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Effects of cathepsins B and L inhibition on postischemic protein alterations in the brain

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Abstract

The effects of selective inhibition of cathepsins B and L on postischemic protein alterations in the brain were investigated in a rat model of middle cerebral artery occlusion (MCAO). Cathepsin B activity increased predominantly in the subcortical region of the ischemic hemisphere where the levels of collapsing mediator response protein 2, heat shock cognate 70 kDa protein, 60 kDa heat shock protein, protein disulfide isomerase A3 and albumin, were found to be significantly elevated. Postischemic treatment with Cbz-Phe-Ser(OBzl)-CHN_2, cysteine protease inhibitor 1 (CP-1), reduced infarct volume, neurological deficits and cathepsin B activity as well as the amount of heat shock proteins and albumin found in the brain. Our data strongly suggests that the decrease in heat shock protein levels and the significant reduction of serum albumin leakage into the brain following acute treatment with CP-1 is indicative of less secondary ischemic damage, which ultimately, is related to less cerebral tissue loss and improved neurological recovery of the animals.

Keywords: Albumin; Cathepsin B; Middle cerebral artery occlusion; Neuroprotection; Protease inhibitor; Rat

Stroke is the most significant neurological disease in the world killing 5.5 million people per year and leaving many of the surviving victims with long term disability [1,2]. Despite its public health significance, the pharmacological treatment of acute stroke is currently limited to clot thrombolysis with intravenous tissue plasminogen activator within 3 h of onset of symptoms. Since a small percentage (<5%) of total stroke patients receive this treatment [3], a more efficacious medical treatment of stroke clearly needs to be developed. Pharmacologic intervention to prevent the death of neurons under the stress of reduced blood supply represents a strategy for treatment of ischemic stroke which could augment the effects of reestablishing blood flow with thrombolysis. Targets for cerebral protection include the enzymatic cascades activated after the onset of neuronal ischemia [4–7]. Numerous biochemical pathways which lead to cellular death have been detailed. In recent years interconnections joining these pathways have become apparent, giving the opportunity to target proteins which can break the chain of reactions leading to early and delayed post ischemic neuronal death. Such a critical pathway is the rapid activation of cysteine proteases including the cathepsins, calpains, and caspases [4–8]. Cathepsins B and L contribute to cerebral injury after focal ischemia with reperfusion [9–13]. Previously, we demonstrated that

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CP-1, a non-toxic cysteine protease inhibitor which is relatively selective for cathepsins B and L, but not the calpains or caspases, is effective at reducing infarct volume and improving functional scores when administered intravenously to rats after 2 h of MCAO and reperfusion [12].

In the present study, the effects of CP-1 on cathepsin B activity and postischemic protein alterations in the brain were investigated. This information may facilitate the identification of protein alterations associated with cathepsin-mediated neuronal injury and provide important insights into the mechanism of CP-1 action.

Materials and methods

Animal model. Male Wistar rats (270–290 g) obtained from Charles River Breeding Co. (Wilmington, MA, USA) were used in the animal experiments and all procedures had been pre-approved by the Institutional Animal Care and Use Committee at Henry Ford Hospital (Protocol # 0655). Two-hour MCAO in rats by the intravascular suture method, drug infusion, measurement of infarct volume and neurological deficits were performed as previously described [12]. CP-1 was synthesized according to Shaw et al. [14]. For studies on cathepsin B inhibition and protein alterations in the brain, the animals were sacrificed after 48 h of survival. At that time, the brains were extracted and separated into right and left, cortical and subcortical regions, immediately frozen in liquid nitrogen and stored at −80 °C.

Preparation of rat brain protein extract. Brain tissue samples collected after the MCAO experiment were minced on dry ice, suspended in cold homogenization buffer (0.25 M sucrose, 1 mM EDTA, and 25 mM 2-(*N*-morpholino)-ethanesulfonic acid, pH 6.5), and homogenized in a Potter–Elvehjem tissue homogenizer. The homogenates were centrifuged at 4 °C for 20 min at 16,000*g*. Aliquots of the supernatants were stored at −80 °C until used for experiments. Protein concentrations were determined using the micro BCA Protein Assay kit (Pierce, Rockford, Ill).

