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Intra- versus extracellular effects of microglia-derived cysteine proteases in a conditioned medium transfer model

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Abstract

Activated microglia release inflammatory mediators that display either beneficial or harmful effects on neuronal survival and signaling. In the present study we demonstrate that exposure to lipopolysaccharide leads to an increase in the lysosomal cysteine proteases, cathepsin B, K, S, and X, in culture supernatants of the microglia cell line BV-2. In addition, we observed an up-regulation of cathepsins in the cytoplasmic fraction in response to stimulation with lipopolysaccharide. Conditioned medium from these cells was toxic to the neuroblastoma cell line Neuro2a. Experiments with membrane-permeable and membrane-impermeable cysteine protease inhibitors suggested that blocking extracellular cathepsins had no effect on microglia-mediated neuron death in this medium transfer model. However, intracellular cathepsins seem to trigger the release of neurotoxic factors. In lipopolysaccharide-stimulated BV-2 cells, inhibition of intracellular cathepsins significantly diminished microglial activation characterized by reduced expression of different proinflammatory cytokines, thereby reducing the neurotoxic effects of the medium. This hitherto unknown intracellular effect of cysteine proteases in activated microglia might connect chronic neuroinflammation with neurodegeneration.

Keywords: cathepsin, cysteine proteases, cytokine, inflammation, lipopolysaccharide, neurodegeneration.

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In the healthy normal brain microglia are present at a resting state. In response to environmental cues such as brain injury or immunological stimuli, they change morphology and acquire an array of functions, including phagocytosis, antigen presentation, and secretion of inflammatory mediators (Perry 2004). There is a strong debate as to which extent this activation process is beneficial or harmful. On the one hand, activated microglia serve diverse beneficial functions essential to neuron survival (Streit 2002), on the other hand, microglia can become overactivated under certain circumstances and produce an excess of cytotoxic factors like superoxide, nitric oxide, and tumor necrosis factor-α (TNF-α) (Block *et al.* 2007). These factors are strong inflammatory stimuli and contribute to neuronal loss during chronic inflammation (Minghetti 2005). Therefore, attempts to halt neurodegeneration in diseases like Morbus Alzheimer or Parkinson's disease include inhibition of microglial activation.

In addition to diverse cytokines, activated microglia secrete certain lysosomal proteases, the cathepsins (CATs) (Ryan *et al.* 1995; Petanceska *et al.* 1996; Liuzzo *et al.* 1999; Kingham and Pocock 2001; Gan *et al.* 2004). Cathepsins belong to the papain superfamily (clan CA, family C1) of cysteine proteases. They are synthesized as inactive pre-proenzymes. The prepeptide is removed during the passage to the endoplasmic reticulum and the pro-CAT undergoes proteolytic processing to the mature enzyme in the lysosomal compartment (Turk *et al.* 2000). Cathepsins, which normally occur mostly in lysosomes, are involved in diverse physiological processes, such as the regulation of enzymes, including key protein kinases and phosphatases, and the induction of specific cytoskeletal rearrangements, which may account for their involvement in intracellular signaling, vesicular trafficking, and structural stabilization (Kirschke and Barrett 1987; Roberts 2005; Stoka *et al.* 2005). Some CATs have also been shown to play a role in diverse pathologies. For example, altered expression or distribution of CATD, CATB, CATE, CATS, and CATX

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Abbreviations used: CATs, cathepsins; CM, conditioned medium; COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; TNF-α, tumor necrosis factor-α.

constitute early pathological features in Alzheimer's disease, tauopathies, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (Bernstein *et al.* 1989; Bernstein and Wiederanders 1994; Mantle *et al.* 1995; Cataldo *et al.* 1996; Kegel *et al.* 2000; Wendt *et al.* 2007, 2008).

In this study we intend to answer the question if CATs are able to interfere with the microglial activation process by exerting intra- or extracellular influences. For this reason we used a coculture model, composed of a neuronal cell line intoxicated with the conditioned medium (CM) of the lipopolysaccharide (LPS)-stimulated microglial cell line BV-2. At the beginning of our studies we investigated whether CATs which have been described to be strongly expressed in microglia or which have been shown to be up-regulated in different pathological conditions are differentially regulated in our model. To further elucidate the function of CATs in our medium transfer cell culture model, we modulated microglial activation and neurotoxicity with the membrane-impermeable cysteine protease inhibitor, E-64 (Barrett *et al.* 1982), its membrane-permeable prodrug, E-64d (Tamai *et al.* 1986), and the CATB inhibitor, CA-074 (Towatari *et al.* 1991) and its membrane-permeable derivative, CA-074Me (Buttle *et al.* 1992). Thus, our study provides a useful approach to distinguish between intra- and extracellular pathways involved in CAT-mediated neuroinflammation.

Materials and methods

Antibodies and reagents

Mouse monoclonal antibody against actin, polyclonal rabbit anti-lysosomal-associated membrane protein 2 (Lamp-2) antibody, LPS, E-64, and CA-074 were obtained from Sigma-Aldrich (Munich, Germany). All other cell culture reagents and media were purchased from Invitrogen (Karlsruhe, Germany). The inhibitors CA-074Me, Z-Val-Val-Nle-diazomethylketone (CATS inhibitor), and E-64d and the substrate Z-VVR-AMC were obtained from Bachem (Weil am Rhein, Germany). Goat polyclonal antibodies against CATK, CATL, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Rabbit polyclonal antibodies against inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were obtained from Cayman Chemicals (Ann Arbor, MI, USA). Monoclonal rat anti-CD11b antibody was purchased from Serotec (Düsseldorf, Germany). Goat polyclonal antibodies against CATX, CATS, and TNF-α, and rat monoclonal antibody against CATB as well as the substrate MCA-R-P-P-G-F-S-A-F-K(Dnp)-OH were obtained from R&D Systems (Wiesbaden, Germany). Horseradish peroxidase-coupled secondary antibodies were purchased from GE Healthcare (Munich, Germany).

