Reaction of small heat-shock proteins to different kinds of cellular stress in cultured rat hippocampal neurons

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Abstract Upregulation of small heat-shock proteins (sHsps) in response to cellular stress is one mechanism to increase cell viability. We previously described that cultured rat hippocampal neurons express five of the 11 family members but only upregulate two of them (HspB1 and HspB5) at the protein level after heat stress. Since neurons have to cope with many other pathological conditions, we investigated in this study the expression of all five expressed sHsps on mRNA and protein level after sublethal sodium arsenite and oxidative and hyperosmotic stress. Under all three conditions, HspB1, HspB5, HspB6, and HspB8 but not HspB11 were consistently upregulated but showed differences in the time course of upregulation. The increase of sHsps always occurred earlier on mRNA level compared with protein levels. We conclude from our data that these four upregulated sHsps (HspB1, HspB5, HspB6, HspB8) act together in different proportions in the protection of neurons from various stress conditions.

Keywords Stress response · Neuroprotection · Small heat-shock protein · Hippocampal neuron

Introduction

The family of small heat-shock proteins (sHsps), a class of molecular chaperones, is comprised of 11 members in human and rodents. They are part of a phenomenon called stress tolerance. After sublethal stress leading to sHsp upregulation

the cell is able to tolerate a subsequent more severe stress condition (Kato et al. 1995; Kitagawa et al. 1990; Murry et al. 1986). They are induced by pathophysiological conditions such as heat, hypoxia, or oxidative stress leading to denaturation of proteins. Under those conditions, they bind in an ATP-independent manner to partially unfolded proteins and prevent their irreversible aggregation (Haslbeck et al. 2005; McHaourab et al. 2009). In addition to these chaperone-like properties, some sHsps are characterized by various specific functions such as anti-apoptotic or anti-inflammatory effects.

While many studies described the cytoprotective effect of sHsps on heart or muscle tissue, less is known about their action in the brain and especially in neurons. HspB1 (Hsp25/Hsp27) is the sHsp whose upregulation has been studied most extensively in neurons after several stress conditions but mostly after ischemia or heat shock (Bechtold and Brown 2000; Hebb et al. 2006; Kalesnykas et al. 2008; Kato et al. 1994, 1999; Krueger-Naug et al. 2000; Lu et al. 2002; Satoh and Kim 1995; Schwarz et al. 2010; Yang et al. 2012). Fewer studies showed the upregulation of HspB5 (α B-crystallin) (Fukushima et al. 2006; Minami et al. 2003; Piao et al. 2005), HspB6 (Hsp20) (David et al. 2006; Niwa et al. 2009), or HspB8 (Hsp22) (Garcia-Lax et al.; Yew et al. 2005) in neurons after stress. In addition, HspB1 and HspB5 have been found to associate with protein aggregates in several neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis, or Parkinson's disease (Bjorkdahl et al. 2008; Braak et al. 2001; Crippa et al.; Kato et al. 1997; Renkawek et al. 1999, 1994; Shinohara et al. 1993; Wilhelmus et al. 2006a, b). Finally, mutations in the genes for HspB1, HspB3, and HspB8 are known to cause hereditary neuropathies (Evgrafov et al. 2004; Houlden et al. 2008; Irobi et al. 2004; Kolb et al. 2010; Tang et al. 2005) or myopathies (Selcen and Engel 2003; Vicart et al. 1998). This suggests a neuroprotective function of small heat-shock proteins.

