Up-regulation of cathepsin B and cathepsin L activities in scrapie-infected mouse Neuro2a cells

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Prion diseases are characterized by the accumulation of an abnormal, protease K-resistant isoform of the prion protein, PrP Sc, which is generated by a post-translational conversion of the protease-sensitive normal cell-surface glycoprotein PrP C involving major conformational changes. The conversion is thought to occur at the plasma membrane or along the endocytic pathway towards the lysosome. PrP Sc aggregates have been found to accumulate in secondary lysosomes. In our study, the activities of two major lysosomal cysteine proteases, cathepsins B and L, were found to be significantly increased in scrapie-infected Neuro2a cells compared with uninfected cells using biochemical and cytochemical methods. We hypothesize that lysosomal proteases may be involved in a ‘second autocatalytic loop’ of PrP Sc formation, acting in concert with the well-known autocatalytic enhancement of PrP conversion in the presence of PrP Sc.

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Smith, 1977). The fluorescent reaction product 4-methoxy-
β-naphthylamine was precipitated by 5-nitro-salicylaldehyde
followed by microscopic evaluation.

In addition, cathepsin B and L activities were measured by
a quantitative fluorometric assay as described previously
(Barrett & Kirschke, 1981; Ulbricht et al., 1995). Briefly,
confluent cultures in 50 cm² flasks were trypsinized, washed
and suspended in ice-cold lysis buffer (400 mM sodium
phosphate buffer, pH 6.0, 75 mM NaCl, 4 mM EDTA-Na₂,
0.25% Triton X-100) followed by incubation on ice for 1 h.
Lysates were homogenized in a Transonic-Bath three times
for 15 s each at 4 °C and centrifuged at 20 000 g for 30 min
at 4 °C. The protein concentration of supernatants (cell
extracts) was determined by the Bio-Rad DC assay and cell
extracts were stored at −80 °C. Prior to starting the reaction,
reaction buffer (for cathepsin B assays: 88 mM KH₂PO₄
12 mM Na₂HPO₄, 1.33 mM EDTA-Na₂, 2.7 mM L-
cysteine, pH 6.0; for cathepsin L assays: 100 mM sodium
acetate buffer, 1 mM EDTA-Na₂, 2 mM dithiothreitol,
pH 5.5) was pre-warmed to 37 °C. Then 100 μl of cell
diluted in lysis buffer (maximally 70–100 μg protein)
was added and mixed, followed by incubation at 37 °C for
5 min. Finally 25 μl substrate solution was added. Fluores-
cence time scans of the reaction mixture in quantitative
assays showed a practically linear course over the first 1000 s
(data not shown). Based on these observations, a 15 min
assay time was chosen as a standard time for all experiments.
A range of protein concentrations was used in the assays in
order to ensure that the reaction did not reach saturation.
All reactions were run at 37 °C for 15 min in a total volume
of 1 ml, in the absence or presence of cathepsin inhibitors
(see below), the latter value defining the non-specific
activity to be subtracted. For cathepsin B assays,
the cathepsin B-specific substrate benzoylcarbonyl-L-
arginyl-L-arginyl-7-amino-4-methylcoumarine (Z-arg-arg-
AMC; 5 μM) was used in conjunction with the inhibitor
trans-epoxy-succinyl-leucylamido-(4-guanidino)-butane (E64; 50 μM), or, alternatively, the
chymotrypsin B- and L-specific substrate benzoylcarbonyl-
L-phenylalanyl-L-arginyl-4-methylcoumarine (Z-phenyl-
alanine-CH₇N₂; 0.5 μM) was used in conjunction with the cathepsin
B inhibitor L-trans-epoxy-succinyl-lle-Pro-OH propylamide
(CA-074; 50 μM). To detect cathepsin L activity,
the substrate Z-phenylalanine-CH₇N₂ (5 μM) was used in conjunction
with the specific cathepsin L inhibitor benzoylcarbonyl-L-
phenylalanyl-L-phenylalanyl-diazomethylketone (Z-phenyl-
alanine-CH₇N₂; 0.5 μM) or, alternatively, in the presence of either
CA-074 or E64, the difference between the two values
defining cathepsin L activity. To eliminate prion infectivity
in the samples before fluorescence measurements, reactions
were stopped by adding 5 vols 6 M guanidine hydro-
chloride, 50 mM Tris/HCl, pH 7.4, and incubating for
20 min at room temperature. The fluorescent product was
quantified in a fluorometer (Hitachi-2000; Ex 370 nm, Em
460 nm) and standardized with amino-methylcumarine.
One unit of enzyme activity represents 1 μmol substrate
turned over min⁻¹.