Cell cultures

BV-2 microglia

The BV-2 immortalized murine microglia cell line has been described previously (Blasi *et al.* 1990). BV-2 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum. Confluent microglial cells were subcultured two to three times per week using 0.25% trypsin. The microglial character of the cells was routinely checked by immunocytochemical evaluation using monoclonal rat anti-mouse CD11b primary antibody.

Neuro2a cells

The mouse neuroblastoma cell line Neuro2a (DSMZ, Braunschweig, Germany) was cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum. Subcultures were prepared as described for the BV-2 cells. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. For western blot analysis, RNA isolation, and CAT activity assays, cultured cells were washed with ice-cold phosphate buffered saline, scrapped off from the dishes, and centrifuged for 5 min at 13 000 *g*. Cell pellets were stored at −20°C until further use.

Activation of microglia

Confluent BV-2 cells were treated with 2 μg/mL LPS in serum-free Dulbecco's Modified Eagle's Medium for 24 h. Successful activation was routinely tested by measurement of nitrite in the culture supernatants using Griess reagent system (Promega GmbH, Mannheim, Germany) and a Microplate Reader (Multiscan RC, Labsystems, Helsinki, Finland). The supernatants were collected, centrifuged (900 *g*, 5 min) to remove any cellular material, and transferred to neuronal cells. The microglial cells and aliquots of the supernatants were harvested for competitive semi-quantitative PCR, activity assays, or western blotting.

Conditioned medium transfer and neurotoxicity assay

To test the neurotoxic effects of soluble factors secreted by activated microglia, confluent Neuro2a cells were cultured in serum-free medium and treated with the supernatants of LPS-stimulated BV-2 cells. To study the effects of different inhibitors, microglia were either pre-treated for 30 min with 40 μM E-64d or CA-047Me prior to a 24-h LPS incubation period (inhibitor pre-incubation) or 40 μM of the membrane-impermeable inhibitors, E-64 or CA-074, were added to the CM from microglia that was already stimulated for 24 h with LPS (inhibitor post-incubation). The resulting culture supernatants were collected, centrifuged to eliminate cell debris, and transmitted to Neuro2a cells for 48 h to induce cell death. Neuronal viability was quantified using the Cell Titer 96® Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions in a Microplate Reader (Multiscan RC, Labsystems). In control assays, Neuro2a cells were directly treated with LPS and/or the different inhibitors. Under these conditions, no cell toxicity was detected (data not shown).

Protein extraction and western blots

For whole cell lysate preparations, cell pellets were homogenized in phosphate buffered saline without Ca^{2+}/Mg^{2+} at 4°C. Homogenized samples were centrifuged for 1 min in a pre-cooled centrifuge at 15 000 *g*. The supernatant was collected and subsequently diluted 1 : 1 in 2× Laemmli sample buffer and boiled for 10 min.

For subcellular fractionation, cell pellets were homogenized in homogenization buffer (250 sucrose, 10 KCl, 1.5 $MgCl_2$, 2 EDTA, and 20 mM HEPES, pH 7.4, containing complete protease inhibitor

cocktail; Roche, Mannheim, Germany) using a glass homogenizer at 4°C. Homogenized samples were briefly centrifuged for 10 min at 600 *g*. Supernatants were collected and underwent an additional centrifugation for 20 min at 12 000 *g* (4°C). The resulting supernatant was saved as cytoplasmic fraction and the remaining pellet, containing organelles like lysosomes and mitochondria, was resuspended in homogenization buffer and saved as lysosomal fraction. All fractions were subsequently diluted 1 : 1 in 2× Laemmli sample buffer and boiled for 10 min.

Protein determination and western blot analyses were performed as described previously (Wendt *et al.* 2007). The following primary antibodies were applied: polyclonal goat anti-mouse CATX (1 : 500), polyclonal goat anti-human CATS (1 : 200), monoclonal rat anti-CATB (1 : 100), polyclonal goat anti-CATK (1 : 100), polyclonal goat anti-CATL (1 : 100), polyclonal goat anti-GAPDH (1 : 200), monoclonal mouse anti-actin (1 : 1,000), polyclonal rabbit anti-mouse lysosomal-associated membrane protein 2 (1 : 400), polyclonal goat anti-mouse TNF-α (1 : 500), polyclonal rabbit anti-mouse COX-2 (1 : 1000), and polyclonal rabbit anti-mouse iNOS (1 : 2000). Each western blot analysis was repeated at least three times. Figures show representative results.

Competitive semi-quantitative PCR

According to the manufacturer's recommendations, total RNA was extracted from cell pellets using Trizol (Invitrogen). Total RNA (1 μg) was used for the RT reaction. The RT reaction was performed by using the RevertAid First Strand cDNA Synthesis Kit and random hexamer primers (both obtained from Fermentas GmbH, St Leon-Rot, Germany) according to the manufacturer's recommendations.

Competitive semi-quantitative PCR was performed as described by Schoenebeck *et al.* (2005) with the following modifications. Each sample contained 2, 4, 8, or 16 pmol of CATX primers (nucleotides 711–730 and 927–946; Acc. No.: NM_022325) or CATS primers (nucleotides 574–593 and 775–794; Acc. No.: AJ002386) and 2 pmol of two β-actin primers (nucleotides 305–324 and 450–469; Acc. No.: NM_007393). The annealing temperature was 55°C.

Determination of CATS and CATX activity

Cell pellets were prepared for enzyme activity tests as described by Wendt *et al.* (2007). Culture supernatants were applied without additional treatments.

