Polyamine Catabolism Is Enhanced after Traumatic Brain Injury

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Abstract

Polyamines spermine and spermidine are highly regulated, ubiquitous aliphatic cations that maintain DNA structure and function as immunomodulators and as antioxidants. Polyamine homeostasis is disrupted after brain injuries, with concomitant generation of toxic metabolites that may contribute to secondary injuries. To test the hypothesis of increased brain polyamine catabolism after traumatic brain injury (TBI), we determined changes in catabolic enzymes and polyamine levels in the rat brain after lateral controlled cortical impact TBI. Spermine oxidase (SMO) catalyzes the degradation of spermine to spermidine, generating H₂O₂ and aminoaldehydes. Spermidine/spermine-N¹-acetyltransferase (SSAT) catalyzes acetylation of these polyamines, and both are further oxidized in a reaction that generates putrescine, H₂O₂, and aminoaldehydes. In a rat cortical impact model of TBI, SSAT mRNA increased subacutely (6–24 h) after TBI in ipsilateral cortex and hippocampus. SMO mRNA levels were elevated late, from 3 to 7 days post-injury. Polyamine catabolism increased as well. Spermine levels were normal at 6 h and decreased slightly at 24 h, but were normal again by 72 h post-injury. Spermidine levels also decreased slightly (6–24 h), then increased by ~50% at 72 h post-injury. By contrast, normally low putrescine levels increased up to sixfold (6–72 h) after TBI. Moreover, N-acetylspermidine (but not N-acetylspermine) was detectable (24–72 h) near the site of injury, consistent with increased SSAT activity. None of these changes were seen in the contralateral hemisphere. Immunohistochemical confirmation indicated that SSAT and SMO were expressed throughout the brain. SSAT-immunoreactivity (SSAT-ir) increased in both neuronal and nonneuronal (likely glial) populations ipsilateral to injury. Interestingly, bilateral increases in cortical SSAT-ir neurons occurred at 72 h post-injury, whereas hippocampal changes occurred only ipsilaterally. Prolonged increases in brain polyamine catabolism are the likely cause of loss of homeostasis in this pathway. The potential for simple therapeutic interventions (e.g., polyamine supplementation or inhibition of polyamine oxidation) is an exciting implication of these studies.

Key words: brain injuries; colocalization immunofluorescence; gene expression; polyamine back-conversion; polyamine quantification; polyamine therapeutic potential; spermine oxidase (SMO); spermidine/spermine-N¹-acetyltransferase (SSAT); time course

Introduction

TRAUMATIC BRAIN INJURY (TBI) IS A SIGNIFICANT public health concern among civilian and military populations. In the United States alone nearly 2 million people suffer TBI each year (Abelson-Mitchell, 2008; Leon-Carrion et al., 2005; Nolan, 2005). It is especially important to point out that neurotraumatic brain damage continues to evolve during the hours, days, months, and perhaps longer after the mechanical

injury. TBI is frequently associated with secondary brain damage caused by hemorrhage, edema, oxidative stress, ischemia, cerebral vascular dysfunction (vasospasm, hyperemia), or thrombosis. Ameliorative treatments would be expected to work either by protecting the brain from the deleterious effects of mechanical and secondary damage or by delaying the onset of secondary injuries (to extend the therapeutic window for more definitive treatments). One critical and understudied pathway affected by brain injuries is

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FIG. 1. Polyamine metabolism and back-conversion pathways.

polyamine metabolism (Fig. 1). A limited number of studies suggest that polyamine catabolism (Fig. 1, bottom) is disrupted after neurotrauma and cerebral ischemia (Gilad and Gilad, 1992; Koenig et al., 1989; Seiler, 2000).

The polyamines spermine and spermidine (Fig. 1, top) are ubiquitous aliphatic cations that have potential for the treatment of brain injuries (Dempsey et al., 2000; Gilad and Gilad, 1992; Koenig et al., 1989; Li et al., 2007; Slemmer et al., 2008; Trout et al., 1995). The products of polyamine metabolism include not only spermine and spermidine, but back-conversion to putrescine, hydrogen peroxide (H_2O_2), and aminoaldehydes (Fig. 1, bottom).