In the cytochemical assay, fluorescence signals resulting
from cathepsin B activity became visible in uninfected
Neuro2a cells by 90 min of incubation with substrate,
whereas in the persistently infected cells they were already
detectable by 30 min, with a time-dependent further
increase in intensity (Fig. 1). In order to see whether or
not this apparent increase in cellular cathepsin B activity in
the infected cells was the result of generally increased protein
content per cell under conditions of infection, we next
performed quantitative fluorometric assays in cell lysates
and normalized the enzyme activity data for total protein
content. These fluorometric assays revealed significant
increases in cathepsin B and also in cathepsin L activity
in ScNB cells compared with uninfected Neuro2a cells (Fig. 2
A, B).

To avoid any artefacts when comparing uninfected with
persistently infected cells due to the commonly performed
recloning of the latter to select for producer cells (Bosque &
Prusiner, 2000), we also performed cathepsin assays on
newly infected cells from a highly susceptible Neuro2a
subline (Zhang et al., 2002) that we had isolated according to
a published protocol (Bosque & Prusiner, 2000) and the
respective uninfected cells. In this system, very similar,
significant increases in cathepsin B and L activities were
found in the infected cells (Fig. 2C, D).

Furthermore, additional assays of cathepsin B were per-
formed using substrate Z-phenylalanine-CH₇N₂ in conjunction
with the inhibitor CA-074 (E) and also of cathepsin L using
the substrate Z-phenylalanine-CH₇N₂ in the presence of either CA-
074 or E64 (F). Again, the data yielded the same pattern, i.e.
significantly elevated cathepsin B and L activities in the

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Fig. 1. Cytochemical detection of cathepsin B activity in unin-
infected Neuro2a (A–D) and ScNB (E–H) cells at assay times of
30 min (A, E), 60 min (B, F), 90 min (C, G) and 120 min
(D, H). For details see text.
infected cells, but the absolute cathepsin L activity values were lower than with the previous assay design, indicating superior specificity for cathepsin L.

The up-regulation of cathepsin activities in neuronal cells following prion infection described here may have important consequences for both pathogenesis and agent replication. Lysosomal hydrolases seem to play a role both in necrosis (Yamashima, 2000) and in the autophagic cell death described in neurons (Kegel et al., 2000). On the other hand, cathepsin B has been implicated in the caspase cascade in apoptosis (Guicciardi et al., 2000; Ishisaka et al., 1999; Kingham & Pocock 2001; Lotem & Sachs 1996; Vancompernolle et al., 1998), and apoptosis appears to be the predominant type of neuronal cell death in prion diseases (Fairbairn et al., 1994; Forloni et al., 1993; Giese et al., 1995; Gray et al., 1999). In view of the above, the possible role of up-regulation of cathepsin activity in the pathogenesis of prion disease merits further study.

Lysosomotropic agents and cysteine protease inhibitors are reported to block PrPSc accumulation in scrapie-infected...
neuroblastoma cells (Doh-ura et al., 2000). Viewing these results together with our present data, we hypothesize that lysosomal proteases might be actively involved in PrPSc formation, which would represent a ‘second autocatalytic loop’ in prion replication, acting in concert with the first, i.e. the well-known autocatalytic enhancement of PrP conversion in the presence of PrPSc (Fig. 3). A possible function for up-regulated cathepsin activities with respect to prion replication might be to disrupt large aggregates of PrPSc, thus creating an expanded population of ‘converting units’, analogous to the sonication step in the ‘in vitro protein-misfolding cyclic amplification’ procedure (Saborio et al., 2001).

How the up-regulation of cathepsin B and L activities by prion infection is mediated is unknown. One intriguing possibility is the involvement of glycosaminoglycans. On the one hand, PrPSc amyloid is known to contain sulfated glycosaminoglycans, which suggests that these may play an essential role in PrPSc formation or stabilization (Snow et al., 1990). Depending on the circumstances, sulfated glycans can either be cofactors (Wong et al., 2001) or inhibitors (Caughey & Chesebro, 2001; Caughey et al., 1991) of PrPSc formation. On the other hand, there is evidence that glycosaminoglycans are involved in the in vivo processing of lysosomal cysteine proteases (Rozman et al., 1999; Ishidoh & Kominnami, 1995) and in cathepsin B regulation (Almeida et al., 2001). Whether or not PrPSc-associated glycosaminoglycans indeed play a role in the up-regulation of cathepsin activity in scrapie-infected cells shown in the present work requires further investigation.

**Fig. 3.** Hypothetical model for the possible involvement of cathepsins in a ‘second autocatalytic loop’ (dashed and dash–dot arrows) involved in the formation of the scrapie-associated isoform of prion protein (PrPSc). The model is supported by the following data: (i) in vitro conversion studies as performed by many groups (continuous bent arrow on the right representing the ‘first autocatalytic loop’ in prion replication); (ii) data by Doh-ura et al. (2000) (dash–dot arrow); (iii) the present paper (dashed arrow). GAG, glycosaminoglycans. Note that the possible involvement of GAG (dotted lines) in the proposed ‘second autocatalytic loop’ is speculative. For details see text.

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**REFERENCES**


Cathepsins B and L in scrapie-infected cells


