**Calcium-Mediated Proteolytic Damage in White Matter of Hydrocephalic Rats?**

**MARC R. DEL BIGIO, MD, PhD, FRCP **

**Abstract.** Hydrocephalus is a pathological dilatation of the cerebrospinal fluid (CSF)-containing ventricles of the brain. Damage to periventricular white matter is multifactorial with contributions by chronic ischemia and gradual physical distortion. Acute ischemic and traumatic brain injuries are associated with calcium-dependent activation of proteolytic enzymes. We hypothesized that hydrocephalus is associated with calcium ion accumulation and proteolytic enzyme activation in cerebral white matter. Hydrocephalus was induced in immature and adult rats by injection of kaolin into the cisterna magna and several different experimental approaches were used. Using the glyoxal bis (2-hydroxyanil) method, free calcium ion was detected in periventricular white matter at sites of histological injury. Western blot determinations showed accumulation of calpain I (μ-calpain) and immunoreactivity for calpain II was increased in periventricular axons of young hydrocephalic rats. Proteolytic cleavage of a fluorogenic calpain substrate was demonstrated in white matter. Immunoreactivity for spectrin breakdown products was detected in scattered callosal axons of young hydrocephalic rats. The findings support the hypothesis that periventricular white matter damage associated with experimental hydrocephalus is due, at least in part, to calcium-activated proteolytic processes. This may have implications for supplemental drug treatments of this disorder.

**Key Words:** Axon; Calpain; Hydrocephalus; Kaolin; Proteolytic enzyme.

**INTRODUCTION**

Hydrocephalus is a common neurological condition characterized by pathological dilatation of the cerebral ventricles. It is usually caused by an obstruction to cerebrospinal fluid (CSF) flow. Axonal damage in periventricular white matter is one of the earliest pathological consequences of ventricular dilatation in humans and animals. The pathophysiology of hydrocephalus-induced brain damage is multifactorial, with contributions by gradual physical stretching and compression of tissues, chronic ischemia, alterations in neurochemical function, and possible accumulation of metabolic waste products (1–3). The damage is progressive with a time course measured in days to months. In order to understand more fully the pathogenesis of brain damage due to hydrocephalus, it would be worthwhile to consider other neurological disorders in which brain damage occurs more rapidly and through a “more simple” mechanism. Such disorders include trauma and stroke. It has been postulated that abrupt physical trauma to brain alters membrane permeability in axons leading to a sequence of local electrolyte disturbances, influx of calcium ion (Ca$^{2+}$), and activation of calpains (4, 5). Calpains belong to the cysteine protease class of cytoplasmic enzymes capable of hydrolyzing a range of substrates including membrane bound receptors and cytoskeletal proteins. Different iso-enzymes are activated at micromolar concentrations of Ca$^{2+}$ (μ-calpain or calpain I) or at millimolar concentrations of Ca$^{2+}$ (m-calpain or calpain II) (6). Proteolytic damage to the axonal cytoskeleton ensues with impairment of axonal transport and ultimately axonal disconnection (7, 8). The same process seems to occur when axons of the optic nerve are more rapidly stretched (9). Following hypoxic-ischemic brain injury, Ca$^{2+}$ influx into axons and neurons plays a role in their destruction through a similar sequence of molecular events (10–12).

Considering that hydrocephalic brain damage is likely caused by what are in effect gradual traumatic and ischemic processes, we hypothesized that Ca$^{2+}$-dependent mechanisms are involved in hydrocephalus-induced axonal damage. We tested this in immature and young adult rats in which hydrocephalus was induced by injection of kaolin into the cisterna magna. The reason for examining the 2 situations is that the pathogenesis might differ somewhat. Young rats develop severe brain distortion with gross enlargement of the cerebral ventricles and head and die within 4–6 wk (13). Adult rats on the other hand develop only mild ventricular enlargement but die within 2 wk, usually because of severely elevated intracranial pressure (14, 15). We first sought to demonstrate the presence of abnormal quantities of soluble ionic calcium in brain using histochemical methods (16, 17). Second we sought evidence that calpains are present and activated in hydrocephalic brains.

