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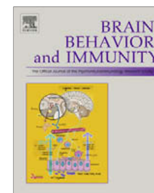

Bid mediates fission, membrane permeabilization and peri-nuclear accumulation of mitochondria as a prerequisite for oxidative neuronal cell death

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ABSTRACT

Mitochondria are highly dynamic organelles that undergo permanent fusion and fission, a process that is important for mitochondrial function and cellular survival. Emerging evidence suggests that oxidative stress disturbs mitochondrial morphology dynamics, resulting in detrimental mitochondrial fragmentation. In particular, such fatal mitochondrial fission has been detected in neurons exposed to oxidative stress, suggesting mitochondrial dynamics as a key feature in intrinsic death pathways. However, the regulation of mitochondrial fission in neurons exposed to lethal stress is largely unknown. Here, we used a model of glutamate toxicity in HT-22 cells for investigating mitochondrial fission and fusion in neurons exposed to oxidative stress. In these immortalized hippocampal neurons, glutamate induces glutathione depletion and increased formation of reactive oxygen species (ROS). Glutamate toxicity resulted in mitochondrial fragmentation and peri-nuclear accumulation of the organelles. Further, mitochondrial fission was associated with loss of mitochondrial outer membrane potential (MOMP). The Bid-inhibitor BI-6c9 prevented MOMP and mitochondrial fission, and protected the cells from cell death. In conclusion, oxidative stress induced by glutamate causes mitochondrial translocation of Bid thereby inducing mitochondrial fission and associated mitochondrial cell death pathways. Inhibiting regulators of pathological mitochondrial fragmentation is proposed as an efficient strategy of neuroprotection.

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1. Introduction

In neurons, mitochondrial function and energy supply is a prerequisite for the function of ion channels, transport proteins, release and recycling of neurotransmitters, calcium buffering, and regulation of apoptosis (Chipuk and Green, 2008; Green, 2005; Kluck et al., 1997; Knott and Bossy-Wetzel, 2008). Mitochondria form a highly dynamic tubular network, and the shape and the size of these organelles are regulated by movements along the cytoskeleton and by frequented fusion and fission events (Bereiter-Hahn and Voth, 1994; Parone et al., 2008; Rube and van der Bliek, 2004). In neurons, such morphological dynamics facilitate the redistribution of mitochondria in response to local changes in the demand for ATP, for example in active synapses or dendrites. Moreover, mitochondrial fission is essential for calcium regulation (Cheung et al., 2007). On the other hand, mitochondrial fusion is needed to exchange mtDNA and other mitochondrial components that may become damaged over time (Okamoto and Shaw, 2005; Chan, 2006; Santel and Frank, 2008). Mitochondrial fusion is therefore important for mitochondrial regeneration and proper function. The rates of fission and fusion are usually balanced but vary between different cell types and may also respond to environmental changes and cellular stress.

Mitochondrial morphology dynamics are controlled by the opposing actions of different dynamin protein family members that are located at or within mitochondria. While many molecules comprising the fission machinery have been identified in yeast, only three mammalian orthologues have been found so far: fission 1 (Fis1), dynamin-related protein 1 (Drp-1), and Endophilin B1 are known to be required for mitochondrial fission in mammals (Karbowski et al., 2004). The mechanisms by which cytosolic Drp-1 becomes activated and recruited to the mitochondria to interact with Fis1 thereby inducing mitochondrial fission, however, remain unclear (Cheung et al., 2007). In contrast to fission, mitochondrial fusion requires both outer and inner mitochondrial components, such as Mitofusin 1 and 2 (Mfn1, 2) and Optical Atrophy protein 1 (Opa1).

Mutations of Mfn2 and Opa1 are associated with severe disturbances in mitochondrial dynamics and hereditary neuropathies, such as Charcot-Marie Tooth disease and autosomal dominant optic atrophy, respectively (Liesa et al., 2009).

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Increasing evidence now also suggests that disturbance of mitochondrial dynamics contribute to neuronal dysfunction and death in various neurodegenerative diseases. For example, rates of mitochondrial fission are significantly accelerated when Cytochrome C is released from mitochondria during apoptosis (Desagher and Martinou, 2000; Wasilewski and Scorrano, 2009) or when mitochondria are depolarized with ionophores (Ishihara et al., 2003). Both, fission and fusion defects may limit mitochondrial motility, decrease energy production, promote oxidative stress and mtDNA deletion, and impair Ca^{2+} buffering, thereby contributing to intrinsic death pathways (Knott and Bossy-Wetzel, 2008; Bossy-Wetzel et al., 2003; Lackner and Nunnari, 2008). Mitochondrial fragmentation in neurodegenerative processes may result from increased mitochondrial fission, decreased mitochondrial fusion, or both.

It has been established that pro-apoptotic Bcl-2 protein family members such as Bax, Bak and Bid mediate mitochondrial dysfunction and associated death signaling in neurons (Arnoult, 2007; Danial and Korsmeyer, 2004; Wang, 2001; Culmsee and Plesnila, 2006), and a number of recent findings suggested that such intrinsic death pathways are associated with extensive mitochondrial fragmentation (Bossy-Wetzel et al., 2003; Karbowski and Youle, 2003; Wang, 2001; Youle and Karbowski, 2005). In the present study, we addressed mitochondrial morphology dynamics in a model of glutamate-induced neurotoxicity in HT-22 cells aiming to elucidate whether Bid was involved in the regulation of mitochondrial dynamics and associated mitochondrial cell death pathways after induction of oxidative stress.