Polyamines function to maintain DNA and chromatin structure and regulate signal transduction and cell growth (Hasan et al., 1995; Heby et al., 1988; Igarashi, 2006; Igarashi and Kashiwagi, 2000). Intracellular polyamine levels are tightly regulated (Gilad and Gilad, 1991, 1992; Ientile et al., 1988; Ingi et al., 2001; Li et al., 2007; Paschen, 1992; Schimchowitsch and Cassel, 2006; Seiler and Bolkenius, 1985; Seiler and Lamberty, 1975; Sparapani et al., 1996). These molecules can also function beneficially as immunomodulators and antioxidants.

Spermine and spermidine have antioxidant properties both intracellularly and extracellularly (Clarkson et al., 2004; Lovaas, 1997; Lovaas and Carlin, 1991; Sava et al., 2006; Zhao et al., 2007). Polyamines are integral membrane components and are indispensable in mitochondrial stabilization (Clarkson et al., 2004; Jensen et al., 1989; Liu et al., 2001; Paschen, 1992; Wood et al., 2006b; Xiong et al., 2005). For example, spermine can prevent the deleterious effects of induced mitochondrial permeability transition by stabilizing mitochondrial membranes (Liu et al., 2001; Tassani et al., 1996;

Weinberg et al., 2000a, 2000b). Moreover, spermine has putative anti-inflammatory functions that regulate macrophage and T-lymphocyte activation and inhibit the production of pro-inflammatory cytokines (Zhang et al., 1997, 1999).

Ornithine decarboxylase is a primary enzyme in polyamine synthesis that has been implicated in polyamine changes in the brain after TBI (Raghavendra Rao et al., 1998; Rao et al., 2000; Schmitz et al., 1993). In contrast, the catabolic products generated by polyamine oxidation (i.e., H_2O_2 and aminoaldehydes) are cytotoxic and have been implicated in the mediation of brain injury (Li et al., 2003; Mello et al., 2007; Seiler, 2000; Takano et al., 2005; Wood et al., 2006a, 2006b, 2007). Dysregulation of the enzymes integral to polyamine catabolism may also be a cause of prolonged disruption of polyamine homeostasis. In the brain, expression of spermidine/spermine N^1 -acetyltransferase (SSAT) and spermine oxidase (SMO) increases during the subacute period (0.5 to 24 h) after brain injuries, and polyamine catabolism consequently increases as well (Adibhatla et al., 2002; Babu et al., 2003; Baskaya et al., 1996; Dogan et al., 1999a, 1999b; Gilad et al., 1993; Koenig et al., 1989; Kontos and Wei, 1992; Li et al., 2007; Nagesh Babu et al., 2001; Schimchowitsch and Cassel, 2006; Seiler, 2000; Temiz et al., 2005). SSAT catalyzes the acetylation of spermidine and spermine and is the rate-limiting step in their back-conversion via N^1 -acetylpolyamine oxidase (APAO) in a reaction that generates H_2O_2 , aminoaldehydes, and putrescine (Fig. 1, bottom). Similarly, SMO catalyzes the degradation of spermine in a reaction that generates H_2O_2 and aminoaldehydes.

Thus, their biomolecular, antioxidant, and immunomodulatory properties make polyamines and their catabolism potentially important factors in the mediation of neuroprotection

and secondary tissue damage after acquired brain injuries. To better understand polyamine metabolism in the pathophysiology of brain injuries, we have begun to identify changes after TBI in specific polyamine catabolites and the enzymes that produce them.