Cathepsin S activity was measured using 20 μM Z-VVR-AMC as a substrate in 4 mM phoshphate-buffered saline/0.01% Brij-35/1 mM EDTA/2 mM freshly prepared dithiothreitol (pH 7.4). In parallel assays, the reaction was followed with or without the specific CATS inhibitor, Z-Val-Val-Nle-diazomethylketone (10 nM) (Shaw *et al.* 1993). Assays were performed in triplicate in black 96-multiwell plates (BD Biosciences, Heidelberg, Germany) at 22°C. Protein concentration was 50 μg/mL in a total volume of 50 μL. Prior to the addition of substrate and starting of the assay, enzyme solutions were incubated for 5 min at 22°C with the CATS inhibitor or diluent. After 60 min of incubation, fluorescence was measured at 355/460 nm in a Mithras Multimode Microplate Reader LB 940 (Berthold Technologies, Bad Wildbad, Germany). The activities of the proteases were calculated on the basis of relative fluorescent units. The difference between non-inhibited and Z-Val-Val-Nle-diazomethylketone-inhibited probes was adequate for CATS activity.

Cathepsin X activity was determined as hydrolysis of MCA-R-P-P-G-F-S-A-F-K(Dnp)-OH as described previously (Wendt *et al.* 2007). Protein concentration was 100 μg/mL in a total volume of 50 μL.

Enzymatic N-deglycosylation

To identify glycoforms of CATX and CATS on western blots, extracted proteins from BV-2 cells were deglycosylated using GlycoProfile™ II (Enzymatic In-Solution N-Deglycosylation Kit) from Sigma-Aldrich according to the manufacturer's recommendations. In each test sample, 5 μg extracted protein was incubated with 0.5 units of peptide *N*-glycosidase F at 37°C for 1 h. Aliquots of each sample were diluted 1 : 1 in 2× Laemmli sample buffer and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In control assays, deglycosylation of RNase B was performed.

Data analysis

Western blot experiments were quantified after normalization of each sample against the endogenous actin or GAPDH level using the image analysis software TINA 2.09 (Raytest, Straubenhardt, Germany). Protein expression in culture supernatants was evaluated by direct comparison of the optical density of the respective bands using the above mentioned image analysis software.

Data were analyzed by means of one-way analysis of variance (ANOVA) to determine statistical significance. All pairwise multiple comparison procedures (Tukey's test) were used for *post hoc* comparisons. If tests on normality and/or equal variances failed, a Kruskal–Wallis ANOVA on ranks (H-test) followed by the Dunn's or Tukey's *post hoc* test was performed. Comparisons between two groups were made using Student's *t*-test or Mann–Whitney test (*U*-test). All statistical analyses were conducted with SigmaStat 3.1 software (Systat Software GmbH, Erkrath, Germany). Statistical analyses were always made within one experimental series consisting of results with cells from the same passage, harvested at the same day. The figures show representative data for at least three independent experimental series consisting of at least three independent experiments each.

Results

Expression of different cathepsins in LPS-stimulated BV-2 cells

First, we addressed the question whether microglial cells modulate the protein expression of different CATs following activation by LPS. For this purpose, BV-2 cells were stimulated with LPS for 24 h. The success of this activation process was confirmed by an increase in nitrite concentration in the cell culture supernatants (Fig. 1a).

Western blot analysis revealed a prominent expression of all analyzed CATs (CATL, CATB, CATK, CATS, and CATX) in the lysosomal fraction and the culture supernatants of BV-2 cells (Fig. 1b–f). The different antibodies detected at

