GENE DELETION OF CYSTATIN C AGGRAVATES BRAIN DAMAGE FOLLOWING FOCAL ISCHEMIA BUT MITIGATES THE NEURONAL INJURY AFTER GLOBAL ISCHEMIA IN THE MOUSE

T. OLSSON, a, J. NYGREN, a K. HÅKANSSON, b C. LUNDBLAD, c A. GRUBB, b M.-L. SMITH a AND T. WIELOCH a

aLaboratory for Experimental Brain Research, Wallenberg Neuroscience Center, Lund University, BMC A13, SE-221 84 Lund, Sweden
bDepartment of Clinical Chemistry, Institute of Laboratory Medicine, Lund University, SE-221 85 Lund, Sweden
cDepartment of Physiological Sciences, Lund University, SE-221 84 Lund, Sweden

Abstract—Cystatin C is distributed in all human tissues and fluids with a particular abundance in the cerebrospinal fluid. Cystatin C is a strong endogenous inhibitor of lysosomal cysteine proteases, such as cathepsin B, L, H and S, that are involved in various biological processes such as degradation of cellular proteins and regulation of enzymes, as well as in pathological processes. Pharmacological inhibition of cathepsins has been shown to reduce neuronal damage after brain ischemia, suggesting that cystatin C is an endogenous neuroprotectant. Cystatin C has also amyloidogenic properties and is co-localized with β-amyloid in degenerated neurons in Alzheimer’s disease, suggesting a role in neuronal degeneration. To test the hypothesis that endogenous cystatin C is neuroprotective during brain ischemia, global and focal brain ischemia was induced in mice with the cystatin C gene knocked out. Following focal ischemia, larger brain infarcts were found in cystatin C knockout mice, probably due to a reduced inhibition of the cathepsins during ischemia. In contrast, brain damage after global ischemia was diminished in cystatin C knockout mice, suggesting that cystatin C has an aggravating effect on selective neuronal damage after global ischemia. © 2004 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: cathepsin B, protein aggregation, hippocampus, delayed neuronal death, inflammation, cysteine protease.

Cystatin C (CC) is a low molecular weight (13 kDa) protein with 120 amino acids containing two internal disulphide bridges, which belongs to family two of the cystatin super-family (extracellular and/or transcellular cystatins). CC is distributed in all human biological fluids and tissues (Abrahamson et al., 1986, 1990; Barrett, 1986; Grubb et al., 1983; Lofberg and Grubb, 1979), and is particularly abundant in the cerebrospinal fluid (Abrahamson et al., 1986). Although CC is mainly found in the extracellular space, there is also indication for an intracellular localization (Pierre and Mellman, 1998). CC is expressed in the brain of rat, monkey and man and is localized predominantly in astrocytes but also in some neurons (Yasuhara et al., 1993), such as the CA1 pyramidal neurons in the gerbil hippocampus (Ishimaru et al., 1996).

CC is a potent endogenous inhibitor of the cysteine proteases, including cathepsin B, L, H and S, that are implicated in various biological processes such as degradation of cellular proteins and regulation of pro-enzymes and pro-hormones (Marks et al., 1986; Taugner et al., 1985). However, CC is also implicated in other cellular functions, unrelated to its protease inhibition, such as regulation of the phagocytic activity and chemotactic response of polymorphonuclear neutrophils (Leung-Tack et al., 1990a,b), and in up-regulation of nitric oxide release from activated macrophages (Verdot et al., 1996). Also, the glycosylated form of CC is involved in neuronal stem cell proliferation (Taupin et al., 2000). Furthermore, in Alzheimer’s disease, CC accumulates in degenerated neurons together with aggregates of β-amyloid (Levy et al., 2001), and its role as a neuroprotectant or mediator of neurodegeneration has been discussed (Deng et al., 2001). CC is upregulated in degenerated neurons in the hippocampal subregion CA1 after global ischemia in the rat (Palm et al., 1995) suggesting a role in the neuronal death after cerebral ischemia.

