Substrate to Calpain after Neonatal Cerebral Hypoxia-Ischemia* 

Calpastatin Is Up-regulated in Response to Hypoxia and Is a Suicide Substrate to Calpain after Neonatal Cerebral Hypoxia-Ischemia* 

Klas Blomgren, Ulrika Hallin, Anna-Lena Andersson, Malgorzata Puka-Sundvall, Ben A. Bahr, Amanda McRae, Takaomi C. Saito, Seiichi Kawashima, and Henrik Hagberg

From the Perinatal Center, Department of Pediatrics, Sahlgrenska University Hospital/Ostra, SE 416 85 Göteborg, Sweden, 2Department of Pharmaceutical Sciences and the Neurosciences Program, University of Connecticut, Storrs, Connecticut 06269-2092, and 3Cortex Pharmaceuticals, Inc., Irvine, California 92618.

In a model of cerebral hypoxia-ischemia in the immature rat, widespread brain injury is produced in the ipsilateral hemisphere, whereas the contralateral hemisphere is left undamaged. Previously, we found that calpains were equally translocated to cellular membranes (a prerequisite for protease activation) in the ipsilateral and contralateral hemispheres. However, activation, as judged by degradation of fodrin, occurred only in the ipsilateral hemisphere. In this study we demonstrate that calpastatin, the specific, endogenous inhibitor protein to calpain, is up-regulated in response to hypoxia and may be responsible for the halted calpain activation in the contralateral hemisphere. Concomitantly, extensive degradation of calpastatin occurred in the ipsilateral hemisphere, as demonstrated by the appearance of a membrane-bound 50-kDa calpastatin breakdown product. The calpastatin breakdown product accumulated in the synaptosomal fraction, displaying a peak 24 h post-insult, but was not detectable in the cytosolic fraction. The degradation of calpastatin was blocked by administration of CX295, a calpain inhibitor, indicating that calpastatin acts as a suicide substrate to calpain during hypoxia-ischemia. In summary, calpastatin was up-regulated in areas that remain undamaged and degraded in areas where excessive activation of calpains and infarction occurs.

Hypoxic-ischemic brain damage is an important contributor to long term neurologic sequelae in term and preterm infants (1–3). The intracellular calcium concentration increases during anoxia/ischemia (4) followed by a secondary phase of cellular calcium overload simultaneous with or slightly preceding development of hypoxic-ischemic neuronal damage (5, 6). Activation of calcium-dependent enzymes is considered to be an early feature in this process (7). Calpains (EC 3.4.22.17) are calcium-activated, nonlysosomal, neutral cysteine proteases proposed to participate in many important intracellular processes, such as turnover of cytoskeletal proteins and regulation of kinase activity and transcription factors (8, 9). The activity of calpains is strictly regulated by calcium concentrations and interaction with calpastatin (the endogenous inhibitor protein), membrane phospholipids, and a multitude of other factors. The ubiquitous distribution of calpains and the complex regulation of their activity indicate that these proteases play important roles under both physiological and pathological conditions. Calpain activation, as judged by the appearance of specific fodrin (10) breakdown products (FBDP) has previously been demonstrated in adult (12–16) and neonatal (17, 18) models of ischemia and hypoxia. Activation of calpains and selective degradation of preferred substrates precede neuronal degeneration, indicating that these proteases are activated during hypoxia-ischemia (HI) and in the early phase of reperfusion after HI, preceding neuronal death (12, 13, 16–19). In vivo administration of inhibitors of calpain activity has been shown to be neuroprotective in adult models (15, 19, 20), including CX295, the inhibitor used in this study (21, 22). Inhibition of calpains may be particularly effective in the perinatal setting, considering the high calpain content in the rapidly growing brain (23) and increased calpain activity following hypoxia-ischemia (24). Furthermore, calpain activation, as judged by the accumulation of FBDP, was especially prominent in the white matter (18), which is interesting in the light of the specific vulnerability of the white matter in the immature brain and its devastating clinical consequences (periventricular leukomalacia).