**MATERIALS AND METHODS**

All animals were treated in accordance with guidelines set forth by the Canadian Council on Animal Care and experiments were approved by the local animal use committee. All efforts were made to minimize suffering and the number of animals used. Three-week-old (weight 43–61 g) and adult (10–12-wk-old, 250–350 g) male Sprague–Dawley rats were bred locally. To induce hydrocephalus, the rats were anesthetized with ketamine/xylazine (90/10 mg/kg IM), the neck was shaved, and
under aseptic conditions a 27-gauge needle was inserted percutaneously into the cisterna magna. Sterile kaolin suspension (0.05 ml; 250 mg/ml in 0.9% saline) was injected slowly. Controls received a sham injection. With this quantity of kaolin, young rats die within 4–6 wk with grossly enlarged ventricles, while adult rats develop only mild ventricular enlargement but die within 2 wk because of severely elevated intracranial pressure (14). Therefore, for these studies young rats were killed 3.5 to 4 wk and adult rats were killed 1 wk after kaolin injection.

Histochemical Detection of Ionic Calcium

Magnetic Resonance Imaging

Magnetic resonance (MR) imaging was performed using a Bruker Biospec/3 MR scanner equipped with a 21-cm bore magnet operating at a field of 7 T (Karlsruhe, Germany) to obtain T2-weighted images of brain in the coronal plane. The widths of the lateral ventricles and cerebrum were measured in the rostral cerebrum immediately anterior to the third ventricle. Frontal horn size was expressed as a ratio determined by dividing the width of the ventricle by the width of the cerebrum. These methods have been previously described in detail (13, 15). Rats were imaged no more than 24 h prior to death.

Detection of Calpains and Cytoskeletal Proteins

For Western blot detection, 3 separate sets of perfusion-flushed brain samples from a total of 30 rats were analyzed. In a previous experiment, frontal cerebral samples, including both cortex and white matter, were obtained from young rats 1–4 wk after kaolin or sham injection (19). Corpus callosum/ periventricular white matter was microdissected from brains of young control (n = 3) and hydrocephalic rats (n = 6) 3–4 wk following kaolin injection. Similar corpus callosum/periventricular white matter samples were taken from adult control (n = 3) and hydrocephalic rats (n = 5) 1-wk postinjection. Tissue samples were homogenized in RIPA buffer and protein concentration was determined by the Lowry method (20). Equal quantities of protein (10 μg) were loaded into 5%–15% acrylamide/ SDS vertical gels and electrophoresed at 100 volts for 2 h. Prestained molecular weight standards were run in parallel. Proteins were transferred to nitrocellulose membrane and blocked in Tris buffered saline with 5% milk, 0.02% sodium azide, and 0.1% Tween-20. Membranes were incubated with mouse monoclonal anti-calpain I (1/500 dilution in the same buffer; kindly donated by Drs. Gail Johnson and John Elce) (21), rabbit polyclonal anti-calpain II (1/1,000 dilution; AB1625, Chemicon International Inc., Temecula CA), rabbit polyclonal anti-brain axon/presynaptic elements spectrin (1/250 dilution; AB992, Chemicon) (22), mouse monoclonal anti-neurofilament 68 kD (1/1,000 dilution; clone NR4; N5139, Sigma), and rabbit antiserum raised against a spectrin cleavage product (Ab38 1/1,000 dilution; kindly donated by Dr. R. Siman [23]) overnight at 4°C. Then membranes were incubated with biotinylated goat antimouse or biotinylated sheep anti-rabbit (1/2,000 dilution; Jackson ImmunoResearch Labs, West Grove PA) followed by streptavidin-peroxidase and chemiluminescence detection. Each experiment was run at least in triplicate. Densitometric readings were obtained from exposed X-ray film and were normalized to control values.

Detection of Calpain Activity In Situ Using Fluorogenic Substrate

Eight adult hydrocephalic rats were killed by pentobarbital overdose 1 wk after kaolin injection, along with 6 adult controls. The heart was perfused briefly with ice cold 0.1 M phosphate buffered saline (PBS) to clear blood from the vasculature, then the brain was quickly removed and cut with a razor blade into 1-mm-thick coronal slices that were placed on a glass slide. N-succinyl-leucine-tyramine 7-amido-4-methylcoumarin (suc-Leu-Tyr-AMC; S3389, Sigma), which is cleaved by active calpain I and II to yield a substrate that fluoresces under ultraviolet
Fig. 1. Photomicrographs showing glyoxal bis (2-hydroxyanil) (GBHA) detection of Ca\(^{2+}\) in adult rat brain. The red precipitate is negligible in periventricular white matter of control rats (A) and cerebrum of hydrocephalic rats (B). However, there are red Ca\(^{2+}\)-chelates in white matter at the angle of the ventricle (C) and in ependymal cells (arrowhead) lining the lateral ventricle (D) of hydrocephalic rats. Abbreviations: v, ventricle; p, pial surface. Magnification: frames A–C, ×40; frame D, ×150.

