Synergistic Activation of Caspase-3 by m-Calpain after Neonatal Hypoxia-Ischemia

A MECHANISM OF "PATHOLOGICAL APOPTOSIS"?

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Received for publication, August 28, 2000, and in revised form, December 14, 2000. Published, JBC Papers in Press, December 21, 2000, DOI 10.1074/jbc.M007807200

The relative contributions of apoptosis and necrosis in brain injury have been a matter of much debate. Caspase-3 has been identified as a key protease in the execution of apoptosis, whereas calpains have mainly been implicated in excitotoxic neuronal injury. In a model of unilateral hypoxia-ischemia in 7-day-old rats, caspase-3-like activity increased 16-fold 24 h postsisult, coinciding with cleavage of the caspase-3 proenzyme and endogenous caspase-3 substrates. This activation was significantly decreased by pharmacological calpain inhibition, using CX295, a calpain inhibitor that did not inhibit purified caspase-3 in vitro. Activation of caspase-3 by m-calpain, but not μ-calpain, was facilitated in a dose-dependent manner in vitro by incubating cytosolic fractions, containing caspase-3 proform, with calpains. This facilitated required the presence of some active caspase-3 and could be abolished by including the specific calpain inhibitor calpastatin. This indicates that initial cleavage of caspase-3 by m-calpain, producing a 29-kDa fragment, facilitates the subsequent cleavage into active forms. This is the first report to our knowledge suggesting a direct link between the early, excitotoxic, calcium-mediated activation of calpain after cerebral hypoxia-ischemia and the subsequent activation of caspase-3, thus representing a tentative pathway of "pathological apoptosis."

The relative contributions of necrosis and apoptosis to the injury that develops after cerebral hypoxia-ischemia (HI) has been a matter of much debate (1). Recent studies suggest that cell death after HI is different from developmentally regulated cell death in most cases and cannot appropriately be described as apoptotic (2–5). Nevertheless, HI cell death shares important morphological and biochemical features with apoptotic cell death, such as activation of caspases and nucleosomal DNA fragmentation (6–17).

Caspases, a family of cysteine proteases with an unusual substrate specificity, requiring an aspartate residue in the P1 position, have been identified as key executors of apoptosis (18). Calpains, another family of cysteine proteases, are calcium-activated and are proposed to participate in the turnover of cytoskeletal proteins and regulation of kinases, transcription factors, and receptors (19, 20). Calpains have mainly been implicated in excitotoxic neuronal injury and necrosis (21–23). Pharmacological inhibitors of calpains and caspases exert cerebroprotective effects (9, 14–16, 24–26). A growing body of literature has emerged, demonstrating functional connections between calpains and caspases (27). Common substrate proteins have been identified, such as fodrin (28–31), calpastatin (32, 33), actin (34), PARP (35), and tau (36). There are reports demonstrating calpain-mediated cleavage of caspase-3 (35, 37) and caspase-7 (38, 39) as well as caspase-8 and -9 (39). Furthermore, the proapoptotic protein Bax was cleaved by calpain during drug-induced apoptosis of HL-60 cells (40), and calpain may be responsible for cleaving the loop region in Bcl-xL, thereby turning an antiapoptotic molecule into a proapoptotic one (41). One study demonstrated synergy between calpains and the proteasome downstream of caspases in constitutive apoptosis of human neutrophils (42), whereas other studies demonstrated an upstream regulatory role for calpains in the apoptosis of neutrophils (43) and thymocytes (44). Recently, Nakagawa and Yuan showed that m-calpain may be responsible for the activation of caspase-12 by the endoplasmic reticulum, indicating a link between calcium dysregulation and apoptosis (41).

Previously, we found that the supposedly calpain-dependent degradation of calpastatin in a model of neonatal cerebral HI followed a biphasic pattern (45), where the second phase closely
followed the activation of caspase-3. Reports demonstrating degradation of calpastatin by caspase-3 (32, 33) prompted us to investigate further the spatial and temporal activation of these two proteases and possible interactions in this model.

