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Opposing roles for caspase and calpain death proteases in L-glutamate-induced oxidative neurotoxicity

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ABSTRACT

Oxidative glutamate toxicity in HT22 murine hippocampal cells is a model for neuronal death by oxidative stress. We have investigated the role of proteases in HT22 cell oxidative glutamate toxicity. L-glutamate-induced toxicity was characterized by cell and nuclear shrinkage and chromatin condensation, yet occurred in the absence of either DNA fragmentation or mitochondrial cytochrome *c* release. Pretreatment with the selective caspase inhibitors either benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (pan-caspase), *N*-acetyl-Leu-Glu-His-Asp-aldehyde (caspase 9) or *N*-acetyl-Ile-Glu-Thr-Asp-aldehyde (caspase 8), significantly increased L-glutamate-induced cell death with a corresponding increase in observed nuclear shrinkage and chromatin condensation. This enhancement of glutamate toxicity correlated with an increase in L-glutamate-dependent production of reactive oxygen species (ROS) as a result of caspase inhibition. Pretreating the cells with *N*-acetyl-L-cysteine prevented ROS production, cell shrinkage and cell death from L-glutamate as well as that associated with the presence of the pan-caspase inhibitor. In contrast, the caspase-3/-7 inhibitor *N*-acetyl-Asp-Glu-Val-Asp aldehyde was without significant effect. However, pretreating the cells with the calpain inhibitor *N*-acetyl-Leu-Leu-Nle-CHO, but not the cathepsin B inhibitor CA-074, prevented cell death. The cytotoxic role of calpains was confirmed further by: 1) cytotoxic dependency on intracellular Ca^{2+} increase, 2) increased cleavage of the calpain substrate Suc-Leu-Leu-Val-Tyr-AMC and 3) immunoblot detection of the calpain-selective 145 kDa α-fodrin cleavage fragment. We conclude that oxidative L-glutamate toxicity in HT22 cells is mediated via calpain activation, whereas inhibition of caspases-8 and -9 may exacerbate L-glutamate-induced oxidative neuronal damage through increased oxidative stress.

Introduction

Oxidative stress plays a major role in the induction of neuronal cell death in a number of disease states (Simonian and Coyle, 1996). The L-glutamate-induced model for oxidative stress in the murine hippocampal HT22 cell line has been shown to be an established model of oxidative neuronal cell death (Tan et al., 1998b; Satoh et al., 2000; Dargusch and Schubert, 2002; Rossler et al., 2004). In this model, increased levels of extracellular L-glutamate reduce cystine uptake by limiting or even reversing a cystine/L-glutamate exchanger, the so-called x_c^- transporter (Murphy et al., 1989). Under physiological conditions, this antiporter, which consists of the two subunits xCT and 4F2, mediates the Na^+-independent transport of extracellular cystine into the cell, in exchange for intracellular L-glutamate (Bannai, 1986; Sato et al., 1999). Intracellular cystine is reduced to cysteine which may then be incorporated into the key intracellular antioxidant molecule, glutathione (Halliwell and Gutteridge, 1999). However following excessive L-glutamate exposure, the exchanger is either blocked or even reversed to deplete intracellular cysteine levels. The consequent reduction in intracellular glutathione levels may then render the cell susceptible to reactive oxygen species (ROS)-induced damage, ultimately leading to oxidative cell death (Tan et al., 1998a). The x_c^- transporter is specific for glutamate (Sato et al., 1999) and, at least in PC12 cells, oxidative glutamate toxicity was not mimicked by either aspartate or glycine (Schubert et al., 1992).