Statistical Analysis

Quantitative data were analyzed to confirm a normal distribution and are presented as mean ± standard error of the mean. For Western blots, the densitometric values were normalized to those of control values and percent changes relative to control are indicated. Statistical analysis consisted of 2-tailed Student t-test or ANOVA with post-hoc Bonferroni-Dunn calculations for inter-group comparisons, as appropriate. Statistical significance is defined as p < 0.05. Software used was StatView 5 (SAS Institute; Cary, NC).

RESULTS

Behavioral changes, ventricle size changes evident on MR imaging, and neuropathological changes in young rats have been detailed in previous publications (13, 19, 24, 26). Following kaolin injection, young rats exhibited delayed weight gain by 1 wk, followed by progressive enlargement of the cerebral ventricles and head, ataxia, and lethargy after 2–3 wk. Adult rats exhibited more rapid deterioration with weight loss, mild enlargement of the ventricles, and lethargy developing in a few days and death occurring 1–2 wk after kaolin injection.

Calcium Ion Localization

In none of the 5 control rat brains of either age was there any red chelation product indicative of free Ca\(^{2+}\), except rarely at sites where the brain surface had been damaged during removal. In 6/6 young and 3/5 adult hydrocephalic rats there was red chelation product in periventricular white matter at the angle of the ventricle, although it was not clear whether the deposits were intra- or extra-cellular. Deposit margins blended into rarified white matter, which lacked red staining. In 3 rats, focal reaction product was seen along the ependyma (Fig. 1). Reaction product was never observed in choroid plexus or cerebral cortex.

Calpain Detection

In control brains at both ages, calpain I-like immunoreactivity was weak and restricted to ependymal cells
Fig. 2. Photomicrographs showing calpain I-like immunoreactivity in young rat brain. In control rats (A) immunoreactivity was detected only in ependymal cells (arrow) lining the lateral ventricles. In hydrocephalic rats (B) immunoreactivity was also observed in large periventricular axons (arrow) and the cytoplasm of scattered small glial cells (arrowhead). Scale bar = 5 μm.

Fig. 3. Western blot detection of calpain I (80 kD) in samples of periventricular white matter from adult control (C) and hydrocephalic (H) rat brains. Three of the hydrocephalic samples exhibit a relative increase in the band intensity.

Calpain I Adult Hydrocephalic Rats

(C) (H)

(Fig. 2A). Calpain II-like immunoreactivity was detected weakly in scattered glial cells of white matter. In young hydrocephalic brains with advanced ventriculomegaly the intensity of both was increased in ependyma and periventricular white matter. White matter labeling was in a pattern suggestive of localization to myelin or axolemma of large (1–2-μm-diameter) axons (24, 26) (Fig. 2B). No changes were evident in adult brains. Western blot detection of calpain I and II in control brains revealed bands at ~80 kD, the expected size for the large calcium dependent catalytic subunit in its inactive state (6). Isolated periventricular white matter homogenates demonstrated significant changes in calpain I content. Optical density of the calpain-I bands on Western blots was increased 128% (p = 0.027) in adult tissue 1 wk after induction of hydrocephalus (Fig. 3) and by 355% (p < 0.001) in young rats 4 wk after induction of hydrocephalus, although there was no change at 3 wk (Fig. 4). This corresponds to the period during which axonal injury increases rapidly (24). Despite testing a variety of electrophoresis conditions, we could not resolve a second calpain band at 76 kD, which is said to be the cleaved active form of calpain I (21). Calpain II was unchanged in periventricular white matter samples of both ages, but
Calpain I - Young Hydrocephalic Rats

Fig. 4. Western blot detection of calpain I (80 kD) in samples of periventricular white matter from young control (C3 and C4) and hydrocephalic rat brains. The band intensity is unchanged 3 wk (H3) after induction of hydrocephalus but is greater after 4 wk (H4) when white matter damage is severe.

Calpain II - Young Hydrocephalic Rats

Fig. 5. Western blot detection of calpain II (80 kD) in samples of cerebrum from young control (C2) and hydrocephalic rat brains 2 wk following kaolin injection (H2). The band is less intense in hydrocephalic brains.

cerebrum homogenates from young hydrocephalic rats exhibited a 41% decrease (p = 0.011) in the optical density of calpain II bands 2 wk after kaolin injection (Fig. 5). In these samples, which included predominantly gray matter, no change was observed for calpain I.