It is generally considered that upon stimulation calpains are translocated to cellular membranes, where they bind to potential substrates. The interaction between calpains, substrates (25), and cellular components such as acidic phospholipids (26) and DNA (27), drastically increase the calcium sensitivity of the proteases, lowering the calcium concentration required for activation down to physiological or near physiological levels. Calpastatin, the endogenous inhibitor protein of calpains, has...
also been demonstrated to bind to membranes, even purified phospholipid vesicles (28). Hence, the subcellular distribution of calpains and calpastatin is crucial for the functions of these proteins. The interaction between calpains and calpastatin is reversible and calcium-dependent in the sense that calpains bind to the inhibitor only after binding calcium (29). The inhibitory activity seems to be specific for calpains, because no other protease tested so far is affected by calpastatin. Interestingly, it has been shown that calpastatin can be easily degraded by calpains in vitro (30, 31) and in cultured cells (32). No conclusive evidence of calpain-induced cleavage of calpastatin in vivo has been presented so far. Using a modified Levine preparation (33, 34) allowed us to study the effects of both hypoxia and hypoxia-ischemia (HI) on the immature brain in the same animal. Previously we found that calpain was translocated to membranes, a prerequisite for activation, to the same extent in both hemispheres, but activation, as indicated by increased fodrin proteolysis, occurred only in the ipsilateral, damaged hemisphere (17, 18). The present work was undertaken to elucidate whether calpastatin is involved in the process of halting calpain activation in the contralateral, undamaged hemisphere.

**EXPERIMENTAL PROCEDURES**

**Induction of Hypoxia-Ischemia—**Unilateral HI was induced in 7-day-old Wistar F rats of both sexes (33, 34). The pups were anesthetized with halothane (3.0% for induction and 1.0–1.5% for maintenance) in a mixture of nitrous oxide and oxygen (1:1), and the duration of anesthe-
sia was <10 min. The left common carotid artery was cut between double ligation of prolene sutures (6–0). After the surgical procedure the wounds were infiltrated with a local anesthetic, and the pups were allowed to recover for 1–2 h. The litters were then placed in a chamber perfused with a humidified gas mixture (7.70% oxygen for induction and 1.0–1.5% for maintenance) in a

**Preparation of Samples and Subcellular Fractions for Western Blotting—**The animals were sacrificed by decapitation, and the brains were rapidly dissected out on a bed of ice, weighed, quickly frozen in ice-pentane and dry ice, and stored at –80 °C. Cortical tissue rostral to the hippocampus (approximately 50 mg) was dissected out from each hemisphere at –10 °C. The tissue was homogenized by sonication in 10 volumes of ice-cold 50 mM sodium borate (pH 8.0) containing 320 mM sucrose, 5 mM EDTA, 0.1 mM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, and 3 mM NaN3. Homogenate samples were mixed with an equal volume of SDS-PAGE buffer and heated (96 °C) for 5 min. Subcellular fractionation was carried out essentially according to Hu and Wieloch (35) and Cotman et al. (36). The remaining homogenate was centrifuged at 800 × g for 10 min at 4 °C, followed by centrifugation of the supernatant at 9,200 × g for 15 min at 4 °C. The resulting pellet (P2), representing a crude synaptosomal fraction, was washed by resuspending it in 500 μl of homogenizing buffer and cen-
trifuging again at 9,200 × g. The washed pellet was resuspended in 250 μl of 3 × SDS-PAGE buffer, heated (96 °C) for 5 min, and stored at –80 °C. The supernatant (S2) was further separated into a particulate membrane fraction (P2) and a cytosolic fraction (S2) by centrifugation at 150,000 × g for 1 h at 4 °C. The S2 was decanted, added to an equal volume of 3 × SDS-PAGE buffer, heated (96 °C) for 5 min, and stored at –80 °C.