Release of lysosomal hydrolytic enzymes, such as cathepsins, have been suggested to be important cytotoxic components in brain ischemic injury (Kohda et al., 1996). Cathepsin B is up-regulated in the primate hippocampus 3–5 days after global ischemia and postischemic treatment with CA-074, a specific cathepsin B inhibitor, and E-64c, an inhibitor of both cathepsin B and L, conferred neuroprotection to the hippocampal subregion CA1 (Tsuchiya et al., 1999; Yamashima et al., 1998; Yoshida et al., 2002). Following focal brain ischemia, induced by occlusion of the middle cerebral artery, the cysteine protease inhibitors stefin A and CP-1 decreased infarct size in the rat (Seyfried et al., 1997, 2001). As a potent cysteine protease inhibitor, CC may therefore serve as an endogenous neuroprotectant. Yet, the role of endogenous CC in cerebral ischemia and neuronal death has not previously been investigated. In light of these results, our aim was to investigate the neuroprotective effect of endogenous CC after cerebral ischemia. We used a mouse strain with a knocked-out CC gene (Huh et al., 1999) in models of transient focal and global ischemia.
**EXPERIMENTAL PROCEDURES**

**Animals**

Male mice, C57BL/6J/DBA of a mixed background strain with a deficient gene for CC, described previously (Hüh et al., 1999), were used. CC knockout (ko) and wild-type (wt) animals were not littermates, but generated from two separate lines bred in parallel. The gene for the agouti color is co-localized with CC ko and therefore ko animals were agouti colored and wt mice were black. Animals used for the experiments were 2–6 months old, and housed under diurnal light conditions with free access to food and water before surgery. All experiments were approved by the Malmo/Lund animal ethics committee and were handled according to the animal protection act of the Swedish Government, and the European Communities Council Directive. Efforts were made to limit the number of animals used and to minimize their suffering. The total number of animals used for global ischemia were ko, n=23 and wt, n=19; and for focal ischemia studies with immunohistochemical analysis; ko, n=10 and wt, n=6 and 2,3,5-triphenyltetrazolium chloride (TTC) staining analysis; ko, n=13 and wt, n=10.

**Immunohistochemistry**

CC ko and wt animals were killed by carbon dioxide asphyxiation. After cutting the cranium open, the brain was carefully extracted and fixed at +4 °C for 4 h in 4% formaldehyde in 0.1 M phosphate buffer at pH 7.4 and further processed for immunohistochemistry. Sagittal cryostat sectioning (12 μm) and immunolabeling were performed with standard procedures.

The monospecific polyclonal rabbit antisera used were raised against CC isolated from human urine (Löfberg and Grubb, 1979). It is known to cross-react with mouse and rat CC (Hakansson et al., 1996). The same antiserum has earlier been carefully validated for detection of human CC (Löfberg, 1982).

Fluorescein isothiocyanate-conjugated secondary antibodies, raised against rabbit IgG were used (Southern Biotechnology Associates, Inc., Birmingham, AL, USA).

Labeling control experiments included using different secondary antibodies, omitting the primary antibodies, and analysis of unlabeled sections for identifying possibly confounding autofluorescence were performed.

The specimens were examined using a Nikon Diaphot 300/Bio-Rad MRC1024 (Nikon Corporation, Japan; Bio-Rad Laboratories, USA) confocal laser-scanning microscope. Images were viewed and processed using Confocal Assistant (copyright Todd Clark Brej) and Adobe Photoshop (Adobe Systems, Mountain View, CA, USA).

**Quantification of CC by enzyme-linked immunosorbent assay (ELISA)**

A previously described ELISA for detection of mouse CC was applied (Håkansson et al., 1996). Briefly, brains from CC ko and wt mice were removed and the tissues homogenized with a Teflon-glass homogenizer in 50 mM Tris-buffer, pH 7.4, containing 0.15 M NaCl, 5 mM benzamidinium chloride, 10 mM EDTA, and 0.1% (w/v) NaN3. Supernatants from homogenates were diluted to suitable concentrations and applied to a microtiter plate (Maxi-Sorp; Nunc, Copenhagen, Denmark) coated with specific antibodies against mouse CC. Sample or calibrator solution was added to each well. After washing, biotinylated specific antibodies for CC were added and incubated for 1 h followed by washing and addition of streptavidin–horseradish peroxidase conjugate (Amerham). After washing, a substrate mixture of 2,2-azino-di-(3-ethylbensothiazolin-sulfonate) and H2O2 was added to the plates. The absorption at 405 nm was measured with a Titerek multiscan spectrophotometer. For measurement of total protein concentration a dye-binding assay was used (Håkansson et al., 1996).