Assay for cathepsin B activity. Cathepsin B activity was determined fluorometrically in an SLM Aminco 8000 fluorimeter (ThermoFisherScientific, Waltham, MA) using Cbz-Arg-Arg-aminomethylcoumarin (AMC) (Calbiochem, San Diego, CA) as substrate. Briefly, 4 μL of rat brain extract were mixed with 1200 μL of the assay buffer (25 mM PIPES, 0.337 mM Na_2HPO_4, 0.441 mM KH_2PO_4, 137 mM NaCl, 5.365 mM α-D-glucose, and 2 mM L-cysteine, 2.5 mM dithiothreitol, pH 7.4) containing 100 μM Cbz-Arg-Arg-AMC. The fluorescence signal emitted by the hydrolyzed AMC fluorophore was monitored at 460 nm with excitation at 370 nm. The reaction solution with or without purified human liver cathepsin B (200 U/mg protein; Calbiochem, San Diego, CA) were used as controls. Specific activity was determined as μmoles of AMC released per min per mg of protein.

Sodium dodecyl sulfate gel electrophoresis (SDS–PAGE) and Western blot analysis. SDS–PAGE was carried out according to Laemmli [15]. Immunoblots were performed according to the technique of Towbin et al. [16]. Rabbit polyclonal anti-collapsin response mediator protein 2 (CRMP-2), rat monoclonal anti-heat shock cognate 70 kDa protein (Hsc70), sheep polyclonal anti-rat serum albumin (RSA) and horseradish peroxidase (HRP)-conjugated donkey anti-sheep antibodies were purchased from Abcam (Cambridge, MA). HRP-conjugated goat anti-rabbit and -mouse IgGs were from Chemicon (Temecula, CA). HRP-conjugated goat anti-rat was purchased from Jackson ImmunoResearch (West Grove, PA). A monoclonal antibody to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained from Imgenex (San Diego, CA). Labeled proteins were detected with the enhanced chemiluminescence (ECL) method according to the manufacturer's instructions (GE Healthcare Biosciences, Piscataway, NJ, USA). Bands were quantified by densitometry using GelDoc 2000 (Bio-Rad, Richmond, VA) and the free open source software ImageJ (http://rsb.info.nih.gov/ij/).

Two-dimensional high performance chromatofocusing (HPCF)/SDS–PAGE. Brain proteins were separated on an HPCF column (2.1 × 250 mm) with a Beckman ProteomeLab PF2D System (Beckman-Coulter, Fullerton, CA) followed by high-throughput SDS–PAGE of the post HPCF fractions using an E-PAGE™ 96 System (Invitrogen, Carlsbad, CA). Two chromatofocusing buffers, Equilibration/Start Buffer (SB, pH 8.5) and Elution Buffer (EB, pH 4.0) (Beckman-Coulter), were used to generate a linear pH gradient at a flow rate of 0.2 ml/h. Proteins (3 mg) in SB were applied to the HPCF column and fractions were collected between pH 8.5 and 4.0 at 0.15-pH intervals. Aliquots (20 μl) of the post HPCF stroke and sham fractions, matched by pH-intervals, were analyzed on a pre-cast E-PAGE™ 96 6% gel according to the manufacturer's instructions. Each E-PAGE™ 96 gel contains 96 sample and 8 marker lanes in a patented staggered well format. After staining the gel with Coomassie® brilliant blue, the 2-D protein maps (pI/molecular weight) were compared to monitor changes in protein expression levels.

Mass spectrometry (MS). Protein bands with ⩾two-fold change in intensity (sham vs stroke) were excised from the Coomassie-stained gels. The gel slices were destained with 50% methanol and 10% acetic acid in deoinized water, and the proteins were treated with NH_4HCO_3, reduced with dithiothreitol, alkylated with iodoacetamide and trypsinized for 4 h at 37 °C with sequencing grade trypsin (Promega, Madison, WI) using an automated in-gel process (ProGest, Genomic Solutions, Ann Arbor, MI). Digests were analyzed by liquid chromatography (LC)/MS/MS using a Magic C18AQ reverse phase column (200 Å, 3 μm; 0.2 × 50 mm; Michrom Bioresources, Auburn, CA) and an LTQ-XL linear ion trap mass spectrometer (ThermoFinnigan, Waltham, MA). Data analyses were performed, using Bioworks and Scaffold software programs that utilize SEQUEST, X!Tandem and ProteinProphet algorithms. MS/MS spectra were searched against a rat protein database. Proteins with 100% probability were identified with at least 2 unique peptides, and as many as 8 (60 kDa heat shock protein). Scaffold calculates the probabilities for the peptide, the joint peptide probability from several search engines, and the protein probability separately for each MS sample.