Materials and Methods

Lateral controlled cortical impact

Animal protocols were concordant with the *NIH Guide for the Care and Use of Animals* and approved by the University of Cincinnati Institutional Animal Care and Use Committee. Sprague-Dawley rats (300–400 g, Harlan Laboratories, Indianapolis, IN) were pre-anesthetized with isoflurane (~4%) and immobilized in a stereotaxic frame. Oxygen and 2.25% isoflurane were administered using a vaporizer with a facemask and flow-through assembly to minimize inhalation of exhaled gas. A craniectomy was performed using a 6-mm trephine at a point midway between lambda and bregma, between the sagittal suture and the left lateral ridge, leaving the dura intact. Animals were randomly assigned to sham or TBI groups. Sham operated controls were surgically prepared for TBI but not injured. Cortical contusion injuries were induced using a pneumatic piston (5 mm diameter, 2.7 mm depth, 3 m/sec, 100 msec). After hemostasis was achieved, the scalp incision was closed and anesthesia discontinued. Ani-

mals recovered pinna, cornea, and righting reflexes within 9 min, and were returned to their cages after a 15-min observation period.

Tissue preparation

For biochemical studies, conscious animals were decapitated at 6, 24, 72, and 144 h post-injury using a sharpened guillotine. Brains were rapidly removed, and uncontused parietal cortex was dissected on ice from both ipsilateral and contralateral hemispheres, frozen on dry ice, and stored at -80°C . Alternatively, some brains were snap frozen on powdered dry ice, coronally sectioned at -8°C into thick (300 μm) slices, briefly thaw mounted, and stored at -80°C for microdissection of discrete brain regions. For histochemical studies, animals were anesthetized briefly with isoflurane, killed by bilateral pneumothorax, and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS (PBSF). Brains were rapidly removed, sectioned coronally at the level of the median eminence, post-fixed in 4% PBSF, cryoprotected for 2–3 days in PBS containing 20% sucrose, and then snap frozen on a metal plate in a bucket of powdered dry ice and stored at -80°C . Brains were coronally sliced using a cryostat microtome at -20°C ; sections (20 μm) were mounted on gelatinized slides at room temperature, and stored at -80°C .

FIG. 2. SSAT mRNA levels are elevated in the cerebral cortex adjacent to the site of injury. **(A)** Northern blot showing continued elevation of SSAT mRNA (1.2 kb) out to 7 days post-injury. SSAT mRNA was detected on Northern blots of total RNA extracted from parietal cortex ipsilateral (top panel) and contralateral (bottom panel) to injury at serial time points after TBI. Sham animals exhibited a small, nonsignificant increase in SSAT over naïve rats. **(B)** Histogram showing the early and sustained changes in SSAT mRNA levels in ipsilateral cortex after TBI (normalized to shams). Results from two independent studies, $n \geq 3$ per time point. Cortex was freshly dissected (A) or microdissected (B) from 300- μm frozen sections. Brain injury induced as described in Methods.

FIG. 3. Polyamine levels change over time in the cerebral cortex adjacent to injury. **(A)** Spermidine (left) and spermine (right) decreased transiently during the first day after TBI, and spermidine levels increased at 72 h post-injury, compared to shams and previous time points. **(B)** Putrescine levels (left) increased rapidly and remained elevated for at least 72 h post-injury. N¹-acetylspermidine (N1-acetylSPD) levels (right) were detectable in ipsilateral cortex at 24 to 72 h post-injury. Dotted lines show naïve values. Two-way ANOVA showed significant time and treatment effects, *post hoc* tests used grouped sham values because no differences were observed between sham groups. Sham operation increased putrescine by only a small fraction of the injury-induced changes. \$*p* < 0.01 vs. shams; **p* ≤ 0.05 vs. shams; ^a*p* ≤ 0.05 shams vs. naïves.

RNA measurements

Extracted RNA (Trizol; Molecular Research Products, Cincinnati, OH) was separated by electrophoresis on 1.2% agarose/formaldehyde gels. RNA loading (30 µg) was confirmed via ethidium bromide stained 18/28S rRNA bands. RNA was transferred to nitrocellulose, UV-crosslinked, and probed with ³²P-dCTP-labelled SSAT cDNA (random-priming with Klenow enzyme; New England Biolabs, Ipswich, MA). The 900-bp SSAT template was derived from reverse-transcribed from rat kidney RNA (Zahedi et al., 2003).