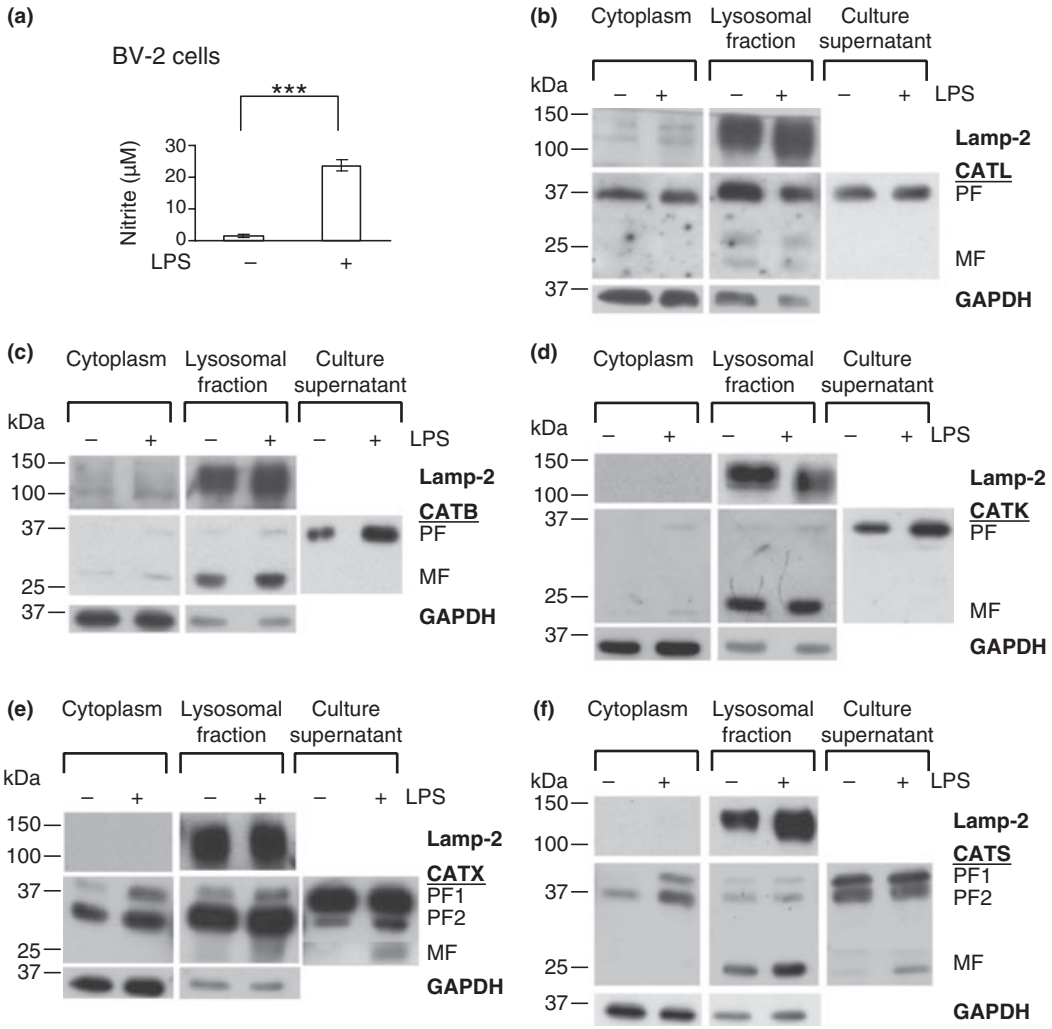

Fig. 1 Protein amounts of different cathepsins are strongly increased in the culture supernatants and the cytoplasm of LPS-stimulated BV-2 cells. **(a)** Determination of nitrite concentration in the culture supernatants of unstimulated (–LPS) and stimulated (+LPS) BV-2 cells. Bars represent the mean ± SEM obtained from 36 independent experiments. Statistical significance ***$p < 0.001$ (*U*-test). **(**b–f**)** Representative western blot analyses of the cytoplasmic and lysosomal fraction and the culture supernatants of stimulated and unstimulated BV-2 cells. Equal amounts of protein (cytoplasmic and lysosomal fraction) or equal volumes (supernatants) were loaded. Because the amounts of mature forms in the culture supernatants were very low, the respective bands became visible at longer exposure times. Expression of GAPDH and Lamp-2 was used as a loading control. MF, mature form; PF, proform.

least two bands for each CAT corresponding to their pro- and mature forms. The proform of CATL (ca. 36 kDa) was found in the cytoplasmic and lysosomal fractions as well as in the culture supernatants of BV-2 cells. Additionally, the CATL antibody detected two lower molecular weight forms corresponding to the 30-kDa murine single-chain CATL and the 24–25 kDa heavy chain of the double chain mature CATL in the lysosomal fractions (Fig. 1b). Activation of BV-2 cells with LPS did not result in an apparent up- or down-regulation of any of the CATL forms in this cell culture model (Fig. 1b). The immunoblots in Fig. 1c and d depict murine CATB and CATK that both migrated as a higher molecular weight form (ca. 35–38 kDa) corresponding to the proform and a lower molecular weight mature form (ca. 23–32 kDa). The mature forms of CATB and CATK were mainly found in the lysosomal fractions, whereas their proforms were most prominent in the culture supernatants of BV-2 cells (Fig. 1c and d). The LPS stimulation slightly increased CATB and CATK protein amounts in the supernatants.

In contrast to the above-described CATs, the analyzed cell culture samples contained two (instead of one) higher molecular weight forms that showed immunoreactivity with anti-CATX and anti-CATS antibodies (Fig. 1e and f). The

size of these forms corresponded to the size of the pre-proforms and the proforms of murine CATS (37 and 39 kDa) and CATX (34 and 37 kDa). To date, there are no publications that can explain the appearance of pre-proforms of lysosomal enzymes in culture supernatants or mature lysosomes. As it was not our intention to raise this question in the current study, we decided to designate the two higher molecular weight forms of CATS and CATX proform 1 and 2. Western blot analyses depicted in Fig. 1e and f revealed that treatment of BV-2 cells with LPS resulted in an increase in the amounts of CATS and CATX in the cytoplasmic fraction. Particularly, the lower molecular weight forms of CATS and CATX, corresponding to the mature enzymes (26 and 29 kDa), were strongly increased in the culture supernatants after treatment with LPS (Fig. 1e and f).

The western blot analyses shown in Fig. 1 were repeated at least three times, and CAT protein expression in stimulated and unstimulated BV-2 cells was quantified (Fig. S1). The quantification experiments revealed that the increase in CATB, CATK, CATX, and CATS protein amounts in the culture supernatants after LPS-stimulation were significant (Fig. S1c–f). CATB, CATK, CATX, and CATS levels were also elevated in the cytoplasm of stimulated microglial cells, with significant effects demonstrated for CATX and CATS proforms (Fig. S1c–f).

In summary, among five different CATs analyzed, the protein levels of four CATs were significantly increased in the culture supernatants of activated microglial cells. In addition, the proforms of CATS and CATX were significantly increased in the cytoplasm after LPS stimulation.

LPS-induced regulation of CATS and CATX

Intrigued by the fact that CATS and CATX intra- and extracellular protein expression and distribution were significantly altered in activated BV-2 cells, we further analyzed the mRNA expression and activity of these CATs in our glial activation model. We could show that LPS treatment did not change the levels of CATX mRNA, whereas the levels of CATS mRNA were increased by LPS (Fig. 2a and b). To examine if the increase in CATS and CATX protein in the medium of activated BV-2 cells also resulted in an increase in proteolytic activity, activity measurements were carried out. Supporting the results of the western blot analysis, LPS treatment significantly increased CATS and CATX activity in culture supernatants and CATS activity in whole cell lysates (Fig. 2c and d).

