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Expression Profile of Bag 1 in the Postmortem Brain

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Abstract

Bag 1 is a protein intimately involved in signaling pathways that regulate cell survival. Here we examined the expression profile of Bag 1 in the brain to consider issues associated with the sampling of anti-apoptotic proteins in a rat model of the human postmortem process. Following a 4 hr postmortem interval, we analyzed the hippocampus of rats maintained at 24 °C or 4 °C storage temperatures using immunocytochemical and Western blotting techniques. Remarkably, postmortem tissue (up to 4 hr) showed a significant and prominent up-regulation of Bag 1 in CA1 and CA3 subfields of the hippocampal formation. Over-expression of Bag 1, however, could only be traced down to a storage temperature of 24 °C. These data suggest that storage temperatures, but not postmortem intervals, significantly affect the expression profile and cellular stability of Bag 1 proteins.

Keywords

Apoptosis; Autopsy; Cerebellum; Hippocampus; Mood disorders; Rat; Storage temperature

Introduction

Bag 1 (Bcl-2-associated athanogene-1) is a protein involved in the regulation of several pathways concerning cell proliferation and cell death (Takayama et al., 1995; Zeiner and Gehring, 1995). As such, Bag 1 interacts with several steroid and growth factor receptors, including those for glucocorticoid and platelet-derived growth factor to promote cell survival (Bardelli et al., 1996; Schneikert et al., 1999). In addition, Bag 1 contains a domain with heat shock 70 (Hsp70) nucleotide-exchange activity, presumably to assist molecular chaperones with the removal of aberrant proteins from the cytosol (McClellan et al., 2005). It seems, therefore, that Bag 1 alleviates cellular stress by linking chaperones (i.e., Hsp70) with the ubiquitin-proteasome system to facilitate degradation of soluble oligomeric species (Luders et al., 2000; Alberti et al., 2002).

The ability of Bag 1 to protect cell function and viability is also seen in the brain where it associates with polyglutamine-expanded huntingtin aggregates, and is induced after traumatic head injury and transient middle cerebral artery occlusion (Hayashi et al., 2000; Seidberg et al., 2003; Jana and Nukina, 2005). Against this background, we examined the expression profile and cellular stability of Bag 1 in rat brains collected after a 4 hr postmortem interval. There is currently much interest in the involvement of Bag 1 expression in psychiatry as this protein is a long-term target for the actions of mood stabilizers (e.g., lithium and valproate) in bipolar

depression (Zhou et al., 2005). Accordingly, it would be valuable to determine potential confounding effects of postmortem factors on Bag 1 immunoreactivity (IR), especially if autopsy-derived human specimens are to be used in psychiatric studies of disease pathophysiology and drug therapy.

Materials and Methods

Adult male Long-Evans rats (250-300 g body weight) from Charles River Laboratories (Stoneridge, NY) were used for all experiments described herein. Animals were group-housed, 2-3 per cage under a 12 hr light:dark cycle (lights on at 0700) and allowed *ad libitum* access to food and water. Brains were collected from three randomly assigned experimental conditions ($n = 3-5$ rats/condition): (a) absolute control animals (no postmortem interval prior to tissue collection); (b) animals maintained at a 4 hr postmortem storage temperature of 24 °C; and (c) animals maintained at a 4 hr postmortem storage temperature of 4 °C. An additional group of rats was also used for histological analysis (i.e., Cresyl Violet staining) of the postmortem hippocampus. The above experimental conditions were carried out between 1000 and 1200 hr of the light cycle. All animal procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and with approval from the NYCOM/NYIT IACUC.