**Antibodies**—The calpastatin antibody was raised against a synthetic 16-mer peptide corresponding to the C-terminal portion of rat calpastatin (37). An antibody that specifically recognizes the N-terminal, calpain-specific proteolytic 147-kDa breakdown product (BDP) of rat α-fodrin (38) was used to detect fodrin cleavage. The calpain antibody (1D10A7) recognizes the large subunit of both m- and μ-calpain (39), with an approximately seven times higher affinity for m-calpain (17). All secondary antibodies, horseradish peroxidase- or biotin-conjugated, were from Vector Laboratories (Burlingame, CA).

**Western Blotting Procedures**—The protein concentration of homogenates, P2, and S2 fractions in SDS-PAGE buffer was determined according to Karlsson et al. (40). Samples corresponding to 20 μg of total protein were electrophoresed on 7.5% SDS-PAGE gels or NOVEX pre-
cast 8–16% Tris-glycine gels (NOVEX, San Diego, CA) and transferred to nitrocellulose (0.45 μM; Schleicher & Schuell, Dassel, Germany) membranes. Immunoreactive species were visualized using horseradish peroxidase-conjugated secondary antibodies, Super Signal, or Super Signal ULTRA chemiluminescent substrates (Pierce) and Fuji RX film (Fuji Photo Film Co., Tokyo, Japan). Films were scanned, and immunoreactive bands were quantified using the software IPLab Gel 1.5f (Scanalytics Corp., Fairfax, VA). Every sample was analyzed 1–8 times, and when multiple determinations were performed the average value was used as n = 1.

**Immunohistochemical Procedures**—Pups were re-anesthetized and perfusion-fixed with Histofix (isotonic, buffered 5% paraformaldehyde (pH 7.2) from Histolab, Vastra Frolunda, Sweden). Following dissection and perfusion-fixation at 4 °C, the brains were placed in 1:1 M phosphate buffer (pH 7.4) containing 150 mM NaCl (phosphate-buffered saline) and 20% sucrose and 0.02% NaN3, overnight at 4 °C and embed-
ed in Tissue-Tek O.C.T. Compound (Sakura Finetek Europe, Zoeter-
woude, Netherlands). Coronal frozen sections (10 μm) were mounted and boiled in 10 mM citrate buffer (pH 6.0) for 10 min (antigen recovery) and blocked in 4% goat serum in phosphate-buffered saline for 30 min at room temperature. The sections were incubated with the calpastatin antibody (1:100 in blocking solution) and the secondary, biotin-labeled antibody (1:250) for 1 h each at room temperature. Visualization was performed using the Vectastain ABC kit (Vector Laboratories, Burling-
emage, CA).

**Quantitative Multiplex RT-PCR**—After decapitation, the brains were rapidly removed and frozen in liquid nitrogen. Total RNA was extracted from each hemisphere using the guanidine isothiocyanate–cesium chlo-
ride method (41), quantified spectrophotometrically at 280 nm, and stored at –80 °C. First strand cDNA synthesis was performed with the Superscript RNase H–Reverse Transcriptase kit (Life Technologies, Inc.) and random hexamer primers (Roche Molecular Biochemicals). Total RNA (2 μg), random primers (500 ng), and RNase-free water to 24 μl. Reverse transcription was carried out for 10 min at 25 °C, followed by 20 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 1 min. The PCR products were subjected to agarose gel electrophoresis, and those of the appropriate size were extracted from the gel and stored at –20 °C. All secondary antibodies, horseradish peroxidase- or biotin-conjugated, were from Vector Laboratories (Burlingame, CA).

**Northern Blotting Procedures**—Oligonucleotide (25-mer) primers were used to synthesize cDNA from total RNA isolated from the forebrain of 7-day-old Wistar F rats using the Superscript RNase H–Reverse Transcriptase kit (Life Technologies, Inc.). The primers were from Kebo Lab (Stockholm, Sweden). The annealing tem-
perature and cycle numbers were chosen such that both the calpastatin and the GAPDH PCR products would be in the linear phase of amplifi-
cation and of similar intensity (data not shown). The PCR products were separated on 1% agarose gels, stained with ethidium bromide, and photographed under UV light. The pictures were scanned, and the bands were quantified using the software IPLab Gel 1.5f (Scanalytics Corp., Fairfax, VA).