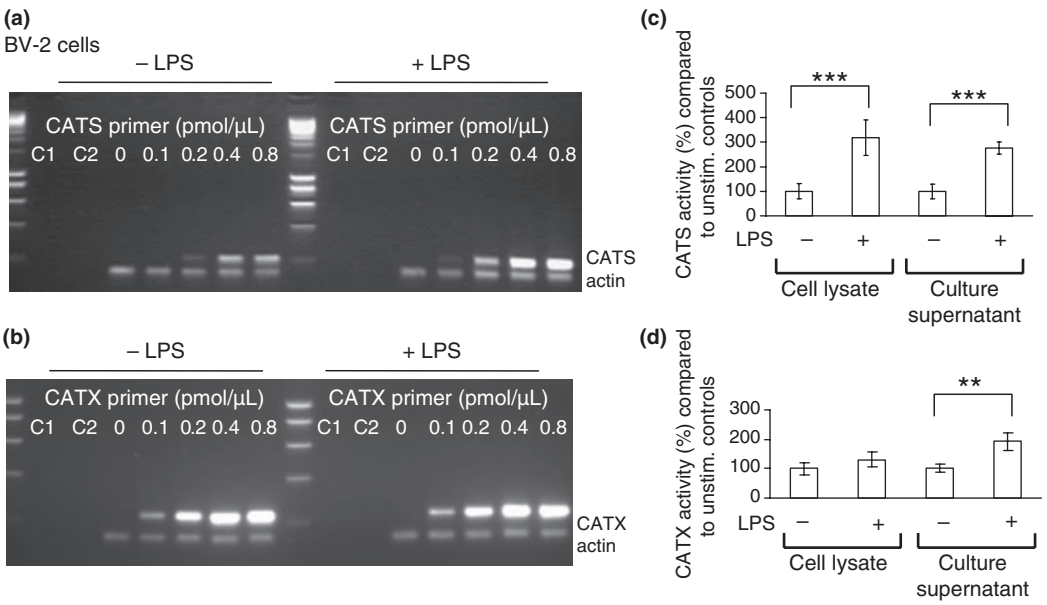

Fig. 2 CATS and CATX activity is increased in activated BV-2 cells. Competitive semi-quantitative PCR analysis of stimulated (+LPS) and unstimulated (–LPS) BV-2 cells. Increasing concentrations of CATS (a) and CATX (b) primers were allowed to compete in PCR reactions with a constant amount of β-actin primers. Different amounts of CATS and CATX RNA in the two RNA preparations are reflected by differences in the increase of the ratios between the CATX or CATS and the β-actin band intensities. C1: water control, C2: cDNA control (without reverse transcriptase). (c) CATS activity measurement of BV-2 whole cell lysate and culture supernatants. Bars represent the mean ± SEM obtained from 11 to 12 independent experiments. Statistical significance $^{***}p < 0.001$ (*U*-test). (d) CATX activity measurement of BV-2 whole cell lysate and culture supernatants. Bars represent the mean ± SD obtained from three independent experiments. Statistical significance $^{**}p < 0.01$ (student's *t*-test).

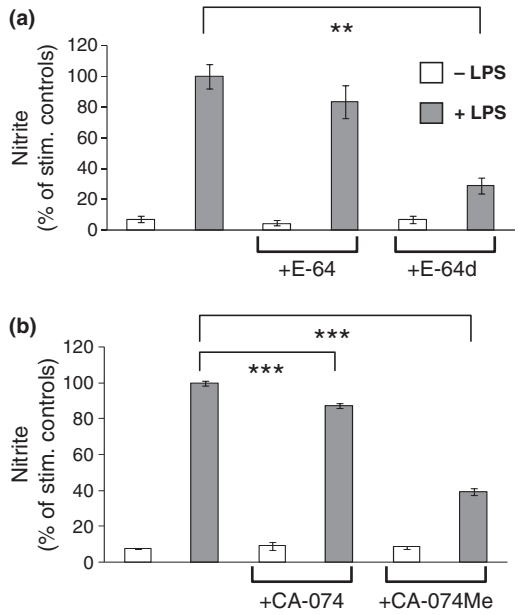

Fig. 3 Inhibition of intracellular cysteine proteases reduces nitrite release from activated microglia. **(a)** Effects of the membrane-impermeable and membrane-permeable cysteine protease inhibitors E-64 (40 μM) and E-64d (40 μM) on nitrite concentrations in the culture supernatants of unstimulated (–LPS) and stimulated (+LPS) BV-2 cells. Bars represent the mean of 15 independent experiments ±SEM. Statistical significance $^{**}p < 0.01$ (H-test). **(b)** Effects of the membrane-impermeable and membrane-permeable CATB inhibitors CA-074 (40 μM) and CA-074Me (40 μM). Bars represent the mean of four independent experiments ±SEM. Statistical significance $^{***}p < 0.001$ (ANOVA). Data are expressed as % of nitrite amount in LPS-stimulated control cells (=100%).

Effects of cysteine protease inhibitors on microglial activation

To further investigate if the up-regulation of CATs influenced glial activation, we pre-incubated BV-2 cells with the cysteine protease inhibitor E-64, its membrane-permeable derivative, E-64d, the CATB inhibitor, CA-047, or its membrane-permeable derivative, CA-074Me. In contrast to the general cysteine protease inhibitors, E-64 and E-64d, CA-074 and CA-074Me were more specific to cysteine CATs (Discussion), among them CATS which was significantly up-regulated in stimulated BV-2 cells. The different inhibitors were added 30 min prior to LPS stimulation. The activation state of microglial cells was verified 24 h after LPS stimulation by measurement of nitrite in the cell culture supernatants. Untreated control groups had very low amounts of nitrite in the culture supernatants (~3 μM), whereas cells treated with LPS alone showed high amounts of nitrite (~30 μM) (Fig. 3). In comparison with the LPS-stimulated control group, the nitrite amount was significantly reduced in the supernatants of activated BV-2 cells pre-treated with E-64d, CA-074Me, or CA-074 (Fig. 3a and b). The effects of the membrane-impermeable inhibitors, E-64 and CA-074, were considerably lower, indicating that intracellular inhibition of cysteine proteases alleviated microglial activation.