**Focal cerebral ischemia**

For focal ischemia studies, a modified model of Hara et al. (1996) was applied. Anesthesia was induced by 2.5% halothane in N2O:O2 (70:30) and continued with 1.5% halothane through an inhalation mask during surgery. Body temperature was maintained at 36–38 °C by the means of a heating pad and a lamp. The skin over the right hemisphere was removed and a flexible optical filament (Probe 318-I; Perimed, Stockholm, Sweden) was fixed by instant glue (Locitite 415 glue and Locitite Tak Pak accelerator 7452) on the skull at the edge of the lateral bone at 2 mm posterior from bregma. The optical filament was attached to a laser Doppler flowmeter (PeriFlux System 5000; Perimed) to later allow measurement of the reduction in cortical cerebral blood flow (CBF) upon arterial occlusion.

A silicon-coated filament (6-0 nylon filament) was introduced into the internal carotid artery through an incision on the external carotid artery. The filament was advanced to block the origin of the middle cerebral artery (MCA), where the placement was confirmed by a reduction in laser Doppler flow. Halothane concentration was lowered to 0.8% during the 40 min period of occlusion.

Following the occlusion period, reperfusion was accomplished by withdrawing the filament. Only mice with an immediate reduction in laser Doppler flow upon occlusion and with reperfusion after occluder removal were included in the study.

After surgery mice were placed in an incubator for 2 h in 35 °C and given 0.5 ml of 5% glucose in phosphate-buffered saline s.c. Overnight the temperature in the incubator was set to 30 °C. The following day animals received additional glucose and were transferred to room temperature. The body temperature was checked at 1 h, 2 h, 24 h and 48 h after reperfusion.

**Assessment of infarct volume with TTC staining**

After 48 h of reperfusion, the animals were anesthetized in 5% halothane, and killed by decapitation. The brains were quickly removed and chilled in ice cold saline for 10 min. Then the brain was placed in a mouse brain-formed matrix. Coronal slices were cut in nine subsequent sections of 1 mm. Slices were incubated for 30 min at 37 °C in saline solution containing 1.0% TTC (Merck; Bederson et al., 1986) for staining of viable mitochondria and thereby surviving brain cells. After TTC staining slices were fixed in 4.0% phosphate-buffered formalin solution. Pictures of fixed slices were digitalized with a CCD camera and the areas of undamaged tissue (TTC stained) in striatum and cortex was calculated using the software National Institutes of Health Image 1.61 (USA). The volume of the infarcted brain area was obtained by first subtracting the undamaged tissue in the hemisphere isplateral to the occlusion, from the uninjured contra-lateral hemisphere at each sectioned brain level and then summing up the areas along the rostro-caudal brain axis.

**Infarction assessment with immunohistochemistry**

Four days after reperfusion, mice were anesthetized with 5% halothane and perfusion fixed in 4% cold phosphate buffered paraformaldehyde (PA) after a short rinse of saline. The brains were removed and postfixated in PA for 24 h followed by saturation in 25% sucrose for at least 24 h. Free floating coronal sections of 40 μm were cut with a distance of 560 μm between each section.

The sections where stained by immunohistochemistry for a neuronal-specific antigen (Neu N) for infarction evaluation. The sections were rinsed in buffer, and 3% H2O2 and 10% MeOH were applied to quench endogenous peroxidase activity. After preincubation with normal horse serum, sections were incubated with
mouse monoclonal Neu N antibody (1:100; Chemicon) overnight at 4 °C. Next day the sections were rinsed and incubated with secondary biotinylated horse anti-mouse antibody (1:200; Vector) and thereafter reacted with avidin–biotin–peroxidase complex (ABC kit; Vector). DAB staining was developed in a nickel intensified peroxidase reaction and infarction was evaluated by the means of a stereological microscope and software (Olympus BX51, Cast2) at ×10 magnification. Infarcted tissue did not contain any Neu N-stained neurons, and was outlined manually in the computer. The infarct volume was calculated similarly as for the TTC stained sections.

