual treatments. In contrast, transferrin internalization in the three groups was significantly higher than in cells

that were neither HS nor treated with GA (Fig. 7). These observations suggest that HS and GA increase transferrin endocytosis by a similar mechanism.

Transferrin endocytosis was increased after expression of Hsp70 A common feature between treatment of cells with GA and HS was the increase in expression of Hsp70. To test the role of Hsp70 in transferrin endocytosis, HepG2 cells were transfected with *Hsp70* gene or an empty plasmid (mock-transfected). Transfection resulted in significant levels of Hsp70 expression in comparison with mock-transfected cells as demonstrated by Western blotting (representative result presented in Fig. 8a). Cells that were transfected with the *Hsp70* gene displayed an increase in transferrin endocytosis as opposed to mock-transfected cells (representative result presented in Fig. 8b), which was proportional to the levels of Hsp70 present in the cells as detected by immunostaining (Fig. 8b). Additionally, blocking Hsp70 expression by siRNA after HS resulted in a significant reduction in Hsp70 expression as measured by Western blotting (Fig. 9a). This treatment also resulted in decreased transferrin endocytosis after HS (Fig. 9b). The reduction in transferrin endocytosis by siRNA was equivalent to the effect of incubation with AcD during HS

Table 1 Recycling time of TfR

Group	Time of TfR recycling (min)
Control	17.3±0.3
GA (5 h)	9.6±2.0*
HS (+24 h)	11.7±1.3*

The time of TfR recycling was evaluated (as described in the [Experimental procedures](#) section) after treatment with GA (5 h) at 37°C or after HS (43°C, 1.5 h) and recovery for 24 h at 37°C. Statistical analysis was performed by one-way ANOVA, followed by Newman-Keuls test

* $p < 0.05$ vs. untreated cells

Fig. 6 HS also resulted in enhanced internalization of transferrin. **a** HepG2 cells were heat-shocked at 43°C for 1.5 h and recovered at 37°C for 0, 3, 8, or 24 h or incubated with GA (1 µg/ml) for 0, 3, 5, or 8 h at 37°C and compared with untreated cells (*control*, **c**). Cells were lysed, and the expression of Hsp70, Grp94, Hsc70, Hsp90, Hsp27, and actin was measured by Western blotting. **b** HepG2 cells were heat-shocked at 43°C for 1.5 h and recovered at 37°C for 0, 3,

6, or 24 h in the presence or absence of AcD (in micrograms per milliliter) or maintained at 37°C (*control*). Then, cells were incubated with FITC-conjugated transferrin, and endocytosis was measured as described before. **c** Alternatively, endocytosis of transferrin was visualized by fluorescent microscopy. Statistical analysis was performed by one-way ANOVA, followed by Newman-Keuls test. * $p < 0.05$ vs. untreated cells

Fig. 7 The combination of GA and HS did not further increase transferrin endocytosis in comparison with individual treatments. HepG2 cells were heat-shocked (43°C, 1.5 h) and recovered for 24 h at 37°C. Then, cells were incubated or not with GA (1 µg/ml) for 5 h. In addition, cells were incubated in similar conditions with GA in the absence of HS. As a control, cells were neither heat-shocked nor treated with GA. Endocytosis of AlexaFluor594-conjugated transferrin was evaluated by fluorometry as described in Experimental procedures. Results are expressed as the endocytic index (IS/OD MTT). Statistical analysis was performed by one-way ANOVA, followed by Newman-Keuls test. * $p < 0.05$ vs. untreated cells

(Fig. 9b). To further substantiate these findings, GA was added to cells in which Hsp70 expression was reduced by siRNA treatment. Indeed, the addition of GA did not cause any increase in transferrin endocytosis in comparison with cells that were treated with GA in the absence of Hsp70 expression attenuation (Fig. 9c). All of these results support the hypothesis that Hsp70 is responsible for the increase in transferrin endocytosis.

Discussion

Fundamental cellular processes are compromised after exposure of cells to extreme environmental stress conditions or toxic agents. The expression of HSP is a common response to control an array of environmental and physiological insults. These proteins participate in the refolding of polypeptides that are denatured as a consequence of the stress, as well as the disposal of damaged macromolecules and toxic metabolites. HSP stabilize multiprotein complexes, cellular membranes, and key cellular processes

Fig. 8 Transferrin endocytosis is increased after transfection with Hsp70. HepG2 cells were transfected with a full-length Hsp70 construct (WT) or an empty vector (EV), and the transfection levels was visualized by Western blotting of cell lysates prepared 48 h after transfection (a). Transferrin internalization in transfected cells was visualized by fluorescent microscopy using FITC-conjugated transferrin (50 μ g/ml, 30 min incubation). A representative experiment is presented. Notice the correlation between elevated Hsp70 levels and higher internalization of transferrin (b)

affected by the stress. Indeed, transcription, splicing, translation, and transport are preserved upon expression of HSP (De Maio 1999). All these actions are directed at preserving cellular integrity. In addition, the uptake of nutrients is a critical component in the recovery of cells from stress conditions to facilitate the return to cellular homeostasis. In the present study, we presented new evidence indicating that endocytosis of cellular nutrients is enhanced during stress conditions, which is mediated by HSP expression, in particular, Hsp70.