Neuroprotective effects of cysteine protease inhibitors

Next, we investigated whether CATs released into the cytosol or the supernatant influenced the neurotoxic potential of activated BV-2 cells. For this purpose, the microglial cells were activated with LPS in the absence or presence of CAT inhibitors for 24 h (inhibitor pre-incubation), and the resulting CM was added to cultures of Neuro2a cells. The viability of the neuronal cultures was assessed 48 h later with the Cell Titer 96 assay (Promega). The viability of Neuro2a cells treated with CM from unstimulated BV-2 cells was defined as 100%. Treatment with CM from LPS-activated BV-2 cells resulted in a significant reduction of the neuronal viability 48 h after medium transfer (Fig. 4a). In parallel assays, we incubated BV-2 cells with both LPS and the membrane-permeable inhibitors, E-64d or CA-074Me, to elucidate the role of cysteine proteases in this effect. The neurotoxic effect of the CM collected from these cell cultures was strongly reduced in the medium transfer model compared with the effect caused by medium from cells that were treated with LPS alone (Fig. 4a). Comparable results were obtained when rat primary cortical neurons were applied instead of Neuro2a cells (data not shown).

On the contrary, when the membrane-impermeable inhibitors, E-64 or CA-074, were added to supernatants from LPS-stimulated BV-2 cells 30 min before adding the supernatant to neuronal cells (inhibitor post-incubation), the reduction in the survival of neuronal cells was very similar to that caused by the supernatant without the inhibitors (Fig. 4b). In this experimental setup cysteine proteases in the CM should be inhibited prior to its addition to Neuro2a cells. As E-64 and CA-074 did not block the neurotoxicity mediated by this medium (Fig. 4b), the neurotoxic effect appeared to be related to intracellular and not extracellular CATs.

Possible pathways influenced by cathepsins

The results of the glial activation and neuronal cell death models described above highlighted the importance of intracellular cysteine proteases in LPS-activated microglial cells. To further investigate the signaling pathways influenced by these proteases, we analyzed the expression of different proinflammatory molecules in activated BV-2 cells by means of western blot analysis. We could show that TNF-α, COX-2, and iNOS were up-regulated after LPS-stimulation (Fig. 5). Pre-incubation with E-64d considerably reduced the expression of these proinflammatory proteins in activated BV-2 cells (Fig. 5). The amounts of active TNF-α secreted from activated BV-2 cells could nearly be abolished by treatment with E-64d (Fig. 5). Experiments with CA-074Me had a comparable outcome whereas pre-incubation with E-64 or CA-074 had only minimal effects (data not shown).

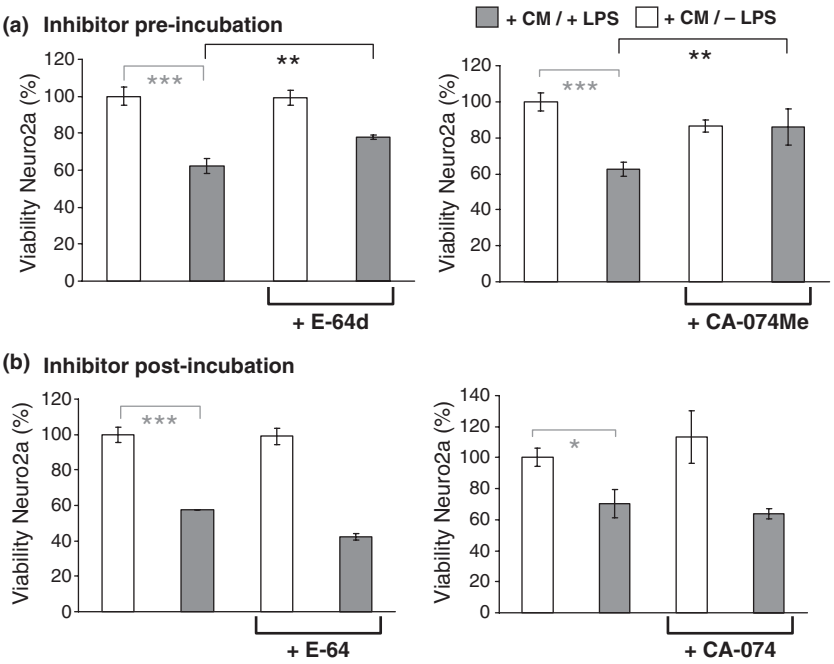

Fig. 4 Effects of cysteine protease inhibitor pre- and post-incubation in a conditioned medium (CM) transfer model. **(a)** Neurotoxic effects of CM from activated microglia on neuronal cells are attenuated by pre-incubation with E-64d or CA-074Me. Cell Titer 96 assay (Promega) 48 h after CM transfer from BV-2 cells to Neuro2a cells. Statistical significance $^{**}p < 0.01$, $^{***}p < 0.001$ (ANOVA). **(b)** Post-incubation of CM from activated microglia with E-64 or CA-074 does not abolish toxic effects on neuronal cells. Cell Titer 96 assay (Promega) 48 h after CM transfer from BV-2 cells to Neuro2a cells. Statistical significance $^{***}p < 0.001$, $^{*}p < 0.05$ (ANOVA). Neuronal survival is expressed as % of controls treated with CM from unstimulated microglia (100%). Bars represent the mean ± SD obtained from three independent experiments.