Caspases represent a ubiquitous family of aspartate-specific cysteine proteases known to play a prominent role in apoptotic cell death (Riedl and Shi, 2004; Kumar, 2007). They are present in cells as inactive zymogens, organized in a hierarchical manner involving upstream initiator caspases and downstream execution caspases. Upon activation of plasma membrane death receptors (e.g. CD95) or following selective mitochondrial cytochrome *c* release, the apical caspases-8 and -9, respectively become activated and in turn pro-

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teolytically activate the execution caspases-3 and -7 (Riedl and Shi, 2004; Kumar, 2007). Previous studies employing caspase inhibitors have provided conflicting results on the possible caspase role in L-glutamate-induced oxidative neuronal cell death. For example, caspase inhibition was found to prevent L-glutamate-induced HT22 in several studies (Tan et al., 1998a; Tan et al., 1998b; Dargusch and Schubert, 2002; Stanciu and DeFranco, 2002) but this was not reproduced in other studies (van Leyen et al., 2005; Zhang and Bhavnani, 2006). Likewise, Ha and Park (2006) reported an increase in caspase-1 and caspases-3/-7 activity following treatment of HT22 cells with L-glutamate (Ha and Park, 2006) whereas no proteolytic caspase-3 activation was detected in another study (van Leyen et al., 2005). Some of these discrepancies may be explained by experimental differences and biochemical endpoints used to assess cytotoxicity. Furthermore, caspases are not the exclusive protease family which may mediate cell death. Similar to caspases, calpains also require proteolytic activation and possess a similar target specificity, exemplified by αII-spectrin (fodrin), actin and poly(ADP-ribose)polymerase which are cleaved by calpains and caspase-3 (Wang, 2000; Friedrich, 2004). Calpain activation has been associated with many neurological disorders including excitotoxicity (Van den Bosch et al., 2002; Das et al., 2005; Takano et al., 2005), traumatic brain injury (Pike et al., 1998), spinal cord injury (Momeni and Kanje, 2005; Arataki et al., 2005) and ischemia (Rami, 2003; Cho et al., 2004; Kawamura et al., 2005). Indeed, their *in vivo* inhibition has been reported to produce successful cyto- and functional protection (Markgraf et al., 1998; Arataki et al., 2005). In the case of L-glutamate-induced HT22 cell death, the role of calpains has also been controversial, with two studies (Tan et al., 1998b; van Leyen et al., 2005) reporting that calpain inhibitors had no effect on the cytotoxic effect of L-glutamate, whereas a third study reported a dependency of L-glutamate-induced cell death on calpain activity (Zhang and Bhavnani, 2006).

In this study we have employed murine hippocampal HT22 cells to re-investigate the role of proteases in L-glutamate-induced oxidative toxicity. We report here that L-glutamate-induced oxidative cell death shares several morphological features of apoptosis, including cell and chromatin condensation. However, no execution caspase activation was observed and cell death occurred in the absence of cytochrome *c* release from mitochondria and nuclear DNA fragmentation. The mechanism of oxidative cell death was ROS- and Ca^{2+}-dependent and was mediated by calpains. Unexpectedly, caspase inhibition exacerbated L-glutamate-induced oxidative HT22 cell death through enhanced ROS production.

Materials and methods

Chemicals. L-Glutamate, Triton X-100, dimethylsulfoxide, dithiothreitol, EGTA, propidium iodide, RNase A, *N*-acetyl-L-cysteine (NAC), cycloheximide and Hoechst 33258 were purchased from Sigma-Aldrich Ltd, (Poole, UK). Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK), *N*-acetyl-Leu-Glu-His-Asp-aldehyde (Ac-LEHD-CHO), Ac-Ile-Glu-Thr-Asp-aldehyde (Ac-IETD-CHO), *N*-acetyl-Asp-Glu-Val-Asp aldehyde (Ac-DEVD-CHO) and Ac-Asp-Glu-Val-Asp-7-amido-4-trifluoromethylcoumarin (Ac-DEVD-AFC) were bought from Bachem (Bubendorf, Switzerland). [L-3-trans-(propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester (CA-074-Me) and *N*-acetyl-Leu-Leu-Nle-CHO (ALLN) were purchased from Calbiochem (Nottingham, UK). Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC) was purchased from Alexis Biochemicals, Birmingham UK. 5-(and -6)-Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA), BAPTA-AM and all cell culture media and reagents were obtained from InVitrogen (Paisley, UK). FuGENE transfection reagent, lactate dehydrogenase (LDH) detection kit and bovine serum albumin were obtained from Roche (Lewes, UK). Monoclonal anti-cytochrome *c* antibody (clone 6H2.B4) and anti-CD95 antibody (Jo2) were purchased from BD Pharmingen (Oxford, UK). Anti-α-fodrin monoclonal antibody was obtained from Affiniti Research Products (Matford Court, UK) Secondary HRP- and FITC-conjugated antibodies were obtained from DAKO Ltd. (Cambridge, UK).