**Northern Blotting Procedures—**Oligonucleotide (25-mer) primers were produced using a synthesizer from Applied Biosystems. The primer sequences were: 5′-CTG CAG GCT TTA GAA CCT CAA CAC C3′ (upstream) and 5′-CTG GAT TCT TTA GAC GCT CCA CCC A3′ (downstream). Primers were designed to amplify a 441-base pair se-
sequence from the coding region of the rat m-calpain gene, bases
Cytosolic Calpastatin—In the major, cytosolic pool of calpastatin, an up-regulation was demonstrated in the contralateral, hypoxic hemisphere (Figs. 1 and 2). The increase was evident already at the end of hypoxic exposure and more prominent after 2–24 h of recovery (171% after 2 h, p = 0.014 and 185%, p = 0.007, respectively, after Bonferroni correction). No significant increase could be detected in the ipsilateral hemispheres. Rather, the contralateral hemispheres displayed 39% (0 h), 112% (2 h), 103% (6 h), 57% (1 days), and 119% (2 days) higher values than the corresponding ipsilateral hemispheres (p = 0.013, 0.037, 0.0007, 0.016, and 0.010, respectively) (Fig. 2). Only intact calpastatin (110 kDa) could be detected in the S3 fraction.

Calpastatin Immunohistochemistry—In tissue sections, the changes in calpastatin immunoreactivity in the parietal cortex followed those demonstrated by Western blotting, i.e. an increase in the contralateral and a decrease in the ipsilateral hemisphere (Fig. 3, panels 1–3). After 2 h of recovery, the number of immunoreactive cells had increased in the contralateral hemisphere compared with controls (Fig. 3, panels 1B and 2B). After 24 h of recovery there was a striking loss of immunoreactive cells in the ipsilateral hemisphere (Fig. 3, panels 1A, 2A, and 3A).

Calpastatin mRNA—The multiplex RT-PCR produced a 320-base pair calpastatin fragment and a 600-base pair GAPDH fragment with similar staining intensities (not shown). The calpastatin mRNA, from the entire hemisphere, did not display any significant differences in the control animals during normal development (PND 7, 9, 13, and 21). During reperfusion calpastatin mRNA was quantified at 2 h and 1, 2, 3, 6, and 14 days of recovery (n = 6 at all time points, except PND 9 (control) and 14 days of recovery, where n = 5). There was a tendency toward up-regulation after HI in both hemispheres, which turned out statistically significant in the ipsilateral hemispheres after 2 h and 6 days of recovery (146%, p = 0.0065 and 150%, p = 0.0039, respectively) and in the contralateral hemispheres after 14 days (204%, p = 0.0446) (not shown). However, considering the inherent limitations of the RT-PCR technique, interpretations should be made with caution because the increases observed were only 2-fold or less.

Calpain—The calpain levels were assayed in pooled samples only; therefore no statistical analyses could be performed. The purpose was to correlate calpain and calpastatin changes, temporally, in identical samples (on the same membranes) and to compare with our earlier findings. The 1D10A7 antibody produced a single, distinct band with an approximate molecular mass of 75 kDa, as previously described (17). The calpain immunoreactivity (per µg total protein) in the P2 fractions was approximately 10% of that in the S3 fractions (data not shown). This ratio is lower than for the membrane and microsomal fraction used previously (corresponding to the P3 fraction), where the immunoreactivity was 20% of that found in the S3 fractions (17). Immediately after HI the calpain levels were up-regulated in the S3 fractions (213 and 151% in the ipsilateral and contralateral hemispheres, respectively), dropping to control levels or below after 2–48 h and increasing again in the ipsilateral hemisphere at 14 days post-HI (146% compared with controls at PND 21) (Fig. 4a). The main change in the P2 fractions was a drastic decrease at 24 and 48 h post-HI to 5 and 6% in the ipsilateral and contralateral hemispheres, respectively, after 24 h (Fig. 4b). In the control animals there was a down-regulation of calpain in the P2 fractions at PND 10 and

![Image](79x515 to 267x729)
PND 21, whereas the S3 fractions displayed an up-regulation at these time points, indicating that the normal development entails a decreased membrane/cytosolic ratio (Fig. 4).