Polyamine measurements

Polyamines were extracted from microdissected fresh frozen brain tissue, essentially as previously described (Kabra et al., 1986; Marcé et al., 1995; Wang et al., 2004). Briefly, polyamines including acetylated derivatives were extracted from tissue samples with 0.6N perchloric acid and centrifuged. The supernatant extract was dansylated using a dansyl chloride solution (10 mg/mL in acetone). Dansylated polyamines were separated by reverse phase high-performance liquid chromatography with spectrophotometric detection.

SSAT and neuN immunohistochemistry

Every sixth section from the entire dorsal hippocampus was chosen for staining. Fixed 20-µm sections were removed from -80°C and antigen retrieval was performed immediately by

incubating them for 1 min at room temperature in 0.05 M boric acid (pH 8.0), removing excess liquid, and microwaving the slides for 10 sec. For horseradish peroxidase staining, sections were further incubated with 3% H₂O₂ in methanol for 20 min. All sections were blocked in 4% Carnation instant nonfat milk (Nestlé, Glendale, CA) then incubated with rabbit polyclonal anti-recombinant human SSAT (1:400; Abcam Inc., Cambridge, MA) for 3 days at 4°C. Sections were washed twice in PBS for 10 min. Secondary antibodies Cy3 goat anti-mouse and fluorescein isothiocyanate (FITC) goat anti-rabbit (both 1:300; Jackson ImmunoResearch, West Grove, PA) were applied to the sections for 30 min in a humidified chamber, and washed twice in PBS with 0.02% Triton-X100 for 10 min and once in PBS. Cover-slips were mounted using mounting media prepared by mixing 0.5 mL 10×PBS, 18.7 µL 60% sodium lactate, 4.4 mL glycerol, and 50 µL EC/NS-Oxyrase (1:1 in glycerol; Oxyrase, Mansfield, OH). Sections incubated without primary antibody and additional sections incubated without secondary antibody were used as controls.

Staining was visualized using a Nikon (Melville, NY) epifluorescence microscope (courtesy of Dr. Keith Crutcher). Entire anatomical regions of interest were photographed separately under identical conditions, and TIFF files were saved using three wavelength cut-off filters for FITC (green for SSAT), Cy3 (red for neuN), and DAPI (violet for nonspecific fluorescence). As is the case with most tissue injuries, autofluorescence was greater in regions proximal to the site of injury and was attenuated during image analyses.

FIG. 4. SSAT immunoreactivity in parietal cortex at 72 h post-injury. (A) Cortical neurons (arrowheads) as well as smaller glial cells (arrows) infero-lateral to the site of injury express low levels of SSAT-ir at 72 h post-injury. (B) Control section with no primary antibody from a cortical field of the same brain as in (A). (C, D) Parietal cortex SSAT-ir ipsilateral (C) and contralateral (D) to injury (same section, same exposure and processing). (E, F) Merged FITC and Cy3 stains. (E) Injured parietal cortex showing merged neuN-ir (red), SSAT-ir (green) staining (double-stained cells are yellow). Several asterisks indicate pyknotic neuronal cells below. (F) Sham parietal cortex showing SSAT-ir (green) and neuN-ir (red). Note the paucity of neuN stain in (E), and the reduced number and intensity of SSAT-ir cells in (F). Scale bars = 50 μm .

Quantification of SSAT signal was performed using an algorithm in Photoshop (Adobe Systems, Inc., San Jose, CA) in which a threshold was set for each field by evaluation of the violet image to visualize autofluorescence. This threshold was applied to the red and green photos, and only then was integrated pixel density (total pixel intensity per 0.45 mm² field) calculated in Photoshop. Integrated pixel densities were then used for statistical comparisons of specific brain regions. Although this algorithm may have resulted in removal of authentic stain, it was very effective in improving contrast (signal to noise) in all brain regions examined. This was confirmed upon staining adjacent sections using horseradish peroxidase with diaminobenzidine for comparisons. One caveat, however, is that this approach may not be appropriate for all studies, especially for detection of reduced staining of a particular target, because it tends to remove more fluorescence from injured than from uninjured tissues.