We previously described that five of the sHsps, i.e., HspB1, HspB5, HspB6, HspB8, and HspB11 (Hsp16.2) are expressed

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in cultured primary rat hippocampal neurons, and only two of them (HspB1 and HspB5) are upregulated at the protein level following heat shock (Kirbach and Golenhofen 2011). We now wanted to answer the questions whether the other three sHsps are induced under other types of stress and if the time course and strength of the response differs. For our experiments, we chose three types of stress. One stress condition was induced by exposure to sodium arsenite. This chemical substance causes malignant transformation and is also a known neurotoxic agent (Repetto et al. 1994). It seems to act by diverse mechanisms, among which are the production of reactive oxygen species (ROS, (Carpenter et al. 2011), a decrease of neuronal nitric oxide synthase (Rios et al. 2009) and induction of apoptosis (Namgung and Xia 2000). Another stress condition known to induce sHsps is oxidative stress, meaning the over-production of ROS. This has been described to play a role in the pathophysiology of Alzheimer's disease (Ebenezer et al. 2010) and is also a hallmark of aging (Butterfield et al. 1999; Navarro and Boveris 2004; Poon et al. 2004). The third stress condition we chose to investigate is hyperosmotic stress, leading to cell shrinkage and thereby inducing several cellular pathways as volume regulation and cytoskeletal reorganization. Hyperosmotic stress is also known to induce apoptosis (Kloskowska et al. 2008) and seems to play a role in inflammatory processes (Brockner et al. 2010). This stress condition is achieved by adding the osmotically active substance sorbitol to the medium which cannot be metabolized by the cell.

In this study, we measured the mRNA and protein levels of all sHsps expressed in cultured rat hippocampal neurons subjected to the above described kinds of stress. We could show that four sHsps (HspB1, HspB5, HspB6, HspB8) were upregulated under those stress conditions. However, they showed differences regarding the time course and strength of regulation, suggesting that sHsps on the one hand act together and on the other hand differ in their expression profile under different stress conditions.

Results

To investigate if different kinds of stress in neurons lead to differences in the upregulation of sHsps, cultured hippocampal neurons were exposed to sodium arsenite and oxidative and hyperosmotic stress with subsequent determination of sHsp mRNA as well as protein amount. In initial experiments, we tested three different concentrations of each substance used to induce cellular stress to find out the optimal dosage not leading to significant cell death allowing a stress response. We assessed cell viability after 72 h of recovery by applying propidium iodide and performed immunostaining for MAP-2 (microtubule-associated protein 2). This allowed us to evaluate the amount of dead cells as well as the integrity of neuronal

dendrites (Electronic supplementary material, Online Resource 1). Thus, we chose a concentration of 100 μ M sodium arsenite, 0.5 μ M H_2O_2 for oxidative stress, and 200 mM sorbitol for osmotic stress displaying clearly sublethal stress conditions.

Expression of sHsp mRNA under different stress conditions

We examined the mRNA level of all sHsps expressed in cultured hippocampal neurons, namely HspB1, HspB5, HspB6, HspB8, and HspB11 up to 24 h of recovery after three different kinds of stress by real-time reverse transcription polymerase chain reaction (RT-PCR). Table 1 shows the primers used.

Sodium arsenite stress

Sodium arsenite stress (100 μ M sodium arsenite for 30 min) resulted in a strong and significant upregulation of HspB1, B5, and B8 mRNA as early as 4 h after stress with its peak ranging between 4 and 7 h persistent until 24 h (Fig. 1). Peak values amounted to 10.74-fold mRNA content compared with control for HspB1, 3.89-fold for HspB5, and 6.64-fold for HspB8. In contrast, HspB6 and HspB11 showed no increase in mRNA amount. HspB11 mRNA was even significantly reduced by factor 0.68 24 h after stress.

Oxidative stress

Under oxidative stress (0.5 μ M H_2O_2 for 30 min), HspB1 and HspB5 were significantly upregulated 7 h after stress, persisting until 24 h (Fig. 1). The upregulation was not as strong as after sodium arsenite stress (peak values of 2.81-fold for HspB1 and 3.29-fold for HspB5). HspB6 and HspB8 mRNA increased slightly without reaching the level of significance. HspB11 mRNA showed no up- or downregulation under this stress condition.