Global cerebral ischemia

The global ischemia model has previously been described in detail (Olsson et al., 2003). Briefly, mice were anesthetized in 2.5% halothane (70% N2O and 30% O2), intubated and connected to a small animal ventilator (model SAR-830-P; CWE, Ardmore, PA, USA) and anesthesia was maintained using 1.0% halothane. Muscle relaxant, 0.25 ml 0.04 mg/ml i.p. vecuronium bromide (Norcuron), was given to inhibit spontaneous breathing movements. The pCO2 from expired air was monitored constantly from 15 min before ischemia until 5 min after the end of ischemia by a capnometer (Cap star 100; Columbus Inst., Columbus, OH, USA). Minute ventilation was adjusted to obtain an end-tidal pCO2 of 1.8%, which in pilot studies corresponds to an arterial pCO2 of 35–40 mmHg in arterial blood. Both common carotid arteries were exposed and encircled loosely with a 4/0 silk thread to enable later occlusion with a microvascular clip. Two flexible optical filaments were fixed to the exposed skull bone above each hemisphere with instant glue, 1 mm caudal to bregma and 4 mm lateral to the midline, and connected to a two-channel laser Doppler flowmeter (Probe 318-I; Periflux System 5000; Perimed) for measurement of cortical CBF.

The cortical CBF was recorded continuously from at least 15 min before ischemia until 5 min after the end of ischemia. Changes in cortical CBF during ischemia (1 and 2 min after occlusion) and 1 and 5 min after start of reperfusion, was compared between groups. The level of cortical CBF during the ischemic episode and in early reperfusion are calculated as percent of starting value (ischemia rCBF/preischemia rCBF)×100. Only hemispheres with a reduction in laser Doppler below 10% of baseline within 1 min after common carotid artery occlusion were regarded as ischemic and were included in the study.

Rectal temperature was monitored continuously before, during and after ischemia and was maintained at 37.2±0.2 °C by a homeothermic blanket (HB 101/2 LSI; Irina S.L., Barcelona, Spain). A customer-designed Plexiglas box with a continuous flow of humidified warm air was placed over the mouse head (heated respiratory humidifier MR 600; Fisher and Paykel Ltd., Auckland, New Zealand), and the brain temperature during ischemia was kept normothermic (37.1±0.1 °C, measured in pilot studies). A time period of 12 min occlusion of both common carotid arteries was used and anesthesia was discontinued 2 min before the end of ischemia. Five minutes after ischemia the laser Doppler probes were removed, and wounds on the skull and in the neck were sutured, and anesthesia discontinued.

Animals remained connected to the respirator until stable spontaneous respiration was established, usually 10–30 min after ischemia, and after disconnection the mice were placed in an incubator at 33.5 °C to maintain a body temperature of approximately 37 °C. The mice were extubated about 10 min after reperfusion. The animals were kept in the incubator for 24 h, and rectal temperature was monitored within the first 2 h after ischemia, then at least two more times during the first day and finally at 24 h of recovery before they were placed in room temperature.

Perfusion fixation and histological preparation

After 4 days of recovery (five ko and five wt mice were fixed on the 3rd day) the animals were anesthetized with 5% halothane in oxygen/nitrous oxide (30:70) and after thoracotomy, the animal was transcardially perfused with 4% buffered formaldehyde at a flow of 3–4 ml/min after a short rinse with saline. The brains were removed and stored at least 24 h in 4% phosphate-buffered formaldehyde at 4 °C before dehydration and embedding in paraffin. Five micrometer coronal sections were cut and stained with Celestine Blue/Acid Fuchsin for evaluation in light microscopy.