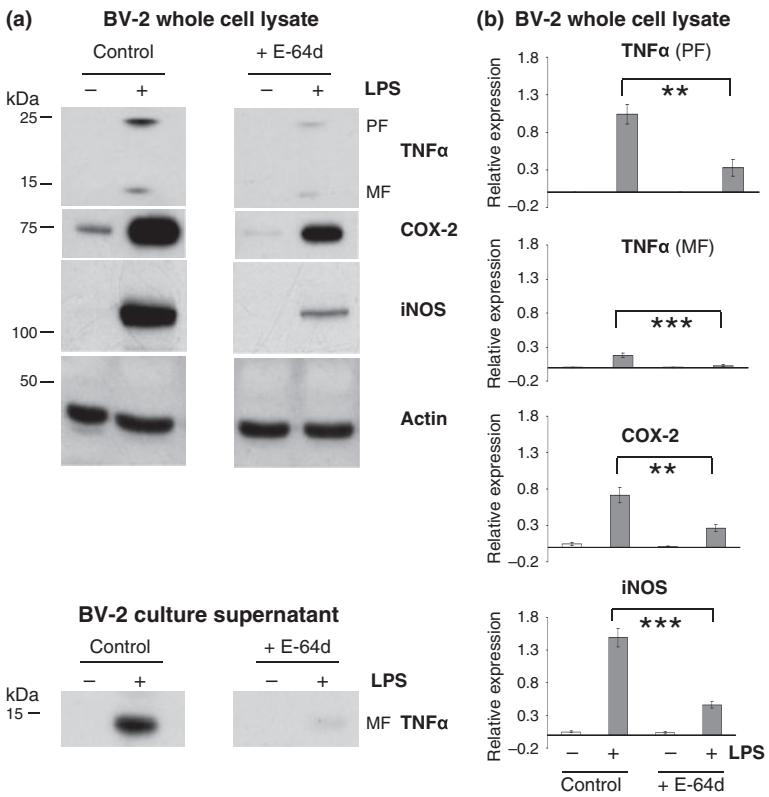

Fig. 5 E-64d decreases the expression of different proinflammatory molecules in activated BV-2 cells. **(a)** Representative western blot analyses of whole cell lysates and culture supernatants of unstimulated (–LPS) and stimulated (+LPS) BV-2 cells after pre-incubation with E-64d (40 μM). Equal amounts of protein and equal volumes of the supernatants, respectively, were loaded. Expression of actin was used as a loading control. **(b)** Quantitative analysis of the protein expression of proinflammatory molecules in unstimulated and stimulated BV-2 cells after pre-incubation with E-64d (40 μM). Bars represent the mean ± SEM obtained from three or four independent experiments. Statistical significance $^{***}p < 0.001$, $^{**}p < 0.01$ (H-test). MF, mature form; PF, proform.

In summary, these data indicated that inhibition of intracellular cysteine proteases reduced the production of nitric oxide and the expression of proinflammatory proteins in LPS-activated microglia. As a result, the neurotoxicity of CM from these cells was diminished.

Discussion

In the present study we have shown that LPS-stimulated microglial cells increase the secretion of the lysosomal cysteine proteases CATB, CATK, CATS, and CATX. In

addition, the protein amounts of CATS and CATX were significantly increased in the cytoplasm of activated BV-2 cells. We also demonstrated that the culture supernatants of LPS-stimulated microglial cells, enriched with CATs, were toxic to neuronal cells. Experiments with membrane-permeable and membrane impermeable cysteine protease inhibitors indicated that this toxic effect was related to the intracellular role of CATs in microglial activation.

Intracellular CATs are discussed as important mediators of inflammatory or apoptotic processes. There are many targets for abnormally distributed cytosolic CATs. Different CATs are able to cleave the proapoptotic factor, Bid, *in vitro*, thus inducing apoptosis (Cirman *et al.* 2004; Boya and Kroemer 2008). However, besides their role in cell death processes, cytosolic CATs may also induce inflammation via the activation of proinflammatory caspases. A direct activation of the proinflammatory caspases 1 and 11 by CATB has been reported (Schotte *et al.* 1998; Vancompernolle *et al.* 1998). *In vivo*, the intracellular effects of CATs may also be crucial in pathological situations characterized by chronic inflammation. Hook *et al.* (2008) showed that E-64d and the membrane-permeable CAT inhibitor CA-074Me improved memory and reduced β-amyloid peptides in a mouse model for Alzheimer's disease.

To draw conclusions from our inhibitor studies about the role of individual CATs in microglial activation, it has to be considered that the applied inhibitors, E-64 and E-64d, are general cysteine protease inhibitors (Barrett *et al.* 1982; Tamai *et al.* 1986). Even though we are intrigued by the significant up-regulation and modified localization of CATS and CATX upon stimulation, E-64 and E-64d also inhibit most other members of the CAT family and cytoplasmic calpains. Therefore, we additionally used the CATB inhibitors, CA-074 and CA-074Me, that are more specific to the CATs investigated in this study. Application of 40 μM of the membrane-permeable inhibitor, CA-074Me, proved to be neuroprotective in our medium transfer model. In contrast to the highly specific CATB inhibitor, CA-074, CA-074Me has been shown to inhibit other cysteine proteases besides CATB. Its effect on CATS is even more potent than that on CATB, with a pK_i of 5 and higher (Montaser *et al.* 2002). On the contrary, intracellular CATX activity was not affected by this inhibitor in BV-2 cells (data not shown). These data point to an important role for CATS in our cell culture model. But to specify all cysteine CATs directly involved in microglial activation further analysis with inhibitors specific for single CATs will be required. Currently, such analysis is hindered by the poor membrane permeability of most of the commercially available CAT inhibitors, rendering an analysis of the effects of intracellular CATs difficult. In addition to the inhibitors mentioned in this study we applied the CATL-specific inhibitor, Z-FF-CHN_2 (Kirschke and Shaw 1981), and the CATS-specific inhibitor, Z-Val-Val-Nle-diazomethylketone (Shaw *et al.* 1993), in our medium transfer experiment. While the CATL inhibitor exerted no neuroprotective effects, the neuroprotective effects of the CATS inhibitor were smaller compared with E-64d (Wendt W., unpublished observations). This may be attributed to the putative poor membrane permeability of the inhibitors. Nevertheless, in the absence of better inhibitors, inhibition of the expression of specific CAT genes using RNA interference might be a more appropriate method to identify single cysteine proteases involved in microglial activation in our cell culture model.