Evidence of Calpain Activation

Application of suc-Leu-Tyr-AMC to adult control rat brain slices resulted in no significant fluorescence under ultraviolet illumination, and brain tissue exhibited no autofluorescence when viewed after buffer application alone. The majority (7/8) of adult hydrocephalic brains exhibited diffuse blue fluorescence in white matter adjacent to the lateral ventricle (Fig. 6) indicative of proteolytic cleavage of the substrate, possibly by calpain. Direct application of substrate solution yielded a more obvious reaction product than that following gel application. For technical reasons explained in the Materials and Methods section, this experiment could not be replicated in the young hydrocephalic brains.

Detection of cytoskeletal degradation products is an indirect indicator of calpain activity following acute brain injury (6). On Western blots the antibody to 68 kD neurofilament detected a strong band at ~68 kD. Although the band was noticeably weaker in samples from some 4-wk hydrocephalic brains than controls (not shown), we did not unambiguously detect breakdown products (27).

On Western blots the antibody to spectrin detected bands at ~240 kD as well as at ~150 kD. The larger represents the full size spectrin molecule and the smaller represents a cross-reacting subproduct (22, 28). Samples from 3/5 young hydrocephalic rats (but no adults) exhibited an additional smeared band at ~145 kD (not shown), which is
the expected size for breakdown products of spectrin (22, 29). However, Western blots using Ab38, which detects spectrin fragments cleaved by calpain (5, 23, 30), failed to confirm the presence of significant quantities of spectrin cleavage product (not shown). Nevertheless, immunohistochemical labeling of frozen brain sections using Ab38 resulted in labeling of scattered axons (3 to 20 per tissue section) in the corpus callosum of 5/5 young hydrocephalic rats and 0/3 control rats (Fig. 7). Similar labeling was not identified in adult hydrocephalic rat brains.

**DISCUSSION**

These experiments using young and adult rats with kaolin-induced hydrocephalus show that Ca$^{++}$ accumulates in periventricular white matter at sites of white matter ischemia (15) or maximal axonal damage (24); that white matter content of calpain I is increased, and that calpain I-like immunoreactivity is increased in periventricular axons; that cleavage of a fluorogenic substrate sensitive to calpain activity is increased in the white matter; and that spectrin-degradation antigens are detectable by immunohistochemistry in periventricular axons of young hydrocephalic rats that suffer severe axonal injury. Together these observations suggest that Ca$^{++}$-activated proteolysis might play a role in the axonal damage that occurs in this experimental kaolin-induced hydrocephalus.

The GBHA histochemical method, as performed in this study, detects only ionized or easily ionizable (i.e. unbound) calcium ion (17, 18). The precise sensitivity is not known, but it is likely that visible reaction product can be detected only when the concentration of Ca$^{++}$ in a 15-μm-thick tissue section exceeds 0.15μg/cm$^2$, which corresponds to roughly 0.25 mmol. The whole tissue staining method used in this experiment is thought to be less sensitive (31, 32). Brain extracellular Ca$^{++}$ is roughly 1.3 mmol, while intracellular Ca$^{++}$ is 10$^5$-fold lower (33, 34). Estimating the extracellular fluid volume at 15% yields a Ca$^{++}$ concentration of ~0.2 mmol in normal whole tissue, in which there was no detectable GBHA precipitate. In hydrocephalic brains Ca$^{++}$ was detected among or within periventricular axons at sites of injury. Intense GBHA precipitate was also observed in residual ependymal cells, which line the lateral ventricle and which possess a variety of calcium binding proteins (35). In hydrocephalus, continuity of the ependymal lining is lost as the ventricles enlarge (2, 36). This might allow unbound Ca$^{++}$, whose concentration is increased in the CSF of patients and H-Tx rats with hydrocephalus (1, 37), to reach vulnerable axons and glia. One could postulate that Ca$^{++}$ gradually accumulates in slowly stretched axons (38, 39), which are subsequently damaged (24). Stretch injury to axons results in altered activity of Ca$^{++}$-ATPase, which in turn predisposes to Ca$^{++}$ increases in the axoplasm (40). Ca$^{++}$ might also accumulate in ischemic oligodendroglia (41). This would help to explain the oligodendroglial dysfunction observed in hydrocephalic brains (26).