Results

Infarct volume

Because our previous dose-response studies demonstrated that treatment with 50 μM CP-1 provided effective neuroprotection [12], a 50-μM solution of the inhibitor solution was given intravenously (15 μl/min) for 4 h immediately after reperfusion following 2 h of MCAO in the present study. Stroke-control animals ($n = 8$) had infusion of vehicle only. All animals survived 7 days, were sacrificed, and the stroke volumes calculated. Infarcted tissues were predominantly in the cortex of the lesioned hemisphere of the brain. No infarction was observed on the non-lesioned side and in sham-operated controls. Physiological parameters of temperature, blood pressure, O_2, PCO_2, and pH were similar among treatment and control groups (results not shown). As shown in Table 1, CP-1 significantly reduced infarct volume at 50 μM ($24 \pm 3.9\%$, percent hemispheric infarct volume ± SD; $P < 0.05$) compared to control ($35 \pm 3.2\%$, $n = 8$ per group; independent *t*-test). This study supports the hypothesis that cathepsin B and/or cathepsin L contribute to cerebral injury after focal cerebral ischemia with reperfusion.

Cathepsin B activity in vivo

Rat brain tissue homogenates from sham, stroke with or without CP-1 treatment groups were assayed for cathepsin

Table 1
Summary of data for infarct volume, cathepsin B activity and ~66 kDa protein levels in right cortex (RC), right subcortex (RSC), left cortex (LC), and left subcortex (LSC) of the animals

Group	Infarct volume ($n = 8$)	Cathepsin B activity (μmoles AMC/min/mg) ($n = 6$)	Relative intensity of ~66 kDa protein band ($n = 6$)
Stroke, untreated	$35 \pm 3.2\%$	LC = 0.14 ± 0.02, RC = 0.18 ± 0.04, LSC = 0.15 ± 0.02, RSC = 0.20 ± 0.03	RSC = 2.23 ± 0.26
Stroke, CP-1 treated	$24 \pm 3.9\%$	LC = 0.11 ± 0.01, RC = 0.11 ± 0.01, LSC = 0.08 ± 0.01, RSC = 0.11 ± 0.02	RSC = 1.60 ± 0.12
Sham-operated	0%	LC = 0.12 ± 0.01, RC = 0.12 ± 0.01, LSC = 0.10 ± 0.01, RSC = 0.12 ± 0.01	RSC = 1.20 ± 0.06
P	$P < 0.05$		

B activity as described in Materials and methods section. Cathepsin B activity was significantly higher ($47 \pm 2\%$ increase, $n = 6$) in the ischemic brain sections compared to sham samples (Table 1 and Fig. 1). When different regions of the brain were compared, it was found that the right subcortex (RSC) of the untreated ischemic brain had more cathepsin B activity than the right cortex (RC), left cortex (LC) and left subcortex (LSC). The animals that received CP-1 had significantly lower cathepsin B activity in all brain regions compared to animals that received vehicle only. Furthermore, it is interesting to note that the total cathepsin B activity measured in the drug treated ischemic brains was slightly lower than the total basal activity in the sham brains (Table 1 and Fig. 1). These results suggest that a 50 μM solution of CP-1, given at 0.9 ml/h for 4 h (1.28 ng/kg), is able to inactivate the cathepsin B activity that is generated in the rat brain after MCAO.