For postmortem intervals, rats were injected (intraperitoneally) with a lethal dose of 25% chloral hydrate (2.5 ml/100 g of body weight) and left undisturbed at specified storage temperatures for 4 hr (see above). Under this lethal drug regimen, death ensues within 3-5 min (Torres et al., 1992). As death occurs over a time-course of less than 24 hr, there is minimal evidence of cerebral hypoxia as described by Hynd et al (2003). After the 4 hr interval, brains were removed from calvaria and immediately fixed in a 4% paraformaldehyde solution (dissolved in 0.1 M sodium phosphate buffer, pH 7.2) for 5 consecutive days at 4 °C. Thereafter, brains were placed in a 30% sucrose buffer solution (dissolved in 0.1 M sodium phosphate) for 3 additional days. Serial coronal sections (50-60 μm) were obtained from the above brains on a sliding microtome and immersed as free floating sections in 0.05 M sodium phosphate cold cryoprotectant (with RNase inhibitors, diethyl pyrocarbonate, 30% ethylene glycol and 20% glycerol), and stored at - 20 °C until conventional histochemical and immunocytochemical assays were performed. The immunocytochemical methods have been described in detail previously and are only briefly summarized below (Torres et al., 2004). Free floating brain sections were incubated for 48 hr at 4°C with a specific rabbit polyclonal IgG directed against mouse Bag 1 (Santa Cruz Biotechnology, CA). The antibody was diluted 1:1000 in potassium-phosphate buffer solution (KPBS) with 1% normal bovine serum and 0.04% Triton X-100. Following several developing phases (including a sensitive antigen retrieval method that uses sub-boiling water-bath heating), brain sections were again washed in KPBS, mounted onto gelatin-chrome-alum-coated slides, allowed to dry overnight, dehydrated through graded concentrations of ethanol, cleared in xylenes, and cover slipped with DPX mountant (Electron Microscopy Sciences, PA).

Specificity for the Bag 1 antibody was instituted in the form of positive and negative controls. Visualization of Bag 1-IR throughout the brain was accomplished under bright-field microscopy using an Olympus microscope equipped with a grid reticule (10 mm^2) at X10 magnification. Bag 1-IR was recognized under bright-field microscopy and topographical distribution and organizational pattern of the protein was characterized and identified using a rat brain atlas (Paxinos and Watson, 1986). Occasionally, the intensity of the immunostaining was heterogeneous. Thus, only neurons showing a strong reaction product were classified as positive IR-cells (see Fig. 1). Data are expressed as means $\pm$ SEM of Bag 1 cell counts. Using a modified version of the fractionator method (West, 1993), Bag 1-labeled neurons were counted (per mm^2) in every 10th serial brain section on coded slides under X10 magnification

using a light microscope with a computer-assisted image analyzer (Neurolucida). Positive cells were counted at two subfields of the hippocampus: cornu ammonis (CA1) and CA3. Here a rostral-medial boundary subsection (Bregma -2.80 mm) was selected for estimates of Bag 1 neurons. The relatively small numbers of Bag 1-IR cells permitted counting of all such neurons in all subfields. Cell counts were subjected to one-way analysis of variance (ANOVA). Holm-Sidak method on pair-wise multiple comparisons was subsequently used for *post-hoc* tests on individual means. Statistically significant differences were defined as $P \leq 0.05$.

For Western blotting, brains were dissected into the hippocampus and cerebellum. Whole samples were then homogenized by sonication in a protein extraction buffer containing 20 mM Tris-HCl, 8% SDS, 0.4% bromophenol blue and a protease inhibitor cocktail (Sigma, St. Louis, MO). The homogenates were centrifuged at 10,000 rpm for 10 min to remove insoluble debris. Protein concentrations were determined by a BCA protein assay kit (Sigma, St. Louis, MO). Blots were carried out using 10 μ g of protein, separated by Tris-HEPES-SDS electrophoresis on 12% polyacrylamide gels. Proteins were then electrophoretically transferred to nitrocellulose membranes. Nonspecific binding on the nitrocellulose was blocked with 5% nonfat dry milk, and proteins were incubated with an antibody against Bag 1 in a 1:1000 dilution. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Pierce, Rockford, IL) was used as the secondary antibody. IR bands were visualized by chemiluminescent substrate (Pierce, Rockford, IL) and exposed to Hyper-film ECL (Amersham Biosciences). Relative immunoblotting optical densities were quantified (as percentage of control) using the UN-SCAN-IT gel analysis system (Silk Scientific, Utah).