MATERIALS AND METHODS

Induction of Hypoxia-Ischemia—Unilateral HI was induced in 7-day-old Wistar F rats of both sexes (46, 47). 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 ligatures, and the carotid suture was made 30 s 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 (70 ± 0.01% oxygen in nitrogen) for 70 min. The temperature in the gas chamber was kept at 36 °C. Following hypoxic exposure, the pups were returned to their biological dams until sacrificed. Control animals were operated and ligated but not subjected to hypoxia. All animal experimentation was approved by the Ethical Committee of Göteborg (approval number 225-97).

In Vivo Calpain Inhibition—Three litters (n = 28) were treated with the calpain inhibitor CX295 (Z-Leu-a-maminobutyric acid-CONHCH2; Cortex Pharmaceuticals, Irvine, CA) or vehicle. The first dose of CX295 in 100 mM NaCl (equivalent to 50 μM DEVD and 50 μM CH2 in 100 mM NaCl, 10 mM EDTA, 1 mM EGTA, 0.2% CHAPS, and 3 mM Na2SO4) was injected subcutaneously immediately after HI. Subsequently, animals were injected with 100 μl of the CX295 solution (equivalent to ~40 μmol/kg or 20 mg/kg body weight) every 3 h for 24 h. Control animals were injected with 100 μl NaCl.

Preparation of Samples—The animals were sacrificed by decapita-
tion, and the brains were rapidly dissected out on a bed of ice, weighed, quickly frozen in isopentane and dry ice, and stored at −80 °C. Cor-
tical tissue rostral to the hippocampus, ~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 Tris-HCl (pH 7.3), containing 5 mM EDTA, aliquoted, and stored at −80 °C. Homogenate samples were mixed with an equal volume of concentrated (3×) SDS-polyacrylamide gel electrophoresis buffer and heated (96 °C) for 5 min.

Inhibition of Purified Enzymes—Recombinant, active human caspase-3 (MBL, Nagoya, Japan), 2.0 μl of reconstituted solution (the absolute amount of caspase-3 is not known), was preincubated with 50 μl of protease inhibitor solution (see below) for 10 min and then mixed with 100 μl of 50 μM DEVD-7-amino-4-methylcoumarin (DEVD-AMC) substrate (Bachem, Bubendorf, Switzerland) in 50 mM Tris-HCl (pH 7.5), 100 μm NaCl, 10 mM EDTA, 1 mM EGTA, 0.2% CHAPS, and 3 mM Na2SO4. Cleavage of DEVD-AMC was measured at 37 °C using a SpectraMax Gemini microplate fluorometer (Molecular Devices, Sunnyvale, CA) with an excitation wavelength of 380 nm and an emission wavelength of 480 nm. DEVD-AMC cleavage was calculated from the Vmax and expressed as relative fluorescence units (RFU)/s/ml. Rabbit lung μ- or m-calpain (48) (480 pmol of AMC produced/min at 37 °C) that was in 5 μl of buffer was preincubated with 50 μl of inhibitor solution (see below) for 10 min and then mixed with 100 μl of 1 mM LY-AMC in 20 mM Tris-HCl (pH 7.5) containing 4 mM CaCl2, 4 mM DTT, 3 mM MgSO4, and 3 mM Na2SO4.

Substrate cleavage was evaluated as described for the caspase-3 assay. Inhibitors were as follows: CX295 from Cortex Pharmaceuticals (Irvine, CA) or against the p17 subunit of calpain (Pierce); and Fuji RX film (Fujifilm, Tokyo, Japan). Films were scanned, and immunoreactive bands were quantified using the software IPLab Gel 1.5f (Scanalytics Corp., Fairfax, VA). Alternatively, membranes were exposed in a LAS 1000 cooled CCD camera, and immunoreactive bands were quantified using the software Image Gauge (Fujiﬁlm, Tokyo, Japan). Every sample was analyzed 1–4 times, and when multiple determinations were performed the average value was used as n = 1. Striping of membranes for reprobing purposes was performed by incubation in 62.5 mM Tris-HCl (pH 6.7), 100 mM β-mercaptoethanol, 2% SDS, at 50 °C for 30 min. All membranes were blotted with the antibody against α-tubulin. Tubulin was used to nor-
malize between samples (10).