Statistics

The number of subjects required for discrimination of >25% changes in group means were calculated for mRNA and protein ($n \geq 4$), biochemistry ($n \geq 3$), and histology ($n \geq 3$) endpoints. Group means and standard error of the mean (SEM) were expressed as mean $\pm$ SEM. Differences in group means were evaluated by analysis of variance with *post hoc* Dunnett (for control comparisons) and Tukey tests (for other group comparisons). A p value of <0.05 was considered necessary to reject the null hypothesis that the group means were equivalent.

Results

Expression of SSAT increases in the damaged cortex after TBI

SSAT mRNA levels were observed to increase in the injured (ipsilateral) but not naïve or uninjured (sham) parietal cortex proximal to the site of injury (Fig. 2). SSAT gene expression increased as early as 2 to 6 h after TBI and remained elevated for up to 7 days post-injury. SMO mRNA also increased ipsilateral to injury but only at 72 h after TBI (data not shown).

Effect of brain injury on cortical polyamine levels

At 6 h post-injury, the neocortical levels of spermidine were down (Fig. 3A, left) and putrescine levels were greatly elevated (Fig. 3B, left) compared to sham levels ($p < 0.05$, Dunnett). By 24 h post-injury, spermidine levels, though still depressed, approached sham values, and continued to increase to elevated levels by 72 h post-injury ($p < 0.01$, Dunnett). At 72 h, levels of spermidine, putrescine, and N¹-acetylspermidine increased in the ipsilateral (Fig. 3) but not in the contralateral (not shown) cortex of rats subjected to TBI. The only measurable alteration in spermine levels was a slight decrease in the injured cortex observed at 24 h (Fig. 3A, right, $p < 0.01$, Dunnett). This coincided with small, but reproducibly elevated N¹-acetylspermidine levels (Fig. 3B, right), observed after 24 h in injured but not sham cortex. Levels of N¹-acetylspermine were below the level of quantification in all tissue samples. Putrescine levels remained elevated for at least 72 h post-injury (Fig. 3B, left). Interestingly, at 72 h, sham spermidine levels were slightly reduced while injured cortex showed recovered and even elevated spermidine levels at this time point.

SSAT immunohistochemistry in the brain after TBI

Lasting changes in SSAT mRNA were observed in the cortex adjacent to the injury site, so we investigated which brain cells might express SSAT (Figs. 4 and 5). To our knowledge, no previous studies have shown SSAT immunoreactivity (SSAT-ir) in rat brain or injured brain tissue. Antibodies against SSAT were used in standard horseradish peroxidase and immunofluorescence labeling paradigms (data not shown). Initially, no signal was detected, so a variety of antigen retrieval techniques were used to stain paraformaldehyde fixed rat brain (20 μm sections). Incubation in sodium borate (100 mM, pH 7.8) with subsequent brief microwave treatment (Methods) was successful in bringing out SSAT staining (Fig. 4A). Control sections incubated without primary (Fig. 4B) or without secondary (not shown) antibody exhibited no SSAT-ir. Initial

FIG. 5. SSAT immunoreactivity in the hippocampus at 72 h post-injury. Hippocampal neurons as well as other cells show SSAT immunoreactivity both ipsilateral and contralateral (not shown) to injury. (A) Merged SSAT-ir (green) and neuN-ir (red) stains: hippocampal neurons beneath the site of injury (upper right) show higher levels of SSAT-ir. Cells in both the corpus callosum and deep cortex (lower right) also stain positive for SSAT. (B–D) Hippocampal pyramidal neurons (B, neuN, arrow), as well as other cell types (C, SSAT, arrows) show SSAT-ir. (D) Merge of (B) and (C) showing neuN and SSAT colocalization (arrowheads). Scale bars = 50 μ m.

studies were conducted to identify SMO staining in the injured brain. After similar antigen retrieval techniques, SMO-ir appeared in neurons and glia throughout the brain (Fig. S1; see online supplementary material at www.liebertonline.com/neu) but was not quantified.