We showed that treatment of cells with two inhibitors of Hsp90 function, GA and RD, as well as exposure to HS conditions, resulted in a time- and ligand concentration-dependent increase of transferrin endocytosis. This enhancement in transferrin endocytosis was not due to an increase in levels of the involved receptor (TfnR) on the cell surface but rather to a rapid internalization and recycling of the transferrin–TfnR complexes. Moreover, we demonstrated that Hsp70 is the major player in the increase of transferrin endocytosis. This assumption is supported by experiments in which cells were transfected with the *Hsp70* gene or its expression was blocked by exogenous addition of siRNA. These observations echo our prior report demonstrating an increase in phagocytosis in cells expressing Hsp70 (Vega and De Maio 2005). Although the role of Hsp70 is evident, we cannot totally discard the possible contribution of other HSP. Prior studies have proposed that Hsp90 is involved in vesicle transport (Lotz et al. 2008). The contribution of Hsp90 to transferrin uptake is less likely, since we observed an increase in endocytosis in the presence of GA, which blocks the function of this protein. GA treatment does not increase transferrin endocytosis after co-incubation with AcD,

suggesting that gene expression rather than inhibition of Hsp90 function is necessary. Moreover, the combination of HS and GA treatment did not result in an additive increase in transferrin endocytosis. Finally, the addition of GA to cells in which Hsp70 was attenuated after transfection with siRNA did not result in an increase of transferrin endocytosis. Although these observations strongly refute a role for Hsp90, we cannot completely discard a possible role for this HSP. Hsp27, as well as other small HSP, has been implicated in the interaction with vesicles. However, the pattern of Hsp27 expression after HS and GA treatment was not identical. We observed a rapid and strong induction

Fig. 9 Attenuation of Hsp70 expression by siRNA reduces the enhancing effect of GA or HS on transferrin endocytosis. Cells were transfected with Hsp70 siRNA or control siRNA. After 48 h of transfection, cells were heat-shocked (43°C, 1.5 h) and allowed to recover at 37°C for 24 h (HS) or maintained at 37°C (control). The level of Hsp70 after siRNA transfection was compared with mock-transfected cells by Western blotting (a). The internalization of FITC-conjugated transferrin was evaluated as described before and compared with cells treated with AcD (10 μ g/ml) before HS (b). HepG2 cells were transfected or not with Hsp70 siRNA (48 h of transfection). Then, cells were treated or not with GA (1 μ g/ml, 5 h), and the internalization of AlexaFluor 594-conjugated transferrin was evaluated as described before (c). Statistical analysis was performed by one-way ANOVA, followed by Newman–Keuls test. * p <0.05 vs. untreated cells; # p <0.05 vs. HS cells

of Hsp27 after HS (3 h into recovery). On the contrary, cells treated with GA required a longer time (16 h) to achieve similar levels of Hsp27. Since the effect of GA on transferrin endocytosis was observed shortly after incubation with this drug (3–5 h), the possible effect of Hsp27 is less evident. Thus, Hsp70 remains as the major candidate to enhance transferrin endocytosis.

Although our observations indicate that vesicle internalization and recycling are the components of the endocytic process that are modulated by Hsp70, the molecular mechanism remains to be fully elucidated. Several possibilities could explain our results. Transferrin internalization is mediated by the classical clathrin-dependent endocytosis process. Thus, it could be speculated that Hsp70 could be involved in the clustering of receptors, invagination of the plasma membrane, and vesicle budding. Perhaps, Hsp70 could also stabilize several components involved in this endocytic pathway, including clathrin cages, adapter, or assembly proteins involved in the formation of the coated pits, GTPases, and dynamin. Another member of the Hsp70 family, Hsc70, has been associated with clathrin-mediated endocytosis. More specifically, Hsc70 is involved in the disassembly of coated pits (Eisenberg and Greene 2007). Hsc70 is very abundant in normal conditions, and its expression is not induced by stress. Previous studies have shown that Hsc70 was necessary for transferrin endocytosis and that mutants of this protein that failed to hydrolyze ATP disrupted transferrin recycling (Newmyer and Schmid 2001). In our studies, the increase in transferrin endocytosis observed after HS or GA treatment does not seem to be related to the presence of Hsc70, since the expression level of this protein is not affected after HS or GA treatment (Fig. 6). This discrepancy is not surprising, since prior studies have shown that these two proteins, Hsp70 and Hsc70, although similar, displayed different roles during translation (Beck and De Maio 1994). In particular, the interaction of Hsp70 and Hsc70 with lipid membranes was different. Moreover, Hsc70 was not observed in cellular membranes in contrast to Hsp70 (Vega et al. 2008). Thus, it is possible that Hsc70 is involved in transferrin endocytosis during normal cellular circumstances, and Hsp70 takes over this process during stress conditions.