**m-Calpain mRNA**—The m-calpain probe distinctly recognized a single band on Northern blots, with an apparent size of 3.5 kilobases (Fig. 5a). There was substantial interindividual variation in the level of m-calpain mRNA, even after normalization to 18 S rRNA. The ratio between the ipsilateral and contralateral hemispheres in each animal, however, proved to be highly reproducible. In control animals this ratio remained close to 100% at all time points, whereas in animals exposed to hypoxia the ratio increased after the insult, being 167% after 48 h ($p = 0.006$) (Fig. 5b).

**In Vivo Calpain Inhibition of Calpastatin Degradation**—Animals treated with CX295 every 3 h for 24 h following the insult displayed significantly less degradation of intact calpastatin in the ipsilateral hemisphere (47% less degradation, $p = 0.003$) (Table I). The ratio of intact calpastatin (ipsilateral/contralateral) was 14.7% in the controls and 54.5% in the CX295-treated animals (85.3 and 45.5% degradation, respectively) (Table I).

**In Vivo Calpain Inhibition of Fodrin Degradation**—The anti-FBDP antibody detected a single band on Western blots with no background. The calpain-specific 147 kDa fodrin degradation product is detectable in neonatal brains of control animals but not in adult animals (17), indicating a higher turnover in the immature, rapidly growing brain producing a steady-state pool of FBDP. As expected, the amount of FBDP was several-fold higher in the ipsilateral hemispheres of animals subjected to HI. The ratio of FBDP (contralateral/ipsilateral) was 21.6% in control animals, injected with vehicle, and 37.6% in animals treated with CX295 subcutaneously ($p = 0.038$) (Table I).
Calpastatin Regulation after Cerebral Hypoxia-Ischemia

Animals were subjected to HI and post-treated with CX295 or vehicle every 3 h for 24 h. The ratios of intact calpastatin (110 kDa) between the ipsilateral and the contralateral hemispheres are displayed in the two top rows. Animals treated with CX295 displayed 47% less degradation of calpastatin (45.4% degradation compared with 85.3% in those receiving vehicle). The ratios of FBDP between the contralateral and the ipsilateral hemispheres are displayed in the two bottom rows. Animals treated with CX295 displayed 47% less degradation of fodrin (68 and 45 kDa) into the cytosol (32) or in adult gerbil hippocampus, where only a minor breakdown product of 15 kDa was detected 7 days after ischemia (44). It has been shown that phosphorylation, probably by protein kinase C, increased the tendency for calpastatin to associate with membranes (45), so this may be a mechanism regulating the subcellular distribution of the inhibitor. The intact, 110-kDa double band probably represents two differently phosphorylated forms. The significance of the 50-kDa CBDP is unclear. Conceivably, it could be aimed at inhibiting calpains by (synaptosomal) membranes. Each calpastatin molecule consists of four inhibitory domains and an N-terminal, noninhibitory domain (domain L) (46), and calpain-cleaved calpastatin fragments as small as 15 kDa have been shown to retain inhibitory capacity (31). It is also possible that different forms of calpastatin, resulting from phosphorylation, dephosphorylation, alternative splicing, or proteolytic cleavage, may have specific roles in modulation of intracellular signal transduction (37, 44, 47–50). Interestingly, the 50-kDa CBDP did not seem to be detected by the antibody in tissue sections only on Western blots. Had the CBDP been detected in tissue sections, the staining would have been much stronger during reperfusion in the ipsilateral than in the contralateral hemispheres. Rather, the tissue sections displayed the same changes as the S3 fraction Western blots, i.e. an up-regulation in the contralateral and a down-regulation in the ipsilateral hemispheres. This could be due to the membrane-bound nature of the CBDP, which may make the epitope(s) inaccessible to the antibody.