Hyperosmotic stress

Under hyperosmotic stress (addition of 200 mM sorbitol for 2 h), HspB1 mRNA levels were significantly increased 4 h after stress, persisting until 24 h (Fig. 1). HspB5 expression increased similarly, but not as strong, reaching significance only after 24 h recovery. HspB8 showed a significant increase at 4 and 7 h recovery while the amount declined again 24 h after stress. The increase was to a similar extent as that in response to oxidative stress (2.57-fold for HspB1, 1.93-fold for HspB5, and 3.75-fold for HspB8). HspB6 revealed only slight but significant increases (around factor 1.3). Interestingly, HspB11 also showed a tendency for increase (1.48-fold) under this stress condition, reaching significance at 24 h after stress.

Table 1 Primer sequences used for real-time RT-PCR

Gene	Primer (5'-3')	Amplicon length
<i>CycA</i>	fwd: AGCACTGGGGAGAAAGGATT, rev: AGCCACTCAGTCTTGGCAGT	248
<i>Rpl13A</i>	fwd: ACAAGAAAAAGCGGATGGTG, rev: TTCCGGTAATGGATCTTTGC	167
<i>HspB1 (Hsp25)</i>	fwd: CGGCAACTCAGCAGCGGTGTCT, rev: CATGTTTCATCCTGCCTTTCTTCGTG	160
<i>HspB5 (αB-crystallin)</i>	fwd: CTTCTACCTTCGGCCACCCTC, rev: GCACCTCAATCACGTCTCCC	164
<i>HspB6 (Hsp20)</i>	fwd: CCATGTGGAGGTCCATGCTCG, rev: GCAGGTGGTGACGGAAGTGAG	195
<i>HspB8 (Hsp22)</i>	fwd: CCGGAAGAACTGATGGTAAAGAC, rev: CCTCTGGAGAAAGTGAGGCAAATAC	166
<i>HspB11 (Hsp16.2)</i>	fwd: ATTATGGCACGAGATGGCTACG, rev: TGTAATCACTAAGGAAGACTTGAGACTG	128

Primer pairs for reference genes cyclophilin A (*CycA*) and ribosomal protein 13A (*Rpl13A*) (Bonefeld et al. 2008) and sHsps

Altogether, our data show that HspB1, HspB5, and HspB8 are induced on mRNA level under all three stress conditions investigated. In contrast, HspB6 mRNA increases inconsistently while HspB11 is the only small heat-shock protein which actually shows a decrease under one stress condition. The strength of the increase of sHsps and the time course vary to some extent under the different stress conditions. Sodium arsenite stress has the strongest and fastest effect on the cells while the response to oxidative and hyperosmotic stress is less pronounced and a little more delayed.

Changes in sHsp protein amount under different stress conditions

The protein amounts of sHsps in response to different kinds of stress were measured by immunoblotting. Figure 2 shows representative blots from at least three experiments at control conditions and 4 to 72 h of recovery after stress. These later time points were included in the experimental design since it is known and was shown by us previously that the upregulation at the protein level occurs later than at the mRNA level.

Sodium arsenite stress

HspB1, HspB5, HspB6, and HspB8 showed a clear upregulation 24 h after stress in all experiments (Fig. 2a). In the case of HspB1, this increase in protein amount persisted until 72 h while the amount of HspB5, HspB6, and HspB8 protein decreased again after 48 h. HspB11 protein levels showed a slight increase between 7 and 24 h after stress. Thus, the increase of HspB1, HspB5, and HspB8 mRNA was reflected by a later and longer-lasting increase in protein amount. Interestingly, HspB6 and HspB11 were upregulated only at protein but not on mRNA level suggesting post-transcriptional regulation.

Oxidative stress

Under this stress condition, HspB1, HspB5, HspB6, and HspB8 protein amount increased around 24 h after stress with a subsequent decrease (Fig. 2b). HspB11 protein levels did not change. These data are in line with the observed alterations of mRNA.