Protein alterations in the rat brain after MCAO

Our goal was to characterize the major postischemic protein alterations that are significantly modulated by cathepsins B and L inhibition in vivo. A significant increase in the level of a ~66-kDa protein was observed in the RSC

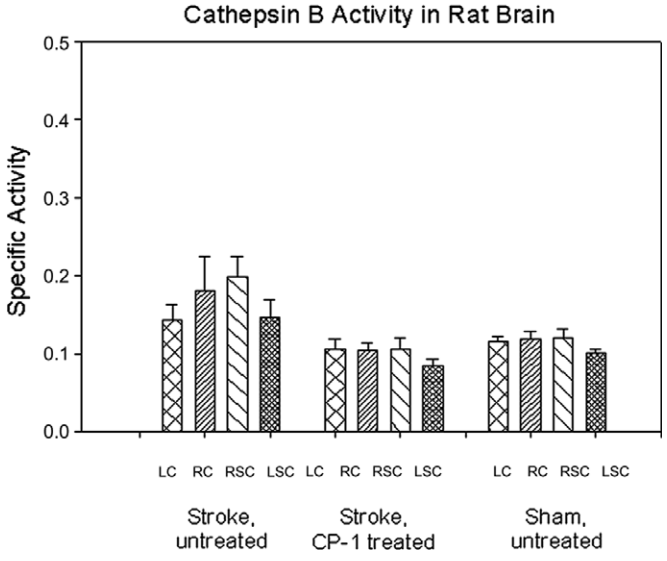

Fig. 1. Cathepsin B activity in the right cortex (RC), right subcortex (RSC), left cortex (LC) and left subcortex (LSC) of rat brain. Activity is expressed in units of enzyme activity per mg of soluble brain protein.

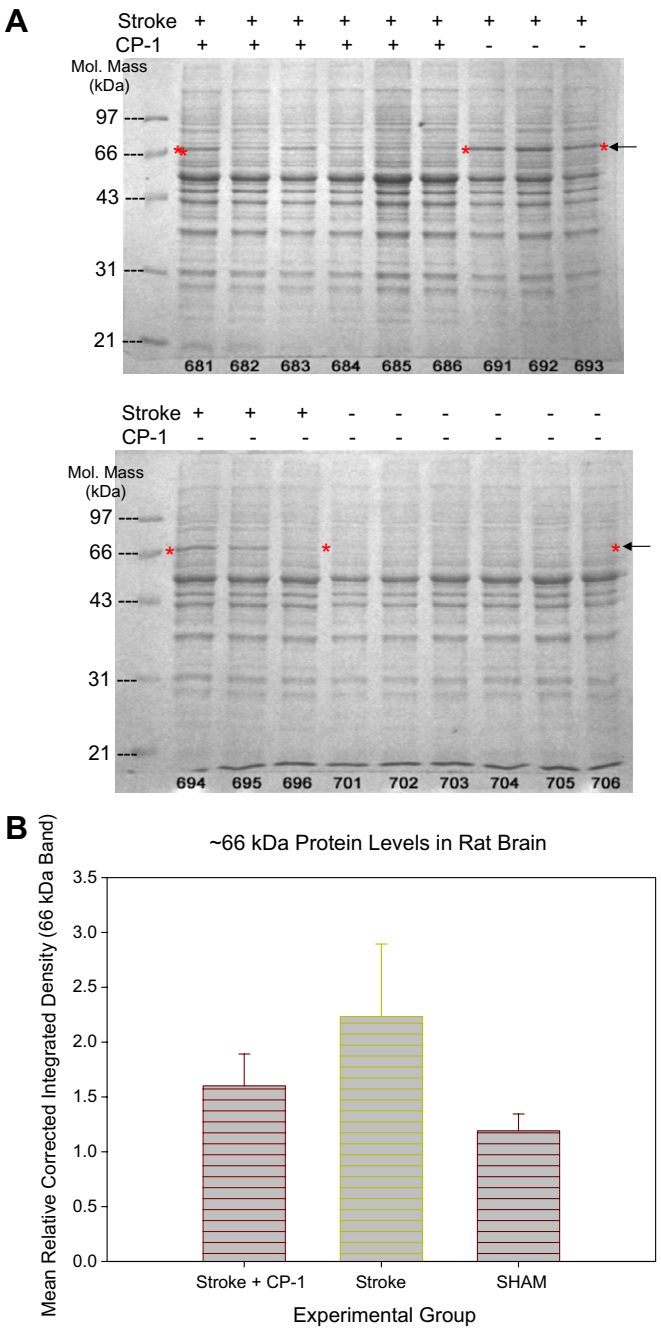

Fig. 2. (A) ~66 kDa protein levels (shown by arrow) in RSC of ischemic CP-1 treated (681–686), stroke-control (691–696), and sham-operated (701–706) animals. (B) Densitometric analysis of the ~66 kDa protein bands in RSC ($n = 6$).