The concept of calpastatin acting as a suicide substrate to calpains has been proposed and demonstrated earlier in vitro (31). Calpastatin is a preferred substrate to calpains, more readily cleaved than fodrin (32), possibly because calpains complex with calpastatin before they have a chance to bind to fodrin and other membrane-associated substrates. To test the hypothesis of calpastatin acting as a suicide substrate also in vivo, a group of animals were treated with a calpain inhibitor, CX295, which has been demonstrated earlier to provide neuroprotection in models of adult brain ischemia (21) and trauma (22). Treatment with CX295 inhibited the degradation of both calpastatin and fodrin, the most widely used marker of calpain activity, indicating that the proteolysis was attributable to calpains. CX295 appeared to be more effective preventing calpastatin depletion than fodrin degradation, because both modes of administration (subcutaneous and intraperitoneal) were effective in inhibiting calpastatin degradation, whereas only subcutaneous injections could decrease the cleavage of fodrin. Generally, intraperitoneal injections produce a rapid, but also shorter lasting, increase in the serum concentration of a drug, reaching a higher peak value than subcutaneous injections. Presumably, the CX295 was present at sufficient serum concentrations for a longer period of the 3-h interval after subcutaneous than intraperitoneal injections. However, degradation of calpastatin and fodrin should be assumed to be inhibited to the same extent. The FBDP is known to have a long


table I

| Ratio          | S.D. | n  | p    |
|----------------|------|----|------|
| Calpastatin    |      |    |      |
| Vehicle        | 14.7 | 7.65| 8    | 0.003|
| CX295          | 54.6 | 29.6| 8    |      |
| FBDP           |      |    |      |
| Vehicle        | 21.6 | 11.8| 8    | 0.038|
| CX295          | 37.6 | 11.0| 4    |      |

animals treated with CX295 intraperitoneally displayed a ratio not significantly different from the controls (not shown).

DISCUSSION

Degradation of Calpastatin—This is the first report to our knowledge demonstrating degradation of calpastatin by calpains in vivo. It has been shown earlier in vitro (30, 31) and in cultured cells (32) and has been implicated in a model of adult, transient forebrain ischemia (44). The degradation was confined to the ipsilateral, damaged hemisphere, and it was discrete, because it produced a specific breakdown product about half the size of the intact molecule. This 50-kDa CBDP seemed to be relatively resistant to further degradation, because it accumulated in the P2 fraction. The CBDP was the major immunoreactive species after 24–48 h of recovery, exceeding the amount of intact 110-kDa calpastatin found in the contralateral hemisphere. A single, membrane-bound CBDP stands in contrast with earlier studies using the same antibody, in epidermoid carcinoma KB cells, where proteolysis of membrane-associated calpastatin resulted in the release of fragments (68 and 45 kDa) into the cytosol (32) or in adult gerbil hippocampus, where only a minor breakdown product of 15 kDa was detected 7 days after ischemia (44). It has been shown that phosphorylation, probably by protein kinase C, increased the tendency for calpastatin to associate with membranes (45), so this may be a mechanism regulating the subcellular distribution of the inhibitor. The intact, 110-kDa double band probably represents two differently phosphorylated forms. The significance of the 50-kDa CBDP is unclear. Conceivably, it could be aimed at inhibiting calpains by (synaptosomal) membranes. Each calpastatin molecule consists of four inhibitory domains and an N-terminal, noninhibitory domain (domain L) (46), and calpain-cleaved calpastatin fragments as small as 15 kDa have been shown to retain inhibitory capacity (31). It is also possible that different forms of calpastatin, resulting from phosphorylation, dephosphorylation, alternative splicing, or proteolytic cleavage, may have specific roles in modulation of intracellular signal transduction (37, 44, 47–50). Interestingly, the 50-kDa CBDP did not seem to be detected by the antibody in tissue sections only on Western blots. Had the CBDP been detected in tissue sections, the staining would have been much stronger during reperfusion in the ipsilateral than in the contralateral hemispheres. Rather, the tissue sections displayed the same changes as the S3 fraction Western blots, i.e. an up-regulation in the contralateral and a down-regulation in the ipsilateral hemispheres. This could be due to the membrane-bound nature of the CBDP, which may make the epitope(s) inaccessible to the antibody.