The mechanism by which Hsp70 accelerates vesicle traffic may be related to the capacity of this protein to interact with membranes. Hsp70 has been reported to incorporate into artificial lipid bilayers opening ion conductance pathways (Vega et al. 2008). Perhaps, these Hsp70 channels may change the ionic content of endocytic vesicles, resulting in faster maturation and targeting of the vesicles to a subsequent compartment. Another possibility is that Hsp70 may be involved in vesicle fusion or in reloading of internal membranes into the plasma membrane. In fact, prior studies have shown that recombinant

Hsp70 induced the aggregation of liposomes in *in vitro* conditions (Arispe et al. 2002). Hsp70 might also modulate the fluidity of the membrane, which has been implicated as a component of the stress response (Vigh et al. 1998). Hsp70 displays a high affinity for phosphatidylserine, which is a major component of the cytosolic side of cellular membranes (Arispe et al. 2004). Thus, a direct interaction of Hsp70 with the budding vesicle is possible.

The increase in endocytosis mediated by HSP is likely an important component of the stress response. Accelerated endocytosis results in a rapid delivery of nutrients and other important macromolecules to cells, which are essential to preserve homeostasis as well as for the recovery from the insult. For example, the internalization of transferrin is necessary for the cellular uptake of iron, which is an important cofactor of many cellular proteins, including mitochondrial oxidative phosphorylation (Aisen and Listowsky 1980). In addition, iron-dependent histone demethylases modulate chromatin structure (Vergara and Thiele 2008). On the other hand, it could also be contemplated that extracellular iron, which may be placed in the environment as a consequence of cell damage or necrosis, could generate reactive oxygen species (ROS) via Fenton reaction. ROS oxidizes lipids, proteins, and/or DNA (Balla et al. 1992). Consequently, the removal of iron from the environment is an important process to preserve cellular integrity and function. In summary, our observations bring a new role for Hsp70 in the stress response. In this case, Hsp70 is involved in the uptake of receptor-mediated ligands, which could be important to maintain cellular integrity and for the delivery of important nutrients for the cell.

References

- Aisen P, Listowsky I (1980) Iron transport and storage proteins. *Annu Rev Biochem* 49:357–393
- Arispe N, Doh M, De Maio A (2002) Lipid interaction differentiates the constitutive and stress-induced heat shock proteins Hsc70 and Hsp70. *Cell Stress Chaperones* 7:330–338
- Arispe N, Doh M, Simakova O, Kurganov B, De Maio A (2004) Hsc70 and Hsp70 interact with phosphatidylserine on the surface of PC12 cells resulting in a decrease of viability. *FASEB J* 18:1636–1645
- Balla G, Jacob HS, Balla J, Rosenberg M, Nath K, Apple F, Eaton JW, Vercellotti GM (1992) Ferritin: a cytoprotective antioxidant strategem of endothelium. *J Biol Chem* 267:18148–18153
- Balogh G, Horváth I, Nagy E, Hoyk Z, Benkő S, Bensaude O, Vigh L (2005) The hyperfluidization of mammalian cell membranes acts as a signal to initiate the heat shock protein response. *FEBS J* 272:6077–6086
- Beck CS, De Maio A (1994) Stabilization of protein synthesis in thermotolerant cells during heat shock. Association of heat shock protein-72 with ribosomal subunits of polysomes. *J Biol Chem* 269:21803–21811