of the ischemic brain compared to the RC, LC, and LSC of the same animal. The ~66 kDa protein levels in the untreated ischemic brain can be ranked in the following order: RSC >> RC > LSC ⩾ LC (see electrophorectogram in *supplementary material*). The ~66-kDa protein level was significantly reduced in the RSC of the ischemic animal that received CP-1. Sham-operated animals had negligible amounts of ~66-kDa protein in all regions of the brain.

Fig. 2A shows the protein profiles of the RSC only ($n = 6$). There was a significant increase in the level of ~66 kDa protein in the ischemic brain (rat #s 691–696) compared to the level in the sham operated rats (rat #s 701–706), which was negligible. Postischemic administration of CP-1 significantly reduced the ~66 kDa protein level (rat #s 681–686). A quantitative analysis of the ~66 kDa protein levels is shown in Fig. 2B.

Identification of rat brain proteins differentially expressed after MCA occlusion

HPCF/SDS–PAGE followed by LC/MS/MS identified dihydropyrimidinase-related protein 2, also known as collapsin-receptor mediator protein 2 (CRMP2), protein disulfide-isomerase A3 (PDI A3), heat shock cognate 71 kDa protein (HSC70), 60 kDa heat shock protein (HSP60) and rat serum albumin (RSA) as proteins in the 60–70 kDa range that were up-regulated in the ischemic brain compared to the sham samples.

The identity and levels of three of the differentially expressed proteins were further confirmed by Western blot analysis. As shown in Fig. 3A (lanes 1 and 2), CRMP-2 (left panel), HSC70 (middle panel), and RSA (right panel) levels increased significantly after ischemia. Postischemic treatment with CP-1 reduced the CRMP-2, HSC70 and RSA levels in the cerebral subcortex (lane 3). A densitometric quantification of the immuno-blots is shown in Fig. 3B. The results shown are for one animal per experimental group, and are representative of the protein profiles observed for the other animals in each group.

Discussion

To the best of our knowledge, the present study represents the first report on the effect of selective cathepsin B and L inhibition on postischemic protein alterations in the rat brain. Previously, we have shown that cathepsin B undergoes increased expression and activation in core neurons early after reperfusion following MCAO in the rat [11]. We now provide new data to show that the enzymatic activity of cathepsin B is elevated predominantly in the right subcortical region of the ischemic brain where the level of several ~66 kDa proteins were found to increase significantly after the ischemic insult. Intravenous administration of CP-1 following MCAO results in the inactivation of cathepsin B in the brain as well as significant reduction in infarct volume and neurological deficits [12, this study]. In addition to its postischemic neuroprotective effect, CP-1 decreased the levels of the ~66-kDa proteins which were upregulated in the periphery of the ischemic core.

The proteins identified in the ~66 kDa band were CRMP-2, PDI A3, Hsc70, Hsp60, and RSA. PDI A3, Hsc70, and Hsp60 are heat shock proteins involved in cellular repair, recovery and survival [17–21] whereas CRMP-2 performs functions related to neurite outgrowth [17,22–25]. The RSA found in the brain extracts could result from contamination of the samples by vascular contents which are unavoidably included in the samples. However, damage to the blood–brain-barrier (BBB) may enhance movement