Evaluation of ischemic damage following global ischemia

The ischemia-induced neuronal damage to the hippocampus, cortex and thalamus was evaluated at three different coronal sections, 1.4, 1.7 and 2.0 mm caudal to bregma (Franklin and Paxinos, 1997). In the hippocampus, neurons in the entire CA1, CA3 and dentate gyrus in each section were counted, as were neurons in the barrel field in neocortex. Normal appearing neurons as well as neurons showing morphological features of ischemic cell death (shrunken cell bodies, eosinophilic cytoplasm and triangulated nuclei) were counted in hippocampus and cortex. The neuronal injury was calculated in percent of the total neuronal cell numbers of the structure in that section. Thalamic damage was evaluated the same sections as hippocampus and cortex using a 0–3 graded scale (0, no damage; 1=0–10%; 2=10–50%; 3=50–100% of the neurons damaged). Damage to striatum was evaluated in sections obtained at 0.6, 0.9 and 1.2 mm rostral to bregma using a 0–5 graded scale (0, no damage; 1=0–20%; 2=20–40%; 3=40–60%; 4=60–80%; 5=80–100% damaged neurons).

Data analysis

The statistic significances of differences in infarct volume between groups were assessed by the Mann-Whitney U test.

For global ischemia, neuronal damage was assessed in individual brain hemispheres (Olsson et al., 2003). The Mann-Whitney U test was used to compare differences in neuronal damage between two groups at a specific coronal level. Unpaired Student’s t-test was used to compare the physiological data and CBF between groups. The χ2 test was used for assessing differences in mortality between groups. A P-value of <0.05 was considered statistically significant.

RESULTS

Absence of CC protein in CC deficient mice

CC protein in brains from CC ko and wt mice was quantified by ELISA. Brain from wt animals (n=11) contained 280±11 ng CC/mg protein, while no measurable quantity of CC protein was found in CC ko animals (n=11). Similarly, using immunohistochemistry, CC protein could be found in all cortical neurons in wt (n=5), while no immunostaining was seen in CC ko mice (n=3; Fig. 1A, B).

Focal ischemia-induced damage increases in CC ko mice

Forty minutes of MCA occlusion led to infarction in both cortex and striatum. The infarct volume was significantly larger in CC ko mice, 33.6 mm3 (n=10, P<0.05) than in the wt animals, 25.5 mm3 (n=5; Fig. 2). Also, in another series of animals, a significantly larger infarct volume was seen in CC ko (n=8, 44% larger infarct) compared with wt
mice \((n/11005, P/11021.05)\) using TTC staining (data not shown). No difference in reduction of cortical blood flow (laser Doppler) upon occlusion (within 5 s) was found between CC ko \((41.4/11006.9.6\%\text{ of baseline})\) and wt animals \((43.0/11006.10.2\%\text{ of baseline})\). Also, in the reperfusion phase, \((5\text{ min of reperfusion})\) the recovery in cortical blood flow was similar in the two groups \((\text{wt, 122.0/11006.46.2%; ko, 124.7/11006.34.2\% of baseline})\). Body temperature at 1 and 2 h of reperfusion was also similar in the groups (data not shown). The mortality was 18% in both the CC ko and wt groups.

**DISCUSSION**

The inhibition of CC gene transcription and the lack of CC protein in the CC ko mice has earlier been demonstrated \((Huh et al., 1999)\), and we now confirm the absence of the CC protein in the brain by immunohistochemistry and ELISA. The absence of CC did not significantly affect cortical CBF, during and after ischemia.

We found that the effect of ischemia on neuronal death was opposite in the two ischemic models. Following occlusion of the MCA, infarct size increased in the CC ko mice compared with the wt animals. In contrast, after global ischemia, damage decreased in CC ko mice in cortical regions, CA3 and dentate gyrus. These two seemingly opposite effects of CC on ischemic brain damage indicates a complex role of CC in the process of ischemic cell death.