Immunohistochemical Procedures—Pups were deeply anesthetized and decapitated. The brains were dissected in 1.1% 3-10% sucrose buffer. The brains were rapidly removed and immersion-fixed at 4 °C for 24 h. After dehydration with graded ethanol concentrations and xylene, the brains were paraffin-embedded and cut into 4-μm coronal sections. Sections were deparaffinized in xylene and rehydrated in graded ethan-
el alcohol concentrations before staining. Immunopositive cells were visualized in a MAP of negative area of parietal cortex 390 × 660 μm in size and outlined with a Neurolucida (Cambridge, UK) system.

Activated Caspase-3—Sections were pretreated with proteinase K (Roche Molecular Biochemicals), 10 μg/ml in PBS for 10 min, at room temperature. Antigen recovery was performed by boiling the sections in 10 mM sodium citrate buffer (pH 6.0) for 10 min. Nonspecific binding was blocked for 30 min with 4% goat serum in PBS. Anti-caspase-3 p17 was applied diluted 1:500 in PBS and incubated for 60 min at room temperature, followed by biotinylated goat anti-rabbit IgG (6 μg/ml in PBS) or fluorescein isothiocyanate-labeled goat anti-rabbit IgG (6 μg/ml) for 60 min. Visualization was performed using vectastain ABC Elite or fluorescence microscopy.

FBP—Antigen recovery and blocking were performed as above. The anti-FBP was applied diluted 1:50 in PBS containing 0.2% Triton X-100 and incubated for 60 min at room temperature, followed by biotinylated goat anti-rabbit IgG (11 μg/ml in PBS) or fluorescein isothiocyanate-labeled goat anti-rabbit IgG (6 μg/ml) for 60 min. Visualization was performed using vectastain ABC Elite or fluorescence microscopy.

Reverse Transcription-PCR—Six pups for each time point were decapitated at 0 h, 1 h, 3 h, 6 h, 12 h, 24 h, 72 h, and 14 days of recovery and snap-frozen in liquid nitrogen. Transcripts were extracted from each hemisphere (52), quantified spectrophotometrically at 260 nm, and stored at −80 °C. First strand cDNA synthesis was performed with the Superscript II RNase H Reverse Transcriptase kit (Life Technologies, Inc.) and ran-

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Synergistic Activation of Caspase-3 by m-Calpain

The caspase-3-like activity (DEVD cleavage), cleavage of the caspase-3 proform and degradation of endogenous caspase-3 substrate ICAD were assessed after treatment with the selective calpain inhibitor CX295. Rat pups were injected every 3 h for 24 h post-HI. The average ratio between the ipsilateral, damaged hemisphere and the contralateral, undamaged hemisphere is indicated for vehicle- and CX295-treated animals. For the cleavage product of caspase-3 (29 kDa), the ratio listed is that between the fragment and the total immunostaining in the ipsilateral hemisphere, because this fragment could not be found in the contralateral hemisphere.

TABLE I
Effects of in vivo calpain inhibition on the activation of caspase-3

| Ratio       | S.D. | n  | p    |
|-------------|------|----|------|
| Caspase-3   |      |    |      |
| DEVD cleavage|      |    |      |
| Vehicle     | 1618.0 | 564.4 | 9 | 0.0004 |
| CX295       | 606.1 | 289.4 | 8 |      |
| 32 kDa      |      |    |      |
| Vehicle     | 72.8  | 18.3 | 11 | 0.005 |
| CX295       | 96.0  | 17.4 | 12 |      |
| 29/29 + 32 kDa|      |    |      |
| Vehicle     | 15.2  | 11.1 | 11 | 0.031 |
| CX295       | 6.88  | 5.46 | 12 |      |
| ICAD        |      |    |      |
| 45 + 32 kDa |      |    |      |
| Vehicle     | 77.5  | 18.2 | 11 | 0.006 |
| CX295       | 99.9  | 14.5 | 10 |      |

Fig. 1. Caspase-3 protein changes during development and 24 h after HI. Caspase-3 immunoblots are shown, demonstrating the following. A, pooled samples of parietal cortex from control animals, demonstrating the total levels of caspase-3 in newborn to adult animals (postnatal days 0–42). B, cortical tissue samples from three different animals all allowed to recover for 24 h after HI, demonstrating the variability in the model. At 24 h post-HI, the degradation of the caspase-3 proform, as well as the DEVD cleavage (not shown), is maximal. The apparent molecular weights of the proforms (33,000 and 31,000) and the proteolytically cleaved fragments (29,000 and 17,000) are indicated on the right. I and C, ipsilateral and contralateral hemispheres, respectively.