To further analyze possible pathways involved in our cell culture model, the expression of different proinflammatory cytokines up-regulated in activated microglial cells was investigated. Intracellular inhibition of cysteine proteases strongly reduced the expression of TNF-α, iNOS, and COX-2 in LPS-stimulated BV-2 cells. Secretion of active TNF-α protein could nearly be abolished by treatment with E-64d, which may be related to the neuroprotective effect in the medium transfer model. The expression of all the above proinflammatory molecules is regulated by nuclear factor κB (NF-κB) (O'Neill *et al.* 2003; West *et al.* 2006). LPS acts via the toll-like receptor 4 as an inducer of phosphorylation and subsequent cleavage of the inhibitor of NF-κB. Thereupon, NF-κB translocates to the nucleus and starts the transcription of different target genes (Hayden and Ghosh 2004). Our results are consistent with the concept that cysteine proteases mediate NF-κB-regulated transcription of proinflammatory cytokines in activated microglia. A potential target for CATs is cleavage of inhibitor of NF-κB. Different studies link cysteine protease inhibition with a reduced activation of NF-κB (Lin *et al.* 1995; Schaecher *et al.* 2004). Furthermore, CATs might be involved in the post-translational processing of proinflammatory molecules. In a recent publication, CATB was shown to be involved in the trafficking of TNF-α-containing vesicles to the plasma membrane in LPS-stimulated macrophages (Ha *et al.* 2008).

Albeit the murine microglia cell line BV-2 has been described as a suitable model for *in vitro* studies on activated microglia (Blasi *et al.* 1990), the expression of CATS and CATX, which are abundant in microglia (Petanceska *et al.* 1996; Journet *et al.* 2000; Garin *et al.* 2001; Wendt *et al.* 2007), had not been analyzed in BV-2 cells before. As expected, we could show that both CATs are prominently expressed in BV-2 cells. In addition to the lower molecular weight mature forms of CATS and CATX we detected two higher molecular weight bands on western blots. The latter might either represent the pre-proforms and proforms of CATS and CATX or a mixture of different proforms. Pre-proenzymes usually do not appear in culture supernatants and mature lysosomes. However, western blot analyses after protein deglycosylation (Fig. S2), protein dephosphorylation or non-reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (data not shown) showed that we neither detected a mixture of glycosylated and non-glycosylated or

phosphorylated and non-phosphorylated forms nor single- or double-chain species of CATS and CATX proforms. Therefore, the higher molecular weight bands may represent the proforms and not fully processed proforms of CATS and CATX, respectively. Processing of pro-CATs does not occur as a one-step process. Heterocatalytic and autocatalytic activation mechanisms have been reported for several pro-CATs and could explain the appearance of various forms (Nishimura and Kato 1987; Nishimura *et al.* 1988; Salminen and Gottesman 1990; Mach *et al.* 1993), which have also been observed for CATS by other authors (Nissler *et al.* 1999).

Our findings that cytoplasmic and extracellular CATs are increased in LPS-stimulated microglial cells indicate that the cysteine proteases are released from the lysosomes into the cytoplasm and the extracellular space. The cytoplasmic fraction, as it was processed in our studies, still contains microsomes. Therefore, it is possible that the increase in CATs observed in the cytoplasmic fraction results from a translational up-regulation at the endoplasmic reticulum. This would be an explanation for the appearance of proforms in the cytoplasmic fractions. On the other hand, a direct release of CATs from endosomes/lysosomes is also conceivable. Leakage of the lysosomal membrane induced by oxidative stress with subsequent release of CATs into the cytosol and/or extracellular space may occur in different pathological situations (Nakamura *et al.* 1989; Stoka *et al.* 2005). Similar observations have also been described for CATB and CATD *in vivo* in the aged and pathological mouse brain (Bernstein *et al.* 1990; Cataldo and Nixon 1990). Furthermore, specialized lysosomes that have acquired the necessary machinery for fusion with the plasma membrane and may release their content including mature lysosomal enzymes into the extracellular space (Blott and Griffiths 2002) have been described. This might explain the appearance of mature CATs in the culture supernatants of activated BV-2 cells. While increased secretion of CAT proforms is a well-known process, for instance, involved in tumor invasion (Nomura and Katunuma 2005), activated macrophages have been reported to secrete mature enzymes (Reddy *et al.* 1995). *In vitro*, such secretion into the extracellular space from activated macrophages or microglia had already been observed for CATB, CATL, and CATS (Ryan *et al.* 1995; Petanceska *et al.* 1996; Liuzzo *et al.* 1999; Nissler *et al.* 1999; Gresser *et al.* 2001; Kingham and Pocock 2001; Gan *et al.* 2004).

In summary, our study provides a novel view on the involvement of cysteine proteases in neurotoxicity caused by CM from LPS-stimulated BV-2 cells, a cell line with many similarities to microglial cells. Although different CATs are secreted during the activation of BV-2 cells, the inhibition of the secreted CATs did not influence neurotoxicity of the cell supernatant. Our data together with results from other authors on the role of CATs in pathological situations underline the significance of a thorough understanding of the role of CATs in inflammatory reactions and the neurotoxic potential of activated microglia.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Quantitative analysis of CATL (a), CATB (b), CATK (c), CATX (d), and CATS (e) protein expression in unstimulated (−LPS) and stimulated (+LPS) BV-2 cells.

Figure S2. Comparison of peptide *N*-glycosidase F-treated (deglycosylated) and untreated protein extracts from BV-2 cells.

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