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![Northern blotting of total RNA from whole hemispheres, using the probe against m-calpain.](image)
half-life (38), thereby masking the inhibitory effect of CX295, whereas intact calpastatin may have a much shorter half-life. 

**Up-regulation of Calpastatin**—Our findings indicate that calpastatin is up-regulated in response to both hypoxia and HI but that the up-regulation is concurrent with extensive degradation of the intact, 110-kDa calpastatin in the ipsilateral hemisphere (85.3% after 24 h of recovery). This up-regulation is consistent with the findings in adult gerbil hippocampus, where an approximately 2-fold increase was detected 4 h after ischemia (44). This up-regulation was sustained in the CA2, where the neurons are less vulnerable. However, in the CA1 and other regions displaying neuronal cell death, the up-regulation was followed by a gradual decrease, below pre-ischemic levels, providing an explanation to why more calpain-induced fodrin degradation is seen in these regions (12, 13) and possibly why they are more vulnerable to ischemic stress. A number of proteins are known to be induced or up-regulated after ischemic stress, such as the heat shock proteins (51, 52), and calpastatin may be one of these proteins, aimed at abating the effects of degenerative cascades. There was a consistent tendency toward a moderate increase (approximately 50%) of calpastatin mRNA in both hemispheres, as judged by the quantitative, multiplex RT-PCR, but RT-PCR is not a good method to detect moderate changes quantitatively. The up-regulation seen on the protein level may be a result of increased translation from already present or newly synthesized mRNA, but it may also be a result of decreased degradation. Decreased susceptibility to proteolysis may be accomplished by alternative splicing or post-translational modification by phosphorylation (37, 45, 53, 54).

**Subcellular Distribution of Calpain**—The relative amounts of calpain in the S3 and P2 fractions changed in a manner consistent with our earlier findings, but there were also important differences. In accordance with earlier findings was the relative decrease in the major, cytosolic pool of calpain post-HI, whereas intact calpastatin in the S3 and P2 fractions changed in a manner similar to that observed in various cell systems, producing 150-kDa fragments similar to those produced by calpains (37, 45, 53, 54).

**Summary**—These results are compatible with our proposed function of calpastatin that upon mild stimulation calpains bind to calpastatin, preventing the proteases from degrading substrates. However, the intramolecular, autocytolytic activation of calpains is not prevented by calpastatin binding (51), and if the stimulus becomes stronger, the protease will degrade its inhibitor and then go on to cleave other substrates. This is supported by our findings in this neonatal model that calpains were translocated to membranes in both the hypoxic (mild stimulation) and the hypoxic-ischemic (strong stimulation) hemispheres, but degradation of calpastatin and fodrin occurred only in the hypoxic-ischemic hemispheres. The most common method to demonstrate calpain activation is to detect specific fodrin cleavage products (12, 13, 16, 17, 38, 56). Our present findings suggest that calpastatin cleavage could be more sensitive for this purpose. However, two reports have recently appeared that demonstrate degradation of calpastatin also by caspases (48, 57). Furthermore, caspases, primarily caspase-3, have been reported to cleave fodrin during apoptosis in various cell systems, producing 150-kDa fragments similar but not identical to those produced by calpains (58–61). The overlapping substrate specificities and possible interactions between these two protease families are interesting and warrant further investigation.

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