- Billecke S, Bender A, Kanelakis K, Murphy P, Lowe E, Kamada Y, Pratt W, Osawa Y (2002) Hsp90 is required for heme binding and activation of apo-neuronal nitric-oxide synthase: geldanamycin-mediated oxidant generation is unrelated to any action of hsp90. *J Biol Chem* 277:20504–20509
- Bukau B, Weissman J, Horwich A (2006) Molecular chaperones and protein quality control. *Cell* 125:443–451
- Carratù L, Franceschelli S, Pardini CL, Kobayashi GS, Horvath I, Vigh L, Maresca B (1996) Membrane lipid perturbation modifies the set point of the temperature of heat shock response in yeast. *Proc Natl Acad Sci U S A* 93:3870–3875
- De Maio A (1999) Heat shock proteins. Facts, thoughts, and dreams. *Shock* 11:1–12
- Dey A, Cederbaum A (2006) Geldanamycin, an inhibitor of Hsp90, potentiates cytochrome P4502E1-mediated toxicity in HepG2 cells. *J Pharmacol Exp Ther* 317:1391–1399
- Dikalov S, Landmesser U, Harrison D (2002) Geldanamycin leads to superoxide formation by enzymatic and non-enzymatic redox cycling: implications for studies of Hsp90 and endothelial cell nitric-oxide synthase. *J Biol Chem* 277:25480–25485
- Eisenberg E, Greene LE (2007) Multiple roles of auxilin and hsc70 in clathrin-mediated endocytosis. *Traffic* 8:640–646
- Gehrmann M, Liebisch G, Schmitz G, Anderson R, Steinem C, De Maio A, Pockley G, Multhoff G (2008) Tumor-specific Hsp70 plasma membrane localization is enabled by the glycosphingolipid Gb3. *PLoS ONE* 3:e1925
- Johnson LS, Presley JF, Park JC, McGraw TE (1994) Slowed receptor trafficking in mutant CHO lines of the End1 and End2 complementation groups. *J Cell Physiol* 158:29–38
- Lindquist S, Craig EA (1988) The heat-shock proteins. *Annu Rev Genet* 22:316–377
- Lotz GP, Brychzy A, Heinz S, Obermann WM (2008) A novel HSP90 chaperone complex regulates intracellular vesicle transport. *J Cell Sci* 121(Pt 5):717–723
- Minowada G, Welch W (1995) Variation in the expression and/or phosphorylation of the human low molecular weight stress protein during in vitro cell differentiation. *J Biol Chem* 270:7047–7054
- Multhoff G (2002) Activation of natural killer cells by heat shock protein 70. *Int J Hyperthermia* 18:576–585
- Multhoff G, Botzler C, Wiesnet M, Muller E, Meier T, Wilmanns W (1995) A stress-inducible 72 kDa heat shock protein (Hsp72) is expressed on the surface of human tumor cells but not on normal cells. *Int J Cancer* 61:272–279
- Necker L, Ivy SP (2003) Heat shock protein 90. *Curr Opin Oncol* 15:419–424
- Newmyer SL, Schmid SL (2001) Dominant-interfering Hsc70 mutants disrupt multiple stages of the clathrin-coated vesicle cycle in vivo. *J Cell Biol* 152:607–620
- Schilling D, Gehrmann M, Steinem C, De Maio A, Pockley AG, Abend M, Molls M, Multhoff G (2009) Binding of heat shock protein 70 to extracellular phosphatidylserine promotes killing of normoxic and hypoxic tumor cells. *FASEB J* 23:2467–2477
- Schulte TW, Akinaga S, Murakata T, Agatsuma T, Sugimoto S, Nakano H, Lee YS, Simen BB, Argon Y, Felts S, Toft DO, Neckers LM, Sharma SV (1999) Interaction of radicicol with members of the heat shock protein 90 family of molecular chaperones. *Mol Endocrinol* 13:1435–1448
- Sreedhar AS, Mihaly K, Pato B, Schneider T, Stetak A, Kis-Petik K, Fidy J, Simonics T, Maraz A, Csermely P (2003) Hsp90 inhibition accelerates cell lysis. Anti-Hsp90 ribozyme reveals a complex mechanism of Hsp90 inhibitors involving both superoxide- and Hsp90-dependent events. *J Biol Chem* 278:35231–35240
- Vega VL, De Maio A (2005) Increase in phagocytosis after geldanamycin treatment or heat shock: role of heat shock proteins. *J Immunol* 175:5280–5287
- Vega VL, Rodriguez-Silva M, Frey T, Gehrmann M, Diaz J, Steinem C, Multhoff G, Arispe N, De Maio A (2008) Hsp70 translocates into the plasma membrane after stress and is released into the extracellular environment in a membrane-associated form that activates macrophages. *J Immunol* 180:4299–4307
- Vergara SV, Thiele DJ (2008) Post-transcriptional regulation of gene expression in response to iron deficiency: coordinated metabolic reprogramming by yeast mRNA-binding proteins. *Biochem Soc Trans* 36:1088–1090
- Vigh L, Maresca B, Harwood JL (1998) Does the membrane's physical state control the expression of heat shock and other genes? *Trends Biochem Sci* 1998:369–374
- Vigh L, Horváth I, Maresca B, Harwood JL (2007) Can the stress protein response be controlled by “membrane-lipid therapy”? *Trends Biochem Sci* 32:357–363
- Zou J, Guo Y, Guettouche T, Smith D, Voellmy R (1998) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell* 94:471–480