(2 μg), random primers (500 ng), and RNase-free water to 24 μl were incubated at 70 °C for 10 min. The mixture was chilled on ice, and 8 μl of 5× first strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl2), 4 μl of 0.1 mM DTT, and 2 μl of 10 mM each of dATP, dGTP, dCTP, and dTTP (Roche Molecular Biochemicals) were added and incubated at 25 °C for 10 min followed by 2 min at 42 °C. RT enzyme (2 μl (400 units)) was added, and the reaction was allowed to proceed for 60 min at 42 °C, followed by 15 min of inactivation at 70 °C. The template cDNA thus obtained was diluted to 100 μl with water and stored at −20 °C. Each subsequent PCR (25 μl) contained 4 μl of template cDNA, a 0.2 mM concentration of each of dATP, dGTP, dCTP, and dTTP (Roche Molecular Biochemicals), a 1 μM concentration of each primer, 1 unit of Taq DNA polymerase (Sigma), and 2.5 μl of 1× PCR buffer (Sigma). Primers were as follows: caspase-3 (GenBank[5] accession number U49930) 5′-TTTTGGAGAAGCAGGACGGCTG-3′ (upstream) and 5′-CACCGGATTCTTCTGTTTGCG-3′ (downstream); GAPDH (GenBank[5] accession number M17701) 5′-ACCAACTTGGAGGAGGACTGTCTG-3′ (upstream) and 5′-GGTTGGAGACCACTGGATTTAATC-3′ (downstream). All primers were from Roche (Stockholm, Sweden). PCR cycling for caspase-3 was as follows: step 1, 94 °C for 5 min; step 2, 24 cycles of 94 °C for 20 s, 62 °C for 20 s, 72 °C for 20 s; step 3, 72 °C for 5 min. PCR cycling for GAPDH was as follows: step 1, 94 °C for 5 min; step 2, 20 cycles of 94 °C for 20 s, 60 °C for 20 s, 72 °C for 20 s; step 3, 72 °C for 5 min. The annealing temperatures and cycle numbers were chosen such that both the caspase-3 and the GAPDH PCR products would be in the linear phase of amplification and of similar intensity (data not shown). The PCR products (412 bp for caspase-3 and 528 bp for GAPDH) were separated on 1.5% 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). The relative amount of caspase-3 mRNA was calculated after normalization to GAPDH, to compensate for errors introduced during the preparation of RNA, the production of cDNA, or the PCR.

Incubation of Endogenous Procaspase-3 with Calpains—Forebrain hemispheres of P7 control animals (n = 7) were homogenized in 10 volumes of 50 mM Tris-HCl (pH 7.3), 5 mM EDTA and centrifuged at 200,000 × g for 45 min to obtain cytosolic (S3) fractions. Aliquots of 100 μl of S3, 3 μl of 100 mM diethylthreitol, 9.0 μl of 100 mM CaCl2, 1.0 μl of 0.5 mM NaOH (to compensate for the drop in pH occurring when Ca2+ ions replace protons in the EDTA molecules), and 58.0 μl of homogenizing buffer were incubated for 30 min at 37 °C. Purified µ- or m-calpain, recombinant caspase-3, purified calpastatin (14 units/ml), calpastatin peptide (5 μg/ml), Sigma), CX295 (1 μM), ZVAD (0.7 μM), or BAF (0.88 μM) was included in some incubations, replacing partly the homogenizing buffer. When inhibitors were added, they were preincubated for 10 min with the enzymes at room temperature before being added to the S3 mixture. The reactions were stopped by adding 8.0 μl of 100 mM EDTA. Aliquots of 50 μl were assayed for DEVD cleavage, and portions equivalent to 90 μg of total protein were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting. The protein concentrations were determined according to Whitaker and Granum (53), adapted for microplates, using a Spectramax Plus plate reader (Molecular Devices). Crude calpastatin (100 units/ml) was purified from rabbit lung via hydrophobic interaction chromatography, as previously described (48) and further purified via trichloroacetic acid precipitation and gel filtration (54).