SSAT staining of injured cerebral cortex at 72 h showed neurons with large nuclei (Fig. 4A, arrowheads) and some smaller nonneuronal, likely glial cells (Fig. 4A, arrows). Injured cortex (Fig. 4A,C,E) showed predominantly neuronal SSAT-ir with more intense staining compared with contralateral (Fig. 4C,D). Uninjured cortex (Fig. 4D,F, same sections as in Fig. 4C,E, respectively) showed low intensity, predominantly nonneuronal SSAT-ir. Note the preponderance of double-stained neurons in the injured cortex (Fig. 4E, yellow cells) and the paucity of single-stained neurons compared to contralateral cortex (Fig. 4F, red cells). Many presumably injured neurons with pyknotic cell bodies and strong SSAT-ir were observed at the injury site (Fig. 4E, asterisks). Shrunken neuronal cell bodies and nuclei are indicative of neuronal apoptosis at this time point (Conti et al., 1998; Gopez et al., 2005).

In the hippocampus (Fig. 5), neurons as well as other cell types showed SSAT immunoreactivity at 72 h after TBI. Many

hippocampal neurons ipsilateral (Fig. 5A) to the site of injury were brightly stained, and contralateral neurons (not shown) exhibited low intensity staining at 72 h post-injury. Groups of pyramidal neurons in Ammon's horn (particularly the CA3 region beneath the site of injury, Fig. 5B–D) showed intense SSAT-ir; weaker staining was also seen in glial cells. Intense staining was also visible in cells of the white matter and deep cortex superior-lateral to the hippocampus, beneath the site of injury (Fig. 5A, top). Other cell types that showed SSAT-ir were small elongated cells (Fig. 5A, bottom center, right) not observed contralateral to injury.

In this study, quantification of SSAT staining in cortex and hippocampus proximal to the site of injury was performed (Bregma -2.0 to -5.0). Two-way ANOVA was performed for SSAT-ir (versus treatment and side) on all brain structures. The density of SSAT-ir increased in the injured versus sham brains; there was also a significant increase on the injured side versus contralateral observations ($p < 0.01$, Tukey; Fig. 6, left). For all brain structures there was a 41% increase in SSAT-ir in the injured compared to sham brains ($p < 0.0001$, Student's *t*-test). Ipsilateral brain areas showed a 58% increase in SSAT-ir over contralateral areas in the injured group ($p < 0.001$,

FIG. 6. Quantification of SSAT immunoreactivity: bilateral increases in SSAT. Increased SSAT-ir in the injured cortex and hippocampus at 72 h post-injury. Cortex shows bilateral increases, while hippocampus shows ipsilateral increases in SSAT-ir. Densitometric analyses using mean integrated pixel density per field, as described in Methods. * $p < 0.01$, Tukey test, § $p < 0.05$, Dunnett.

Tukey), while contralateral brain areas showed a significant 24% increase in SSAT-ir in the injured group ($p < 0.005$, Tukey), compared to shams.

When injured cortical structures were grouped together (Fig. 6, center), SSAT-ir increased significantly; however, there was no significant difference between ipsilateral and contralateral sides for the entire cortex. Individual cortical regions did show some side-related differences (Table 1) and an apparent graded decrease in SSAT-ir with increasing distance from the site of injury. No changes in SSAT staining were detected in any hypothalamic regions evaluated (ipsilateral, contralateral, and midline, not shown). In the hippocampus, significant increases in SSAT-ir were seen in injured compared to sham animals ($p < 0.01$, Tukey); and ipsilateral staining in the injured group was significantly increased compared to contralateral ($p < 0.04$, Dunnett). Individual regions of Am-

mon's horn showed significant increases ipsilateral to injury, namely in injured CA3 and dentate gyrus (Fig. 6, right).