Results

Some experimental procedures that merit consideration are summarized as follows (see Fig. 1). Brains collected from rats immediately after death (i.e., absolute control animals), showed a relative paucity of neurons labeled positive for Bag 1 in CA1 and CA3 hippocampal subfields (Fig. 2). Visualization of the IR with an antibody to Bag 1 revealed both a nuclear and scattered cytoplasmic expression of endogenous Bag 1 (Figs. 1 and 2). This particular distribution of Bag 1 is consistent with that seen in transfected rat nigro-striatal cell lines (Kermer et al., 2002).

The delay between death and brain collection is referred to as the postmortem interval. Typical postmortem intervals implemented in human brains range from 4 to 36 hr (Whitehouse et al., 1984; Barton et al., 1993). In studies using rat brains, the postmortem intervals range from 4 to 6 hr (van Zwieten et al., 1991; Torres et al., 1992). Here, we examined the effects of 4 hr postmortem interval on the expression profile of Bag 1 in rats maintained at 24 °C. Under this experimental condition, we observed a significant up-regulation of Bag 1-IR in CA1 ($F_{(2-14)} = 42.8$, $P \leq 0.001$, compared with absolute control rats and animals maintained at a storage temperature of 4 °C) and CA3 ($F_{(2-14)} = 123.2$, $P \leq 0.001$, compared with absolute control rats and animals maintained at a storage temperature of 4 °C) hippocampal subfields (Figs. 3 and 4). The sub-cellular distribution of endogenous Bag 1 was similar to that seen in absolute control hippocampus. The protein was targeted to the nucleus and cytosol and under no circumstances did we observe Bag 1-IR confined to the processes (axons and dendrites) of granule and pyramidal neurons.

Next, we examined the effects of 4 hr postmortem interval on the expression profile of Bag 1 in rats maintained at 4 °C. Under this storage temperature, we observed a pronounced down-regulation of Bag 1-IR in CA1 and CA3 hippocampal subfields ($P \leq 0.001$, compared with absolute control animals and rats maintained at a storage temperature of 24 °C; one-way ANOVA followed by pair-wise multiple comparison procedures, Holm-Sidak method; Figs. 3 and 4). Thus, while postmortem intervals of 4 hr do not significantly affect the overall autopsy

levels of Bag 1, storage temperature can influence the expression profile of the above anti-apoptotic protein.

To validate our findings further, we carried out Western blotting analysis on rat brains maintained postmortem at either 24 °C or 4 °C storage temperatures. Representative results of Western blots are shown in Fig. 5. Hippocampal homogenates collected from rats immediately after death (i.e., absolute control animals) showed a distinctive band with a MW of ~32 kDa. This isoform corresponds well with one of the three bands identified in rodent tissue (Zhou et al., 2005). An identical band of ~32 kDa was also detected in the hippocampus of postmortem rats maintained at either 24 °C or 4 °C storage temperatures. Quantitative analysis [relative optical density (OD) levels] of hippocampal homogenates obtained from rats maintained at a storage temperature of 24 °C showed a significant up-regulation of Bag 1 compared with OD levels from absolute controls or OD levels determined from animals maintained at 4 °C ($F_{(2-14)} = 41.1$, $P \leq 0.001$; Fig. 6). It should be noted that the rate of differences in Bag 1-IR between brains maintained at 24 °C and 4 °C could not be determined unambiguously due to the difficulty of dissecting CA1 and CA3 hippocampal subfields for Western blotting. Nevertheless, these findings confirmed and extended our earlier results seen by immunocytochemistry depicting up-regulation of Bag 1.