Incubation of m-Calpain with Caspase-3—Aliquots (50 μl) of m-calpain were incubated for 15 min at room temperature under conditions where the enzyme was half-maximally activated (0.36 mM Ca2+). Increasing amounts of active caspase-3 (1–60 units per incubation, where 1 unit is defined as the amount of caspase-3 that will release 1.0 pmol of AMC/min/ml in the DEVD-cleaving assay described above) were included to see if caspase-3 could increase the m-calpain activity directly. Calpain activity was measured as described above.

Statistics—Student’s unpaired t test or analysis of variance with Scheffé’s post hoc test were used.

RESULTS

Caspase-3-like Activity—DEVD-cleaving activity was detectable in neonatal brain samples and increased several fold in the ipsilateral compared with the contralateral hemispheres, in accordance with earlier findings (9), and this increase displayed a maximum 24 h post-HI (not shown). The average ratio between the ipsi- and contralateral hemispheres was signifi-

Fig. 2. Correlation between the enzymatic activity and the presence of the 17 and 29 bands. A simple regression graph to demonstrate the correlation between the relative amounts of the 17- and 29-kDa bands, respectively, and the caspase-3-like activity (DEVD cleavage) in the same samples. The filled circles represent the 17/29 ratio (p = 0.011), and the open squares represent the 29/(29 + 32) ratio (p = 0.0003).

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significantly decreased after calpain inhibition, from 1618% in the vehicle-treated animals to 606.1% in the CX295-treated animals (p < 0.0004) (Table I).

Caspase-3 Protein—The H-277 antibody against caspase-3 displayed a distinct double band with apparent molecular weights of 33,000 and 31,000, respectively (Fig. 1). The amount of caspase-3 protein dropped sharply as the brain growth spurt leveled out, particularly the 33-kDa band, the total level being more than 85% lower at P42 than P7 (Fig. 1A). The 33-kDa band was also preferentially depleted after HI (Fig. 1B). From 2 h up to 72 h after HI, the ipsilateral hemispheres also displayed one or two additional bands with apparent molecular weights of ~29,000 and 17,000, respectively (not shown). These bands were never found in the contralateral hemispheres. The appearance of the 29- and 17-kDa bands occurred parallel to a decrease of the 33/31-kDa proform (called

|                    | Caspase-3 | m-Calpain | µ-Calpain |
|--------------------|-----------|-----------|-----------|
| CX295              | >1.65 mM  | 50 nM     | 50 nM     |
| ZVAD               | 60 μM     | 15 μM     | 15 μM     |
| BAF                | 60 μM     | >3.0 mM   | >3.0 mM   |

**TABLE II**

The IC₅₀ values for the protease inhibitors used

The inhibitor concentrations at which the proteases display half-maximal activity in vitro are shown. All of the inhibitors act on the active site cysteine but with quite different specificities. CX295 was tested up to 1650 μM for caspase-3, and BAF was tested up to 3000 μM for µ- and m-calpain without any detectable inhibitory effect.

**FIG. 3.** Caspase-3 mRNA changes during recovery after HI. Shown is the amount of caspase-3 mRNA, determined by reverse transcription-PCR, in pooled RNA (n = 6 for each time point). P7, P8, P10, and P21 indicate the postnatal day of control animals. The time of recovery after HI is indicated in hours (h) or days (d). Black columns represent the ipsilateral and the gray columns represent the contralateral hemispheres. Because the assay was carried out on pooled samples, statistical significance testing was not possible.

**FIG. 4.** Degradation of endogenous caspase-3 substrates after HI. Shown is the degradation of the three endogenous caspase-3 substrates, ICAD (A), PARP (B), and fodrin (C), on immunoblots of cortical homogenates from animals allowed to recover for 24 h post-HI, a time point when the caspase-3 activity was maximal. The contralateral (C) and ipsilateral (I) hemispheres are indicated. Both the long (45) and short (32 and 31) forms of ICAD were cleaved, but no specific degradation products could be seen (A). The 85-kDa breakdown product of PARP appeared in parallel with a decrease of the intact 116-kDa form (B). The 120-kDa breakdown product of fodrin is considered to be produced by caspase-3, whereas the 145- and 150-kDa bands are generated by calpains (C).