2. Experimental procedures

2.1. Cell culture and induction of neuronal cell death

HT-22 cells were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Karlsruhe, Germany) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine (PAA Laboratories GmbH, Germany). Glutamate (1–5 mM) was added to the serum containing medium and cell viability was evaluated 18 h later.

2.2. Plasmids and gene transfer

The pIRES-tBid vector, mGFP vector and control vectors were generated as described previously (Kazhdan et al., 2006; Duvezin-Caubet et al., 2006). For plasmid transfections 7×10^4 HT-22 neurons were seeded in 24-well plates. Antibiotic containing growth medium was removed and replaced with 900 μl antibiotic free growth medium. Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) mGFP, pIRES-tBid plasmide or empty vector pcDNA 3.1+ were dissolved separately in Optimem I (Invitrogen, Karlsruhe, Germany). After 10 min of equilibration at room temperature each DNA solution was combined with the respective volume of the Lipofectamine solution, mixed gently, and allowed to form plasmid liposomes for further 20 min at room temperature. The transfection mixture was added to the antibiotic-free cell culture medium to a final concentration of 1 μg DNA, and 1.5 μl/ml Lipofectamine 2000 in HT-22 neurons. Controls were treated with 100 μl/ml Optimem I only, and vehicle controls with 1.5 μl/ml Lipofectamine 2000.

2.3. Immunostaining and confocal laser scanning, fluorescence microscopy

For detection of mitochondrial morphology changes during cell death, HT-22 neurons were transfected with the mGFP plasmid, respectively. Twenty-four hours after transfection HT-22 cells were seeded in collagen A-coated Ibitreat μ-slide 8-well plates (Ibidi, Munich, Germany) at a density of 1.7×10^4/well for endpoint analysis on a fluorescence microscope (DMI6000, Leica, Germany).

Alternatively, mitochondria were visualized by MitoTracker Green/Red according to the manufacturer's protocol (Invitrogen, Karlsruhe, Germany). Endpoint pictures were taken after fixation with 4% PFA and DAPI counterstaining of the nuclei 18 h after onset of treatment. Images were acquired using a fluorescence microscope (DMI6000, Leica, Germany) and a confocal laser scanning microscope (LSM 510, Carl Zeiss, Jena, Germany) equipped with an UV, an argon, and a Helium/Neon laser delivering light at 364, 488, and 543 nm, respectively. Light was collected through a 40×1.3 NA, 63×1.4 NA, or 100×1.3 NA oil immersion objectives. DAPI fluorescence was excited at 364 nm and emission was achieved by using the 385 nm long pass filter. Mitotracker Green and mGFP were excited at 488 nm and 543 nm and emissions were detected using 505–530 nm band pass (green) and 560 nm long pass filters (red), respectively.

2.4. Evaluation of mitochondrial morphology

HT-22 cells were transfected with mGFP as described before. After 24 h the cells were reseeded on collagen A-coated Ibitreat μ-slide 8-well plates (Ibidi, Munich, Germany) and treated for 18 h. Endpoint pictures were taken after fixation with 4% PFA and DAPI counterstaining of the nuclei 18 h after onset of treatment. Counting of four different types of mitochondrial states was done in at least four independent experiments with 500 cells. From at least 6 independent pictures per experiment, mitochondrial length was calculated by Image J software (NIH, Bethesda, USA).

2.5. Evaluation cell viability and apoptosis

For morphological analysis of cell viability, transmission light microscopy of living HT-22 neurons growing as monolayers was performed using an Axiovert 200 microscope (Carl Zeiss, Jena, Germany) equipped with a Lumenera Infinity 2 digital camera (Lumenera Corporation, Ottawa, Canada). Light was collected through a 10× 2.5 NA objective (Carl Zeiss, Jena, Germany), and images were captured using phase contrast. Digital image recording and image analysis were performed with the INFINITY ANALYZE software (Lumenera Corporation, Ottawa, Canada). Quantification of cell viability in HT-22 cells was performed in 96-well plates by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction at 0.25 mg/ml for 2 h (Liu et al., 1997). The reaction was terminated by adding dimethylsulfoxide after freezing the plate without media at −80 °C for at least 1 h and absorbance was then determined at 590 nm versus 630 nm (Fluostar OPTIMA, BMG Labtech, Offenburg, Germany). Apoptotic cell death was detected by annexin-V/propidium iodide staining and subsequent flow cytometry analysis. Cells were harvested 18 h after glutamate-treatment by using Trypsin/EDTA, washed once in PBS and stained according to the manufacturer's protocol (Annexin V-FITC Detection Kit, PromoKine, Promocell, Germany). Apoptotic and necrotic cells were determined using FACSScan (BD Bioscience, Germany). Annexin V-FITC was excited at 488 nm and emission was detected through a 530 ± 40 nm band pass filter. Propidium iodide was excited at 488 nm and fluorescence emission was detected using a 680 ± 30 nm band pass filter. To exclude cell debris and doublets, cells were appropriately gated by forward versus side scatter and pulse width, and 1×10^4 gated events per sample were collected. Surviving cells did not show any staining whereas annexin V staining indicated apoptosis and cells positive for both annexin V and propidium iodide were regarded necrotic.

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