Cell culture and apoptosis assays. HT22 cells were cultured in DMEM containing 10% FBS, 2 mM L-glutamine and 50 U/ml penicillin and 50 μg/ml streptomycin under a humidified atmosphere (5% CO_2, 37 °C). Unless indicated otherwise, 8×10^3 cells were seeded into 24-well plates and incubated overnight, prior to media renewal and treatment of the cells with the compounds of interest in DMEM containing 2% FBS. Where indicated, the buffering of the cytosolic Ca^{2+} levels was achieved by pre-incubating HT22 cells with 20 μM BAPTA-AM for 30 min prior to the treatment with L-glutamate. For the measurement of cell viability, aliquots of the media were removed at the indicated time points and assayed for lactate dehydrogenase (LDH) release, according to the manufacturer's instructions (Roche, Lewes, UK). Each sample of LDH was compared to the total, Triton X-100 releasable LDH from the same well at the end of each experiment. Morphological evidence for chromatin condensation was obtained by the DNA-specific dye Hoechst 33258 (2 μg/ml) as previously described (Jones et al., 1998). DNA fragmentation was measured by flow cytometric analysis of propidium iodide-stained cellular DNA as previously reported (Macanas-Pirard et al., 2005). In brief, DNA fragmentation was analyzed by flow cytometric detection of hypodiploid DNA. Cells were detached by trypsinization, combined with medium containing floating cells, and centrifuged at 100 g for 5 min. The pellets were fixed in ice-cold 70% (v/v) ethanol in PBS overnight at 4 °C by gradual addition while vortex mixing. The cells were subsequently stained with 10 μg/ml propidium iodide and treated with 1 mg/ml RNase for 30 min at 37 °C before analysis using a Beckman Coulter Epics XL flow cytometer (argon laser, excitation wavelength 488 nm). A minimum of 20,000 events were acquired in list mode while gating the forward and side scatters to exclude propidium iodide-positive cell debris and analyzed in FL-3 for the appearance of the sub-G1 peak.

Caspase and calpain activity assays. *In situ* caspase-3 activity was analyzed using a previously described probe consisting of DsRed and EYFP linked by a 19 amino acid chain containing the caspase-3 cleavage site Asp-Glu-Val-Asp (called pFRET-casp-3) (Elphick et al., 2006). In brief, cells were transfected with pFRET-casp3 by using FuGENE 5 transfection reagent (Roche, Lewes, UK) according to manufacturer's instructions. Following a 72-hour incubation, transfected cells were then exposed to L-glutamate and confocal laser scanning microscopy images were subsequently recorded (Zeiss 510 META) using a 488 nm argon laser excitation, with the emission channel split at 545 nm enabling separation of EYFP (<545 nm) and DsRed (>545 nm) as described previously (Elphick et al., 2006). The proteolytic processing of pro-caspase-3 was assayed by flow cytometric detection of HT22 cells immunostained for active caspase-3 with a phycoerythrin-conjugated anti-caspase-3 antibody (1:20), according to the manufacturer's instructions (BD Pharmingen, Oxford, UK) (Macanas-Pirard et al., 2005). For the assessment of caspase-3/-7 (DEVDase) and calpain activities, HT22 cells were seeded in 175 cm^2 flasks and treated with L-glutamate as described above. At the indicated time points, cells were harvested, resuspended in caspase lysis buffer (40 mM sucrose, 50 mM NaCl, 5 mM EGTA, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.0), freeze-thawed three times and the supernatant collected (15,000 ×g, 30 min). Eighty μg of sample protein was assayed for DEVDase activity with 40 μM Ac-DEVD-AFC (λex=355 nm; λem=480 nm) in caspase assay buffer (100 mM HEPES, 10% sucrose, 0.1% CHAPS, 10 mM dithiothreitol, pH 7.25) (Jones et al., 1998). Additionally, the cells were resuspended in calpain assay buffer (115 mM NaCl, 1 mM KH_2PO_4, 5 mM KCl, 2 mM $CaCl_2$, 1.2 mM