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ScienceDirect
Journal of Chemical Neuroanatomy 32 (2006) 191–195
Journal of CHEMICAL NEUROANATOMY
www.elsevier.com/locate/jchemneu

Expression profile of Bag 1 in the postmortem brain

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Received 26 April 2006; received in revised form 8 September 2006; accepted 8 September 2006
Available online 13 October 2006

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 h postmortem interval, we analyzed the hippocampus of rats maintained at 24 or 4 °C storage temperatures using immunocytochemical and Western blotting techniques. Remarkably, postmortem tissue (up to 4 h) 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.
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Keywords: Apoptosis; Autopsy; Cerebellum; Hippocampus; Mood disorders; Rat; Storage temperature

1. 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 h 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.

2. Materials and methods

Adult male Long-Evans rats (250–300 g body weight) from Charles River Laboratories (Stoneridge, NY, USA) were used for all experiments described herein. Animals were group-housed, 2–3 per cage under a 12 h 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 h postmortem storage temperature of 24 °C; and (c) animals maintained at a 4 h postmortem storage temperature of

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doi:10.1016/j.jchemneu.2006.09.003

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 h 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 h (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 h, there is minimal evidence of cerebral hypoxia as described by Hynd et al. (2003). After the 4 h 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 five consecutive days at 4 °C. Thereafter, brains were placed in a 30% sucrose buffer solution (dissolved in 0.1 M sodium phosphate) for three 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 h at 4 °C with a specific rabbit polyclonal IgG directed against mouse Bag 1 (Santa Cruz Biotechnology, CA, USA). 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, USA).

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 ×10 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 ± S.E.M. 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 ×10 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 $P \leq 0.05$.

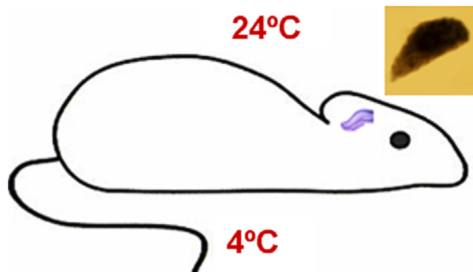

Fig. 1. Schematic diagram of the experimental steps instituted for studying postmortem variables and Bag 1 expression. Adult rats were sacrificed and maintained at depicted temperatures for 4 h. After this postmortem interval, the hippocampus (Cresyl Violet insert) was processed for immunocytochemistry 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 inset).

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, USA). 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, USA). Blots were carried out using 10 μg 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, USA) was used as the secondary antibody. IR bands were visualized by chemiluminescent substrate (Pierce, Rockford, IL, USA) and exposed to Hyperfilm ECL (Amersham Biosciences). Relative immunoblotting optical densities were quantified (as percentage of control) using the UN-SCAN-IT gel analysis system (Silk Scientific, Utah).

3. 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 h (Whitehouse et al., 1984; Barton et al., 1993). In studies using rat brains, the postmortem intervals range from 4 to 6 h (Van Zwieten et al., 1991; Torres et al., 1992). Here, we examined the effects of 4 h 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 pyramidal neurons.

Next, we examined the effects of 4 h 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 h do not significantly affect the overall autopsy levels of

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