Hyperosmotic stress

The HspB1 and HspB5 protein amounts increased after hyperosmotic stress with a peak around 24 h recovery, decreasing again after this (Fig. 2c). The increase of HspB6 protein amount was seen earlier and more persistent, starting to decrease only after 48 h of recovery. In contrast, HspB8 protein amount increased continuously from 7 up to 72 h after stress. HspB11 showed a slight increase in some experiments and tend to decrease 72 h after stress. Interestingly, the HspB11 antibody detected a doublet. In *Chlamydomonas*, HspB11 (IFT25) was shown to be a phosphoprotein with the upper band representing the phosphorylated protein (Wang et al. 2009). Two predicted phosphorylation sites are conserved between *Chlamydomonas* and rat, thus, HspB11 might be phosphorylated in rat neurons.

In summary, we found that HspB1, HspB5, HspB6, and HspB8 protein amount increased in rat hippocampal neurons subjected to all three kinds of stress investigated with some differences in the time course. Mostly, the protein amount of these four sHsps peaked around 24 h after stress, followed by a decrease. However, HspB1 was constantly increased up to 72 h after sodium arsenite stress and HspB8 after hyperosmotic stress. This indicates different modes of action of these sHsps under these stress conditions. In contrast, HspB11 showed an inconsistent reaction. In most experiments, the HspB11 protein amount was unchanged while in some an increase or a decrease was measured. It remains to be determined what role this small heat-shock protein plays in the stress response of neurons.

Fig. 1 Changes of mRNA amount in cultured rat hippocampal neurons after different kinds of stress

Fig. 2 Changes of protein amount in rat hippocampal neurons after different kinds of stress

Discussion

sHsps in neurons after different kinds of stress

Small heat-shock proteins play a major role in the development of cellular stress tolerance in the brain. This phenomenon, which has been described after many different types of stress, is also known for cross-tolerance, meaning that after preconditioned with one kind of stress the cell tolerates also others (Kirino 2002; Plamondon et al. 1999). Studies on sHsps in neurodegenerative diseases (Muchowski and Wacker 2005) as well as studies on hereditary distal motor neuropathies caused by mutations in some sHsp genes

(Mymrikov et al. 2011) may suggest a neuroprotective function of sHsps. However, so far, most studies investigating the role of sHsps in neuroprotection focused on one selected sHsp instead of a comparative analysis of all sHsps. This is the first study investigating systematically the response of all expressed sHsps to three different kinds of stress in hippocampal neurons, i.e., sodium arsenite and oxidative and hyperosmotic stress. Sodium arsenite stress was chosen to mimic an exposure to a neurotoxic chemical substance. Oxidative stress was investigated as it is well known to be involved in neurodegenerative diseases (Ebenezer et al. 2010) and also plays a part in ischemia (Levrant et al. 2003). Finally, hyperosmotic stress was chosen because neurons in the brain have to cope with osmotic imbalances in blood plasma (Loyher et al. 2004).

Previously, we found that rat hippocampal neurons express HspB1, HspB5, HspB6, HspB8, and HspB11, but sublethal heat shock led only to the upregulation of HspB1 and HspB5 proteins (Kirbach and Golenhofen 2011). In the present study, we could demonstrate that not only HspB1 and HspB5 but also HspB6 and HspB8 protein amount was upregulated in cultured hippocampal neurons in response to all three above-mentioned stress conditions. It is interesting that such diverse conditions led to a relative consistent reaction. In the literature, HspB1 is the one sHsp studied most extensively so far. It has been found upregulated in neurons after ischemia (Kalesnykas et al. 2008; Kato et al. 1994; Lu et al. 2002), heat shock (Bechtold and Brown 2000; Krueger-Naug et al. 2000; Satoh and Kim 1995; Schwarz et al. 2010), hypoxia (David et al. 2006), oxidative stress (Schwarz et al. 2010), kainic acid-induced seizures (Kato et al. 1999), exposure to electromagnetic fields (Yang et al. 2012) or toxic substances (Clark et al. 2011), and during axonal regeneration (Hebb et al. 2006). HspB5 has previously been found upregulated in neurons after ischemia (Minami et al. 2003; Piao et al. 2005), olivary hypertrophy (Fukushima et al. 2006), heat shock, and oxidative stress (Schwarz et al. 2010). Interestingly, HspB5 is recruited to dendrites and synapses in response to phosphorylation (Schmidt et al. 2012). Little is known about the regulation of HspB6 and HspB8 in neurons. HspB6 was described to be upregulated after ischemia (Niwa et al. 2009) and hypoxia (David et al. 2006) while for HspB8 an increase was found after proteasome inhibition (Yew et al. 2005). Taking all these data on single sHsps together with our findings, it seems obvious that neurons upregulate more than one sHsp under stress conditions and that HspB1, HspB5, HspB6, and HspB8 seem to act together in protecting them from damage. Focusing on only one sHsp therefore means missing a major part of the sHsp response in neurons.