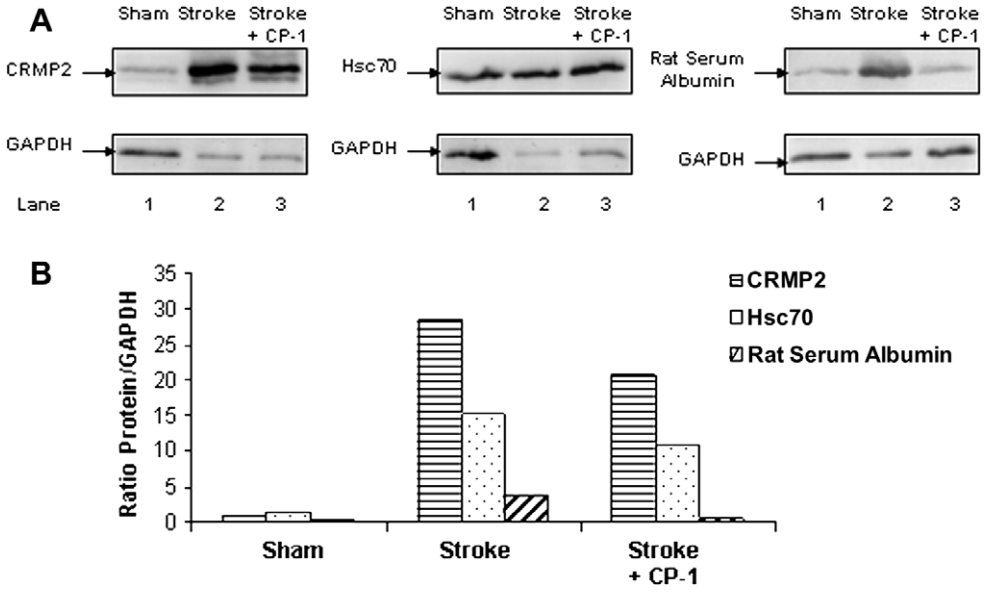

Fig. 3. (A) Western blot analysis for CRMP2 (left panel), HSC70 (middle panel) and RSA (right panel) levels in the RSC of sham-operated (lane 1), ischemic-control (lane 2) and ischemic CP-1 treated (lane 3) rat brains. (B) Densitometric analysis of immunoblots.

of proteins between brain and blood in either direction [26]. Western blot analysis showed that the amount of RSA in the right cerebral subcortex of the stroke-control animals was significantly higher than that found in the same brain region of the CP-1-treated ischemic and sham-operated animals. Therefore, a major portion of the RSA found in the brain may have its origin as a component of extravasated plasma due to damage to the BBB following cerebral ischemia [27,28]. Serum albumin has been examined in CSF and serum/plasma as a marker of BBB integrity with variable findings [26,29]. While CRMP-2 and the heat shock related proteins may participate in a protective response in the vicinity of the ischemic core at 24 h after injury, it is likely that the amount of RSA measured at 48 h in post-ischemic brain samples correlates with the severity of secondary cerebral injury. The reduced levels of CRMP-2, heat shock proteins and RSA at 48 h following acute treatment with CP-1 in this study is indicative of less secondary damage after MCAO, which ultimately, is related to less cerebral tissue loss and improved neurological recovery of the animals. Specific inhibition of cathepsins B and L acutely after ischemia/reperfusion probably blocked some of the early effects of focal ischemia that contribute to BBB damage and severity of vasogenic edema.

A number of proteomic studies have been published in recent years that investigated alterations in protein expression relevant to acute and chronic CNS diseases [30–36]. However, out of the two stroke-related proteomics studies published, there was only one that examined protein alterations in the brain after experimental stroke [17]. The other study investigated the proteome of human brain microdialysates obtained from the unlesioned contralateral side of three stroke patients [36]. Further, none of the studies examined the effect of a pharmacological agent on the protein alterations observed in specific regions of the brain following focal cerebral ischemia. Understanding the mechanisms of action temporally of neuroprotectants such as selective cathepsin B and L inhibitors is critical for their effective and eventual therapeutic application, since the pathways in which they are involved may serve different functions at different times after ischemia. Within the initial hours after ischemic injury, the cysteine protease pathways may be destructive, whereas, beyond several hours, but within 24 h, the pathways set in motion by ischemia (heat shock proteins and CRMP) may participate in recovery of cells that survived the initial period of necrosis and early apoptotic cell death [22–25]. Early neuroprotection followed by subacute neurorestorative therapies may optimize the effective treatment strategies for ischemic stroke.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.11.104.

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