**FIG. 5.** Immunolocalization of caspase-3 and calpain activity in tissue sections. A, parallel tissue sections, stained with the antibodies against the FBDP produced by calpains or active caspase-3 (p17), from animals allowed to recover for 3 and 24 h, respectively. Staining was found almost exclusively in areas with tissue damage, as judged by the loss of MAP 2 (not shown). B, the number of cells immunopositive for FBDP or active caspase-3 was counted in a MAP 2-negative area and expressed as positive cells/mm² ± S.D. (n = 6 for each time point). The number of FBDP-positive cells was highest already at 3 h post-HI, whereas the number of cells positive for active caspase-3 increased during recovery and peaked at 24 h post-HI.
and short (32-kDa) forms of ICAD were degraded, but no specific degradation products could be detected using this antibody (Fig. 4A). The depletion of ICAD in the ipsilateral hemisphere led to a decreased ratio (long and short forms) between the ipsilateral and the contralateral hemispheres. This ratio was significantly increased after calpain inhibition, from 77.5% in the vehicle-treated animals to 99.9% in the CX295-treated animals ($p = 0.006$) (Table I). Depletion of the PARP 116-kDa band was paralleled by an increased 85-kDa band (Fig. 4B).

Degradation of fodrin produced the calpain-dependent 145- and 150-kDa as well as the caspase-dependent 120-kDa cleavage products (Fig. 4C).

**Caspase-3 and FBDP in Tissue Sections**—The caspase-3 p17 antibody was found to be specific for activated caspase-3 in tissue sections, and staining was found only in areas with tissue damage, as judged by the loss of MAP 2 staining and colocalization with DNA damage (6). The number of cells stained by the p17 antibody increased during recovery and was greater at 24 h than at 3 h post-HI (Fig. 5, A and B). The FBDP antibody also produced staining only in areas displaying loss of MAP 2, but it stained a larger number of cells during early (3 h) than late (24 h) recovery after HI (Fig. 5, A and B). Double labeling fluorescence microscopy revealed extensive colocalization of activated caspase-3 and FBDP during early recovery (Fig. 6).

**Activation of Caspase-3 by m-Calpain in Vitro**—Incubation of S3 fractions at 37°C for 30 min did not change the DEVD cleavage or the appearance on caspase-3 immunoblots (Figs. 7, A and B). When m-calpain was added, a 29-kDa band appeared, seemingly identical to the one seen after HI in vivo (Fig. 7C). This band did not appear when an equivalent amount of µ-calpain was used or when m-calpain was used together with calpastatin peptide, purified calpastatin, CX295, ZVAD, or BAF (Fig. 7A). During incubation, just like during HI in vivo, it was primarily the 33-kDa, rather than the 31-kDa, band of the proform that was detected (Figs. 1 and 7A). This was not changed by calpain inhibition (calpastatin peptide, purified calpastatin, or CX295) or by caspase inhibition alone (BAF) but could be prevented by simultaneous caspase and calpain inhibition (ZVAD) (Fig. 7A). The caspase-3-like activity increased 49% after incubation of S3 fractions from seven different hemispheres with m-calpain ($p < 0.0001, n = 7$), and this increase could be abolished when calpastatin peptide was included in the incubation (Fig. 7B). When µ-calpain was included in the incubation, the activity was not significantly increased ($p = 0.1081$) (Fig. 7B). When increasing amounts of recombinant, active caspase-3 were added to cytosolic fractions, the active caspase-3 added could be seen as a 17-kDa band on immunoblots, as expected (Fig. 8). However, an increasing amount of active caspase-3 also promoted the m-calpain-dependent formation of the 29-kDa band from the 32-kDa proform (Fig. 8). The appearance of the 29-kDa band was inhibited by including CX295 (Fig. 8). When increasing amounts of active caspase-3
were added to the S3/m-calpain mixture, the DEVD-cleaving activity increased more than when caspase-3 was added in the absence of m-calpain. This synergistic increase could be abolished by including CX295 in the incubation (Fig. 8). Caspase-3 could not activate m-calpain, because incubating m-calpain (half-maximally activated) with increasing amounts of caspase-3 did not alter the calpain activity (not shown).