To assess the effects of a 4 hr postmortem interval and storage temperatures on Bag 1-IR in brain regions other than the hippocampus, we collected cerebellar homogenates from absolute control animals and rats maintained postmortem at 24 °C or 4 °C. As demonstrated above, a specific band of ~32 kDa was visualized in the cerebellum of all rats regardless of postmortem intervals or storage temperatures (Fig. 5). Unlike the hippocampus, however, Bag 1 OD levels in this brain region were not significantly different for any of the experimental conditions (absolute control = 100 ± 7.5 ; postmortem interval at 24 °C = 85 ± 16.6 ; postmortem interval at 4 °C = 95 ± 10.9 ; $P \geq 0.05$; one-way ANOVA). These results suggest that the effects of storage temperature on Bag 1-IR are specific, at least, to the hippocampus.

Discussion

Human brains obtained at autopsy are often used for studying psychiatric and neurological disorders of unclear etiology. The correct interpretation of neurochemical data must take into account postmortem intervals and storage temperatures (Torres et al., 1992; Bahn et al., 2001; Hynd et al., 2003; Torres et al., 2004). The approach outlined here provides a means with which to examine these two factors in the context of profile expression and cellular stability of an anti-apoptotic protein. In this study, the hippocampus was selected for expression analysis of Bag 1 because increasing data suggest that this brain region underlies the pathophysiology of severe mood disorders (Manji et al., 2001). Further, the hippocampus is a primary neural target for glucocorticoid hormones secreted by the adrenal glands in response to stress (Nestler et al., 2002). In general, we conclude that valid measures of Bag 1-IR may be made in the postmortem hippocampus, provided that storage temperature variables are controlled (see below).

Our results show that postmortem intervals of at least 4 hr do not significantly affect the stability of Bag 1 in the rat hippocampus. However, it is clear that storage temperature has a greater impact on Bag 1 profile expression than postmortem variables. The cause of this differential sensitivity to storage temperature is unknown, but may stem from the fact that postmortem phenomena trigger the expression of anti-apoptotic proteins to maintain cell viability (Sanoudou et al., 2004; Torres et al., 2004). This hypothetical scenario is reasonable at a storage temperature of 24 °C, with Bag 1 suppressing cell death. At a storage temperature of 4 °C, however, Bag 1 appears to undergo a profound metabolic depression reminiscent of a torpor state for reducing energy costs (Carey et al., 2003). Regardless, our data provide *ex vivo*

evidence for a crucial role of temperature in Bag 1 kinetics. This evidence indicates that despite expectations of a stable expression process of Bag 1 at two storage temperatures, this protein goes through a very active translational phase during the first 4 hr postmortem. This finding parallels that of Hsp70 which is also up-regulated in the postmortem rat hippocampus (Torres et al., 2004; Kafel et al., 2006). These data suggest that during the onset of terminal phase (i.e., agonal state) and brain death, certain proteins (e.g., Bag 1 and Hsp70) linking chaperones and the ubiquitin-proteasome system are more susceptible than others to various pre- and postmortem variables. Further, there may be selective vulnerability of different brain regions to various pre- and postmortem factors as Bag 1 in the cerebellum was not significantly affected by 4 hr of postmortem delay. This latter finding is consistent with the view that the cerebellum is not affected by reduced metabolism and that certain cerebellar proteins (e.g., phosphatases) have an intrinsic resistance to hypothermia as well (Planel et al., 2004).