Accumulation of free Ca$^{++}$ in white matter of hydrocephalic rats might be capable of activating calpain I (42). Most published data concerning calpain activation
in brain relate to acute changes, which occur minutes to hours following traumatic or ischemic damage (6, 21, 29, 30, 43–46). Within 30 min of traumatic brain injury, spectrin cleavage products, presumed to result from calpain activity, can be localized by immunohistochemistry along the axolemma (5). In these disorders the insult is abrupt and synchronous and occurs in a large volume of tissue. Conversely, in hydrocephalus the insult to white matter is prolonged over a period of days to weeks and occurs at different points along scattered axons. Using the Ab38 antibody we found that only scattered axons displayed spectrin breakdown products (5). Therefore, one would expect difficulty detecting calpain activation in tissue homogenates. Accordingly, we could not demonstrate in homogenized bulk tissue from hydrocephalic brains cleaved calpain, which is said to be indicative of activation (21), nor could we convincingly demonstrate neurofilament or spectrin cleavage. Nevertheless, cleavage of the fluorogenic peptide suc-Leu-Tyr-AMC indicates that proteolytic activity of calpains (25) is increased in white matter of hydrocephalic rats. Although it was not focally restricted like the Ca\(^{++}\) deposits, the peptide is a small soluble molecule (MW 551) that might diffuse through edematous white matter (13) leading to a homogenous distribution. Alternately it might indicate that proteolytic activity is more widely distributed than the foci of high Ca\(^{++}\) concentrations. It is worth noting that calpain activity in damaged axons might not necessarily be harmful. There is evidence that it is necessary for sealing of damaged axons by allowing cytoskeletal remodeling (47).

Normally calpains are abundant in myelin (48). In this experiment, periventricular axon profiles became immunoreactive for calpain only after induction of hydrocephalus, consistent with the postulate that activated calpains migrate to membrane-associated sites (49). Ventriculomegaly was associated with increased calpain I detection in white matter. Similarly, increased cellular immunoreactivity for calpain has been documented in rat brain following transient ischemia (50). Chronic upregulation of calpain mRNA and protein accumulation have been implicated in the spinal cord neuron degeneration that occurs in MND mutant mice (51). Chronic exposure to ozone, which generates oxygen radicals, is associated with increased calpain activity in rat brainstem (52). A variety of stimuli induce calpain production by cultured C6 glioma cells (53). The hydrocephalus-associated depletion of calpain II from cerebra of young hydrocephalic rats might reflect the rats’ nutritional status, which is known to be suboptimal (13). It has been previously shown that 48-hour starvation is associated with decreased calpain II activity in rat brain (54). Alternately, calpain II depletion might reflect the generalized impairment of myelin and synapse development that occurs in these young hydrocephalic rats, because calpain II is involved in myelination and synaptogenesis (24, 26). Calpain I, which was increased in hydrocephalic brains, does not seem to be capable of degrading calpain II (55).

In this experiment there was an apparent age-dependent difference. Specifically, young hydrocephalic brains seemed to accumulate spectrin-cleavage products and calpain to a greater degree than mature hydrocephalic brains. This can likely be explained on the basis of the magnitude of physical injury with gross distortion occurring in young animals but not in the adults. We have previously shown that despite the development of periventricular edema at both ages (13, 15), damaged axons in the periventricular white matter are not abundant until the ventriculomegaly is severe (24). This suggests that Ca\(^{++}\)-dependent destructive mechanisms among periventricular axons most important when ventriculomegaly leads to severe axonal stretching and are less important in acute adult-onset hydrocephalus wherein raised intracranial pressure and generalized white matter ischemia occur (15).

In conclusion, these data suggest that Ca\(^{++}\)-activated proteolytic mechanisms might contribute to the axon injury that occurs in periventricular white matter of young hydrocephalic rats with severe ventricular enlargement. This could be prompted by trauma in the form of axonal stretching (38, 56, 57) or by local hypoperfusion (11, 12), which both occur in hydrocephalic brains (2, 3). Similar mechanisms are implicated in the neuronal and axonal damage that occurs following brain ischemia and cerebral trauma (6, 43). Unlike ischemic and traumatic injury, wherein the damage occurs over a period of seconds to minutes, in hydrocephalus the damage is gradual and progressive over a period of days to months. Demonstration of the mechanism(s) of axonal injury associated with hydrocephalus is not simply an academic exercise. Pharmacologic modification of this process prior to surgical shunting of CSF would potentially benefit patients in whom a shunt cannot be placed immediately (58, 59).

Note added in proof:

Gene expression changes were assessed in hydrocephalic brain by obtaining pooled frontal cerebrum RNA samples 1 week after kaolin injection from 3 adult hydrocephalic rats and 3 adult control rats. The samples were hybridized in parallel to 2 microarray membranes with rat DNA probes (GF300 rat GeneFilters® microarray; Research Genetics, Huntsville, AL). The probing was repeated in triplicate. Comparison of gene expression revealed, among other changes, 2–2.8-fold overexpression of calpain I mRNA and slight underexpression of calpain II mRNA. These data confirm our protein content observations.
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