The increased infarct size after focal ischemia in CC ko is in accordance with the notion that activation of the cathepsins may contribute to cell death after ischemia. For example, inhibition of cathepsins with stefin A or CP-1 in the rat was protective \((Seyfried et al., 1997, 2001)\). The prolonged period of ischemia during transient MCA occlusion \((40\text{ min})\) could allow cathepsins to be released from their localization in lysosomes, where they function in the degradation of cellular constituents. It has been proposed that that the rise of intracellular calcium during ischemia, activates micro-calpain located at the lysosomal membranes which breaks down lysosomal membranes releasing cathepsins into the cytoplasm, causing degradation of cellular components \((Yamashima et al., 1996)\). In addition, since CC is mainly a secreted protein, the loss of cathepsin inhibition could lead to destruction of the extracellular matrix by cathepsins. Consequently, we suggest that the increased damage in CC ko mice is an effect of increased protease activity due to the absence of endogenous protease inhibition by CC.
In contrast, following global ischemia the effect was the opposite, and the loss of CC conferred less ischemic brain damage. This is also in contrast to what would be expected from the protease inhibitory function of CC, since pharmacological inhibition of cathepsins following global ischemia in primates is neuroprotective (Tsuchiya et al., 1999; Yamashima, 2000; Yamashima et al., 1998; Yoshida et al., 2002). The body temperature, known to influence brain damage (Boris-Moller et al., 1989; Olsson et al., 2003), was similar between the two experimental groups in recirculation phase 0–2 h, but for 2–8 h of reperfusion, the body temperature was 0.7–0.9 °C lower in the ko mice. It is less likely that this small temperature change would affect cell damage. Furthermore, we cannot exclude that the deletion of the CC gene does not affect the maturation time of the cell death process, and that if the animals were allowed to recover for a longer time than 4 days, the difference would have been obliterated.

The deactivation of a gene, e.g. that of CC, may activate compensatory mechanisms that could cause unexpected effects on the outcome following an ischemic insult, as we recently observed in an adenosine A1 receptor deficient mouse strain (Olsson et al., 2004). Still, several studies suggest the notion that CC may in fact contribute to or cause neuronal death. For example, injection of CC into rat hippocampus caused neuronal loss in the dentate granule cell layer (Nagai et al., 2002). Furthermore, using the same CC ko mouse strain as used in the present investigation, it was shown that cell death was less prominent in the ko animals following kainic acid induced status epilepticus, especially in CA3 subfield (Pirttila et al., 2002).

One explanation to the detrimental effect of CC on neuronal survival following ischemia could be its propensity to form protein aggregates. CC has amyloidogenic properties at physiological concentration and under denaturing conditions, in which CC tend to dimerize (Ekiel and Abrahamson, 1996), and form protein aggregates (Janowski et al., 2001). In vivo, CC is co-expressed in protein aggregates with β-amyloid in neurons from brain of Alzheimer’s disease patients (Levy et al., 2001). In an

Fig. 3. Neuronal damage in individual brain hemispheres after 12 min of global ischemia and 4 days of reperfusion in CC ko animals (filled circles, n=23) and wt (open circles n=15). (A) The neuronal injury in CA1, CA3, dentate gyrus (DG) and cortex at bregma −1.7 is expressed in percent of total amount of cells in respective regions. (B) The striatal damage measured as damage score (0–5) at bregma +0.9. Bars represent median values. * Denotes statistical significance (P<0.05) between ko and wt animals, Mann-Whitney U test.
Oxidative stress and protein aggregates accumulate in CA1 pyramidal cells during recovery after global ischemia (Olsson et al., 2003). Ischemia-stress could possibly change the circumstances for protein aggregation including CC, and contribute to cell death. In addition, CC may have immunomodulatory effects following global ischemia. Resident microglia are activated during the first hours of reperfusion following ischemia, and in the CA1 region they become activated macrophages later in reperfusion (Gehrmann et al., 1992, 1995). CC is known to enhance the immunological response and nitric oxide formation of macrophages (Verdot et al., 1996). Hence, in the CC deficient mice the contribution of macrophages to neuronal death following global ischemia may be diminished.

Yet another explanation could be that CC has a differential effect on different brain cells, i.e. glia cells and neurons. Thus following focal ischemia, all cellular elements degenerate in the brain infarct including glia cells and vessels, while following global ischemia, cell death is selectively neuronal. The net effect of CC in glia and neural cells following ischemia would then result in either increased or decreased damage.

In conclusion, the present study shows that endogenous CC is protective in focal ischemia, probably through its inhibitory effect on the cathepsins. In contrast, following global ischemia, cell death could partially be due to other unfavorable properties of CC that may overcome its inhibitory effect on the cathepsins.

### Acknowledgments

The Swedish Science Council (Project No. 08644) and the Bergendahl foundation supported this work. The authors are grateful to Johan Wasselius for help with the immunohistochemistry.

### REFERENCES