Fig. 7. Increased caspase-3 activity and production of a 29-kDa fragment after incubation with m-calpain. A, caspase-3 immunoblot of a cytosolic fraction (S3) incubated with exogenous calpains (m or μ) and protease inhibitors as indicated. The first two lanes show S3 prior to (lane 1) and after (lane 2) incubation at 37 °C for 30 min. Inhibitors were as follows: calpastatin peptide (CP), calpastatin full-size protein (CS), CX295 (CX), ZVAD (Z), and BAF (B). B, the average change in caspase-3-like activity (DEVD cleavage) ± S.D. in the cytosolic fractions (S3) from seven different brain samples incubated with m-calpain (m), m-calpain + calpastatin peptide (m CP), or μ-calpain (μ), respectively. The value for the control samples is the average of the DEVD cleavage in the samples after incubation for 30 min at 37 °C, compared with the activity without incubation prior to the activity assay, demonstrating that the incubation does not change the activity (−1.7 ± 13.3%). ***, p < 0.0001; n.s., not significant, compared with S3 alone, using analysis of variance and Scheffe’s post hoc test (p = 0.31 and 0.11 for m-calpain + calpastatin peptide and μ-calpain, respectively). C, a caspase-3 immunoblot demonstrating that the 29-kDa band produced during hypoxia-ischemia (HI) in vivo has the same apparent molecular weight as that produced by incubating the proform of caspase-3 with m-calpain (S3). When the two samples were mixed (HI+S3) the 29-kDa band still appeared as a single, distinct band.

DISCUSSION

In Vitro—The cleavage of the proform from 32 to 29 kDa was clearly calpain-dependent, because it could be inhibited by calpastatin, and calpastatin does not inhibit any other known protease. Furthermore, m-calpain, but not μ-calpain, could perform this cleavage when equal amounts of the two isozymes were used. When larger amounts of m-calpain were used, however, limited cleavage, producing a 29-kDa band, could be seen (not shown). This is the only report to our knowledge demonstrating a functional difference in substrate specificity between the two ubiquitous calpains. Recent studies have demonstrated cleavage of caspase-3 by calpain, producing an ~45-kDa cleavage product (35, 37), but another report failed to demonstrate such cleavage (38). Wolf et al. (37) also identified the cleavage site in the prodomain of caspase-3. In these three reports, only μ-calpain was used, not m-calpain, which may explain why none of them could find any functional effects. One recent study demonstrated that calpains are able to cleave and inactivate caspase-7, -8, and-9, indicating that calpains may act as negative regulators of caspase processing (39). None of these studies used calpastatin to verify the specificity for calpain. Wolf et al. were the first to demonstrate that ZVAD inhibits calpains (37), which is important to bear in mind when using this drug as a caspase inhibitor. We found that CX295 was selective for calpains and that BAF was equally selective for caspases, which provided us with tools to discriminate between calpain and caspase activity. However, the effects on other cysteine proteases (e.g. cathepsins) have not been investigated. Because purified caspase-3 proform was not available to us (the recombinant caspase-3 used is activated immediately upon synthe-
sis), we used the cytosolic fraction from P7 control brains, containing large amounts of this zymogen. This means that nuclei, mitochondria, and cellular membranes were eliminated, but the possibility cannot be excluded that additional cofactors may have been present, influencing the processes studied. The 32-kDa proform consisted of a distinct double band on immunoblots (33 and 31 kDa), and it is noteworthy that it was primarily the 33-kDa band that was depleted during HI or in vitro incubations. This indicates that there are at least two subpopulations of the caspase-3 proform and that the recruitment of proform to active form is different for these two proforms in these paradigms. It is not known whether the two proforms are the result of pre- or posttranslational modification. The caspase-3-like activity (DEVD cleavage) was positively correlated with the formation of both the 29- and the 17-kDa bands, indicating that the 29-kDa band may be an intermediate form of the process of forming the two active subunits. The finding that m-calpain-induced production of this 29-kDa form enhanced caspase-3 activity indicates that the further two, caspase-dependent cleavages necessary to form the active forms may be facilitated by this initial cleavage in the prodomain. This was further supported by the finding that both m-calpain and caspase-3 had to be active to achieve this effect, i.e. that the initial m-calpain-dependent formation of the 29-kDa form was followed by caspase-dependent cleavage into the active subunits. It was excluded that the effect observed was due to caspase-mediated activation of m-calpain, because the activity of m-calpain incubated under conditions where the activity was half-maximal could not be changed by the addition of active caspase-3 (not shown). The synergism was further demonstrated by showing that a higher amount of caspase-3, with a constant amount of m-calpain, enhanced the calpain-dependent formation of the 29-kDa band, as well as the caspase activity, more than when caspase-3 alone was added (Fig. 8).