Posttranscriptional regulation of sHsps

Concerning the time course of the stress response, we found the peak of protein amount around 24 h of recovery after stress for most sHsps followed by a subsequent decrease. This seemed largely independent of the kind of stress applied. Interestingly, we observed the increase on mRNA level earlier (mostly already after 4 h of recovery) as at the protein level (starting only after 7 h of recovery). This discrepancy was observed by us also after heat shock (Kirbach and Golenhofen 2011). It is well known that exposure to stress leads to a translational arrest followed by a selective translation of stress related transcripts (DeGracia et al. 2008; Fahling 2009; Lindquist 1986). Mizzen and Welch (1988) observed that this translational arrest lasted up to 5 h after heat shock, a time frame fitting to our observation.

In two cases, a prolonged increase of protein amount up to 72 h of recovery was measured: for HspB1 after sodium arsenite and for HspB8 after hyperosmotic stress. Interestingly, the mRNA amount in both cases already started to decrease after 24 h of recovery. This hints at a posttranscriptional regulation mechanism involved in the stress response. It was recently shown in heart muscle that HspB6 is regulated by the microRNA miR-320 (Ren et al. 2009). MicroRNAs are small non-coding RNAs which are incorporated in the RNA-induced silencing complex, bind to the 3'UTR of target mRNAs and inhibit their translation (Boyd 2008). This microRNA could therefore play a role in the regulation of HspB6 in neurons. Whether microRNAs also play a role in the regulation of HspB1, HspB5, and HspB8 remains to be investigated.

HspB11—a special case

The fifth sHsp expressed in rat hippocampal neurons, HspB11, showed an inconsistent regulation in our experiments. Mostly, no change in protein or mRNA amount was observed after stress while in some instances up- or downregulation was detectable. This is in concordance with our previous finding after heat shock where no upregulation was observed (Kirbach and Golenhofen 2011).

HspB11 was first described by Bellyei et al. (2007) as a novel sHsp (Hsp16.2). They reported a homology to the alpha-crystallin domain and also a cytoprotective effect by stabilization of the mitochondrial membrane. However, it is still under debate if this protein belongs to the sHsp family at all, as the described homology with the alpha-crystallin domain was not confirmed by a more detailed approach (Kappe et al. 2010). Our data also suggest that HspB11 does not act primarily as a chaperone after stress but may have a constitutive function. It has been implicated to be a part of the intraflagellate transport complex B of primary cilia (Follit et al. 2009) where it seems to be required for Hedgehog

signaling (Keady et al. 2012). It would therefore be of interest if it aids in cilia signaling in neurons.