Discussion

Relevance of polyamine findings to TBI

Currently, no medical treatments have proven effective at improving functional outcomes after TBI. Catabolism of polyamines after brain injury depletes spermine and spermidine levels (Aizenman, 1995; Kontos, 1985; Paschen, 1992; Samanta et al., 1998; Seiler, 2000; Xiong et al., 2005). Aberrant polyamine homeostasis can lead to secondary injury both through depletion of cellular spermine or spermidine, and via generation of toxic metabolites (putrescine, reactive aminoaldehydes, and H_2O_2 ; Fig. 1, bottom). Depletion of polyamines leads to disruption of mitochondrial membrane

TABLE 1. GRADED SSAT EXPRESSION IN CORTEX PROXIMAL AND DISTANT TO THE SITE OF INJURY

Cortical brain region	Side	% Change	Comparison	p^a
Parietal cortex, superior adj. (layers I and II)	Ipsilateral	234 ± 18	Injured vs. sham	<0.02
	Contralateral	238 ± 9	Injured vs. sham	<0.02
Parietal cortex, deep adj. (sup. to corpus callosum)	Ipsilateral	205 ± 13	Injured vs. sham	<0.001
	Contralateral	174 ± 5	Injured vs. sham	<0.02
Parietal cortex, inferior adj. (layers I and II)	Ipsi + Contra	137 ± 12	Injured vs. sham	<0.02
Perirhinal/entorhinal cortex	Ipsi + Contra	165 ± 9	Injured vs. sham	<0.005
Piriform cortex	Ipsi + Contra	133 ± 12	Injured vs. sham	<0.05

Results are integrated pixel density as percent sham $\pm$ SEM.

^aTukey test.

Adj., adjacent to the site of injury; sup., superior; Ipsi + Contra, both sides grouped together, no significant difference when compared individually.

potential and the induction of apoptosis, whereas administration of polyamines may reverse both of these. Polyamine catabolism after brain injuries increases production of the toxic and pro-oxidant metabolites putrescine, aminoaldehydes, and H_2O_2 (Fig. 3; Adibhatla et al., 2002; Dogan et al., 1999a; Layton et al., 1997; Seiler, 2000; Zini et al., 1990). The critical role of polyamines in mediating membrane and DNA integrity, mitochondrial calcium buffering, and oxidative stress suggests that therapeutic targeting of polyamine metabolism could benefit the injured brain.

Increased polyamine catabolism after TBI

TBI initiated an early (2–6 h) and sustained (7 day post-injury) increase of SSAT mRNA in the neocortex ipsilateral to injury (Fig. 2). In addition, SSAT-ir brain cells ipsilateral to injury appeared to stain more intensely than in the comparable contralateral regions (Figs. 4–6). Moreover, concomitant increases in SSAT activity were revealed by increased spermidine, putrescine, and acetylated spermidine levels (Fig. 3) for at least 3 days post-injury. These results indicate that SSAT expression increased in neural tissues after TBI. Taken together, these findings confirm that increased polyamine back-conversion activity disrupted polyamine homeostasis in the traumatically injured brain.

Likewise, after peripheral tissue injuries, polyamine homeostasis is altered, with enhancement of the catabolic arm of the pathway (Barone et al., 2005; Dogan et al., 1999a; Nagesh Babu et al., 2001; Shappell et al., 1993; Zahedi et al., 2003). Thus, enhanced polyamine catabolism may also be associated with worsened brain injury, neuroinflammation, and neuronal cell death.

Injury-related increases of SSAT-ir in the cortex at 72 h post-injury appeared both in neurons and frequently in non-neuronal cells (likely astrocytes, microglia, and macrophages). Whereas in the hippocampus, increases in SSAT staining appeared to be somewhat cell type specific at 72 h, predominantly staining neuronal cells. Interestingly, we observed ipsilateral increases in hippocampal SSAT and bilateral increases in the cortex. This implies a local, neuron-specific effect of injury on SSAT in the hippocampus (in the regions most vulnerable to neuronal death), while cortical SSAT expression may be controlled globally, e.g., by changes in cytokines or other blood-borne factors.