It is generally thought that both agonal (i.e., premortem) and postmortem states can yield a strong influence on proteins subsequently measured in autopsy tissue (Barton et al., 1993; Hynd et al., 2003). For instance, rapidity of death, nature of the agonal state and disease phenotype are particularly relevant premortem factors affecting the stability of specific proteins. Similarly, postmortem variables such as tissue pH, storage temperature and postmortem intervals can also modify overall neurochemical integrity. In this regard, experimental brains are normally cooled to 4 °C to minimize degradation of certain species of mRNA in individual tissue samples (Bahn et al., 2001; Hynd et al., 2003). Here we have found an apparent effect of a postmortem variable (e.g., storage temperature) on Bag 1 expression. These results are consistent with the idea that temperature has a greater impact on mRNA integrity than postmortem interval (Bahn et al., 2001). Thus, storage temperature should be controlled for in human and animal postmortem brain studies, particularly those analyzing a broad spectrum of molecular chaperones.

What could be the relevance of these findings to brain diseases? It is noteworthy that there is evidence linking aspects of low body temperature (~29 °C) and cell dysfunction in animal models of neurodegenerative diseases (Planel et al., 2004). More specifically, abnormal tau hyperphosphorylation in the hippocampus is associated with hypothermia. Whether increases in Bag 1 activity can also be traced to changes in temperature *in vivo* is not known. The observation that administration of lithium and valproate increases the expression of Bag 1 by inhibiting the glucocorticoid receptor in the rat hippocampus (Zhou et al., 2005), raises the possibility that hypothermia might be an additional factor in modulating the activity of Bag 1.

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Fig. 1.

Schematic diagram of the experimental steps instituted for studying postmortem variables and Bag 1 expression. Adult rats were sacrificed and maintained at depicted temperatures for 4 hr. After this postmortem interval, the hippocampus (Cresyl Violet insert) was processed for immunocytochemistry and Western blotting. Note that Bag 1-IR is visualized as a brown reaction product in the nucleus and scattered parts of the cytoplasm (box insert).

Fig. 2.

Representative dark-field photomicrographs of hippocampal neurons testing positive for Bag 1. Note the differential effects of storage temperature on Bag 1-IR. Both CA1 and CA3 subfields of the hippocampus (Bregma -2.80) show a similar pattern of Bag 1 expression irrespective of storage temperature. Control (i.e., absolute control) denotes hippocampus derived from an animal without a postmortem interval. Rats maintained at depicted storage temperatures had rectal temperatures of ~24 °C and ~4 °C, respectively. Control animals had rectal temperatures of ~37 °C (i.e., physiological temperature). X40 magnifications.

Fig. 3.

Quantitative comparison of Bag 1 cells in the CA1 subfield of the rat hippocampus. Data are means $\pm$ SEM. Absolute control denotes cell counts from animals without a postmortem interval (physiological temperature). 24 °C and 4 °C denote storage temperatures during a postmortem interval of 4 hr. * $P \leq 0.05$ when compared with absolute control cell counts.

Fig. 4.

Quantitative comparison of Bag 1 cells in the CA3 subfield of the rat hippocampus. Data are means $\pm$ SEM. Absolute control denotes cell counts from animals without a postmortem interval (physiological temperature). 24 °C and 4 °C denote storage temperatures during a postmortem interval of 4 hr. * $P \leq 0.05$ when compared with absolute control cell counts.

Fig. 5.

Representative Western blots of Bag 1 in the hippocampal formation and cerebellum. The most salient band migrated at ~32 kDa and is roughly equivalent to one of the three isoform bands identified in rodent tissue (the other two being ~50 and ~36 kDa). Molecular mass markers (in kDa) are shown on the left. Bag 1 levels in the hippocampus are more susceptible to storage temperature than those of the cerebellum. Control (i.e., absolute control) denotes hippocampus and cerebellum derived from an animal without a postmortem interval.

Fig. 6.

Western blot analysis of Bag 1 protein levels in the rat hippocampus. Data are means $\pm$ SEM. Absolute control denotes protein levels from animals without a postmortem interval (physiological temperature). 24 °C and 4 °C denote storage temperatures during a postmortem interval of 4 hr. * $P \leq 0.05$ when compared with absolute control levels. NS = not significant when compared with absolute control levels.