In conclusion, we could show that four small heat-shock proteins (HspB1, HspB5, HspB6, HspB8) are induced in rat hippocampal neurons after sodium arsenite and oxidative and hyperosmotic stress whereas only HspB1 and HspB5 were upregulated after heat shock. We found variations in the time course of the responses which might be due to posttranscriptional regulation. These four sHsps seem to act together to protect neurons from adverse conditions. It is not clear if this protection is mediated by their chaperone activity alone or by their individual specific functions. The controversial sHsp HspB11 does not seem to play a role in the stress response under these conditions.

mRNA amount of HspB1, HspB5, HspB6, HspB8, and HspB11 relative to control after sodium arsenite and oxidative or hyperosmotic stress (first, second, and third columns, respectively). Data from at least three independent experiments are presented as mean $\pm$ SEM. The non-parametric Mann–Whitney *U* test was used for statistical analysis, *p < 0.05.

Rat hippocampal neurons prepared from embryonal hippocampi (E18–E19) of Sprague–Dawley rats as described previously (Kirbach and Golenhofen 2011) were seeded at a density of 30,000 cells/cm² in Petri dishes and grown in Neurobasal^{TM} medium supplemented with B27® (Life Technologies GmbH, Darmstadt, Germany) for 14 days. At DIV14, cells were exposed to sodium arsenite stress (100 μ M sodium arsenite, 30 min), oxidative stress (0.5 μ M hydrogen peroxide, 30 min), or hyperosmotic stress (200 mM sorbitol, 2 h). After stress, cells received conditioned medium from cultures without stress of the same preparation. After the indicated time points of recovery cells were lysed and total RNA isolated, DNase treated and reverse-transcribed as described (Kirbach and Golenhofen 2011). Real-time PCR was performed as described previously (Kirbach and Golenhofen 2011) with 2 μ l 1:10 diluted cDNA and the QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany). Primers used for amplification are listed in Table 1. Original fluorescence *F*₀ was calculated according to Rutledge and Cote (2003). The geometric mean of both reference genes (cyclophilin A and Rpl13A) was used for normalization. The resulting values were then calculated in relation to the control sample of the respective stress experiment, which was set to 1.

Protein amount of HspB1, HspB5, HspB6, HspB8, and HspB11 after (A) sodium arsenite stress, (B) oxidative stress, and (C) hyperosmotic stress. Representative immunoblots from at least three independent experiments are shown. Equal amount of protein loading was controlled by PonceauS staining which is shown exemplarily for the HspB1 blot after arsenite stress.

Cells were cultured and stressed as described in Fig. 1. After the indicated time points of recovery, cells were lysed with Laemmli buffer and protein concentration determined by

amido black staining (Dieckmann-Schuppert and Schnittler 1997; Heinzel et al. 1965). Immunoblots were carried out as described (Kirbach and Golenhofen 2011) with equal amounts of protein (20 µg per lane) after separation with SDS-PAGE on 15 % acrylamide gels. Membranes were stained for 2 min with 0.5 % PonceauS, 3 % trichloroacetic acid to control equal protein loading and transfer. For the detection of sHsps, the following antibodies were used: rabbit anti-HspB1 (1:1000, Stressgen, SPA-801), rabbit anti-HspB5 (1:500, Stressgen, SPA-223), rabbit anti-HspB5 (1:5000, Lifespan, LS-C105475), goat anti-HspB6 (1:1000, R&D Systems, AF4200), rabbit anti-HspB8 (1:750, Cell Signaling, 3059), rabbit anti-HspB11 (1:1000, kind gift of Prof. Dr. Bellyei, Dept. of Oncotherapy, University of Pécs, Hungary). All antibodies were incubated in blocking solution containing 5 % milk except for the anti-HspB5 antibody from Lifespan which was incubated with 5 % bovine serum albumin or 1 % milk. Secondary antibodies were horseradish-peroxidase labelled goat anti-rabbit IgG (1:5000, Jackson Immuno Research Laboratories) or rabbit anti-goat IgG (1:2000, Dako). Washing steps were performed using TBS with 0.05 % Tween20. Detection of bound antibodies was carried out with the enhanced chemoluminescence system (Pierce ECL Western Blotting Substrate, Thermo Fisher Scientific Inc.) on X-ray films (Hyperfilm ECL, GE Healthcare).

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