**In Vivo**—The sequential activation of first calpain and then caspase-3 after HI was demonstrated to occur in the same cells and in only areas displaying loss of MAP 2, indicative of tissue damage (Figs. 5 and 6). This provides us with an attractive model of calcium-dependent enhancement of caspase-3 activation during the second phase of neuronal degeneration. It has been shown earlier that calpain activation after ischemia occurs in two phases, an early phase immediately following the insult and a second, more extensive phase coinciding with cellular degeneration (21, 55). This biphasic degradation of fodrin was less obvious in our model (56), but the degradation of calpastatin was clearly biphasic (45). It is not clear if calpains or caspase-3, or both, were responsible for the calpastatin cleavage, because both enzymes have been demonstrated to cleave this protein (32, 33). The caspase-3 activity in control animals was low, and it is possible that this enhanced activation of caspase-3 after HI is essentially different from that occurring during developmentally regulated apoptosis. There are numerous reports of increased apoptosis-related parameters (where caspase-3 holds a pivotal position) detected after cerebral ischemia (e.g. Refs. 7, 8, and 10–12) as well as reports of neuroprotection after administration of caspase inhibitors (e.g. Refs. 9 and 14–16), but the present results indicate that these may be, at least partly, secondary to calcium-dependent events occurring early after the insult, thereby offering a possible link between Ca\(^{2+}\) dysregulation and "pathological apoptosis." This is supported by our finding that the NMDA receptor antagonist MK-801 attenuated caspase-3 activation after neonatal HI (57). Furthermore, recent studies have demonstrated that Ca\(^{2+}\) dysregulation following endoplasmic reticulum stress can lead to the activation of caspase-12 (58), possibly mediated by m-calpain (41).

Hu et al. (59) found that 90% of damaged cortical neurons were immunopositive for active caspase-3 in P7 rats after HI, but only 1% were immunopositive in P60 animals. Ni et al. (13) found high levels of caspase-3 mRNA in the fetal and neonatal brain but low levels in the adult brain. Chen et al. (11) also reported low levels of caspase-3 mRNA and protein in the adult brain. Both of these reports demonstrated an up-regulation in the hippocampus after 24–72 h of reperfusion after focal ischemia in the adult brain. Our data support these findings, with decreasing amounts of caspase-3 mRNA and protein during normal development, but neither the mRNA nor the protein were found to be substantially up-regulated after HI in the immature brain. The constitutive expression of both calpains (60) and caspase-3 is high in the developing brain, indicating that these proteases may be particularly important targets for neuroprotective strategies in the perinatal setting.

In summary, this is the first report to our knowledge demonstrating a functional difference in the substrate specificities of the two ubiquitous calpains (\(\mu\)- and m-calpain) as well as facilitated activation of caspase-3 by m-calpain. Caspase-3 has been identified as a key protease in the execution of apoptosis, whereas calpains have mainly been implicated in excitotoxic neuronal injury. Our data suggest a direct link between the early, excitotoxic, calcium-mediated activation of calpains after cerebral HI and the subsequent activation of caspase-3, thus representing a tentative pathway of "pathological apoptosis." This mechanism should be more important in the immature brain because of the high levels of both calpains and caspase-3 during development.

**Acknowledgments**—We are very grateful to Dr. Donald W. Nicholson (The Merck Frosst Center for Therapeutic Research, Quebec, Canada) for supplying the antibody against active caspase-3 and to Dr. Xiaodong Wang (University of Texas Southwestern Medical Center) for supplying the antibody against ICAD/DPFF45.

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Synergistic Activation of Caspase-3 by m-Calpain after Neonatal Hypoxia-Ischemia: A MECHANISM OF "PATHOLOGICAL APOPTOSIS"?
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J. Biol. Chem. 2001, 276:10191-10198.
doi: 10.1074/jbc.M007807200 originally published online December 21, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M007807200

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