SSAT catalyzes the acetylation of spermidine and spermine that are then oxidized by polyamine oxidase to putrescine generating H_2O_2 and acetylaminopropionaldehyde in the process (Fig. 1). SMO degrades spermine in a reaction that also generates H_2O_2 and 3-aminopropanal. Spermine loss in particular may contribute to membrane injury, DNA damage, decreased mitochondrial calcium buffering capacity, and increased vulnerability to oxidative stress (Clarkson et al., 2004; Lovaas and Carlin, 1991; Sava et al., 2006). We also observed increases in SMO mRNA (unpublished results) for 3 to 7 days after TBI. The late induction of SMO correlated very well with spermidine increases, suggesting that SMO activity might be elevated at later times post-injury. Thus, oxidation of essential polyamines may also be considered a source of secondary tissue damage, increased inflammation, and apoptotic cell death in the injured brain.

It is possible that the elevation of SSAT in response to environmental stressors may be adaptive (Gilad et al., 2001; Kaasinen et al., 2000, 2003). However, our findings suggest that prolonged dysregulation of SSAT gene expression/enzyme activity may be detrimental to the injured brain. One potential mechanism by which SSAT may worsen brain injury is through its interplay with ornithine decarboxylase (ODC) resulting in increased polyamine flux (Raghavendra Rao et al., 1998). ODC, the first step in *de novo* polyamine synthesis, increases acutely in the brain after injury (Raghavendra Rao et al., 1998).

Interestingly, increased expression and activity of SSAT may increase the expression and activity of ODC (Kramer et al., 2008). Moreover, blockade of ODC improved outcomes in rat models of cerebral ischemia and reperfusion (Kindy et al., 1994; Temiz et al., 2005) and after TBI (Baskaya et al., 1996; Rao et al., 2000; Schmitz et al., 1993). Thus, an alternative interpretation of the observations of increased spermidine and putrescine, together with measurable acetylspermidine, is that both SSAT and SMO increase with a concurrent increase in ODC. This would result in increased putrescine, not as a result of back-conversion (SSAT/APAO), but due to the predicted increase in ODC activity. And since spermidine would actually be increasing, feedback inhibition could prevent conversion to spermidine (and spermine).

Yet another mechanism through which enhanced polyamine back-conversion may contribute to cerebral injury is through production of toxic metabolites (Fig. 1). Increases of aminoaldehyde (Fig. 3) and pro-oxidant metabolites, such as H_2O_2 and 3-acetyl-aminopropanal, are damaging to vulnerable brain tissue (Li et al., 2003; Mello et al., 2007; Seiler, 2000; Takano et al., 2005; Wood et al., 2006a, 2006b, 2007). Therefore, the onset of polyamine back-conversion and polyamine synthesis after brain injury, together with concomitant elevations of oxidative metabolites and acetylated polyamines, likely instigate secondary tissue injury. Thus, treatments that retard polyamine catabolism and reduce these cytotoxic oxidative byproducts may be expected to reduce secondary tissue damage in brain regions that demonstrate enhanced polyamine catabolism after TBI.

Conclusions

Based on these findings, we hypothesize that increased polyamine catabolism in brain regions associated with the functional deficits observed after TBI with concomitant disruption of polyamine homeostasis leads to a neurotoxic environment contributing to secondary injury as well as worse outcomes. We have initiated a series of studies to determine the role of polyamines and their catabolism in the pathophysiology of TBI and the potential of approaches that increase tissue polyamine levels or inhibit their catabolism as therapeutic measures for prevention of secondary tissue injury in the treatment of TBI. We predict that improved functional recovery will be associated with prolonged increases of spermine and spermidine, as well as decreased putrescine, aminoaldehyde, and oxidant levels in somatosensory cortex and hippocampus. Furthermore, we will begin to examine if approaches that counteract polyamine catabolism lessen the secondary tissue damage after TBI by reducing oxidative tissue injury and the apoptotic response.

Author Disclosure Statement

No competing financial interests exist.

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