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Function of BID – a Molecule of the bcl-2 Family – in Ischemic Cell Death in the Brain

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Key Words
Cerebral ischemia · Apoptotic cell death · Cytochrome c release · BID · Knock-out mice

Abstract
Mitochondrial mechanisms, particularly the release of cytochrome c, play a role in the death of nerve and glial cells in cerebral ischemia. We have currently investigated whether BID, a proapoptotic molecule of the bcl-2 family and promoter of the release of cytochrome c is expressed in the brain, activated by cerebral ischemia in vivo, and contributes to ischemic cell death. We found BID in the cytosol of mouse brain and of primary cultured mouse neurons and showed that neuronal BID is a substrate for caspase 8. BID was cleaved in vivo 4 h after transitory occlusion of the middle cerebral artery. Further, $BID^{-/-}$ mice had a significant attenuation of infarction (–67%) and significantly lower release of cytochrome c (–41%). The findings indicate that the proapoptotic molecule BID may contribute to the demise of nerve cells from cerebral ischemia by release of cytochrome c and activation of caspase.

Introduction

Programmed cell death is a feature of chronic and acute neurodegenerative diseases including Alzheimer's and Huntington's disease, amyotrophic lateral sclerosis (ALS), and stroke [1, 2]. It is mediated, in part, by caspases, a family of cysteine proteases that cleave and disassemble proteins essential for cell survival [3]. Caspases are activated during cerebral ischemia [4–6], and ischemic cell death is significantly attenuated by caspase inhibition [7] or caspase gene deletion [8].

The sequence of events leading to caspase activation has been well characterized in non-neuronal cells [9]. In these cells, BID, a 22-kDa cytosolic member of the Bcl-2 family of proapoptotic proteins [10] provides one mechanism by which TNF/Fas family death receptor activation is linked to downstream events [11]. These death receptors activate caspase 8, which cleaves BID to its truncated active form (tBID; 15 kDa)[12]. tBID targets the outer mitochondrial membrane and induces conformational changes in BAK and BAX [13] and by so doing, triggers cytochrome c release into the cytosol [14]. There cytochrome c together with APAF-1 and caspase 9 form the apoptosome complex, which results in activation of cas-

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pase 3 and other effector caspases and ultimately cause cell death [15]. The importance of BID in death receptor mediated programmed cell death and in amplification of upstream cell death signals is demonstrated by the fact that BID knockout mice are strongly resistant to death receptor induced hepatocellular apoptosis [16].

Very little is known about BID expression and its role in brain, or in fact, whether a similar death pathway involving BID exists in neurons. However, expression of cell surface death receptors of the TNF/Fas family [17–19], cleavage of caspase 8 [5] and caspase 3 [6], and release of cytochrome c from mitochondria [20], have all been described individually in ischemia. Given these similarities, we hypothesized that BID may figure as a mediator upstream of mitochondria in neuronal death after cerebral ischemia. To explore this hypothesis, we used a well-characterized model of ischemic cell death (middle cerebral artery occlusion, MCAo). We determined whether BID knockout mice were protected against ischemia, and examined the relationship between the presence of activated BID (tBID) and cytochrome c release in adult mouse brain.

Part of the original findings of this study have already been published elsewhere [21].

Methods

Cytosolic and Mitochondrial Proteins

Tissues from adult C57Bl/6 mice (20–25 g) were homogenized in 10 volumes of hypertonic buffer [22]. Supernatants containing cytosolic proteins were isolated by centrifugation at 20,000 g. tBID was enriched by immunoprecipitation (150 μg protein incubated with anti-BID/tBID antibody at 1:500 dilution over night). All procedures were performed at 4°C.

Western Blot

10–20 μg protein/lane were separated and blotted as previously described [23]. The blot was probed with anti-Bid (rabbit polyclonal, 1:1,000), or anti-cytochrome c (clone 7H8.2C12, 1:200, BD PharMingen, Franklin Lakes, N.J., USA) at 4°C overnight. Membranes were then exposed to horseradish-peroxidase-conjugated anti-rabbit IgG for 1 h. Antibody binding was detected using the ECL system (Amersham, Arlington Heights, IL). Equal protein loading of gels was assured by Coomassie Blue or α-tubulin staining. Relative optical density of protein bands was quantitated by using the Bio-Rad Multi-Analist Version 1.0

Transgenic Mice

BID-deficient mice ($BID^{-/-}$) were generated as previously described [16] and backcrossed 7–8 generations into its C57Bl/6 background strain. $BID^{-/-}$ mice were born at the expected Mendelian frequency and showed no apparent developmental abnormalities. As controls wild type animals ($BID^{+/+}$) from the same litter ('littermates') were used for all experiments. The macro- and microscopic brain morphology of uninjured $BID^{-/-}$ mice did not differ from wild type controls. Only male mice matched for age (6 weeks) and weight (20–25 g) were used for experiments.

Transitory Focal Cerebral Ischemia

Cerebral ischemia was induced by an intraluminal filament as previously described [24]. Successful occlusion and reperfusion was demonstrated by Laser-Doppler flowmetry (PF2B; Perimed, Stockholm, Sweden) in each animal. Functional outcome was assessed on a 5-point scale (0 = no deficit, 1 = weakness of the contralateral forepaw, 2 = circling, 3 = loss of righting reflex, 4 = no motor activity).

Cytochrome c Assay

Cytochrome c in cytosolic fractions of brain homogenates was measured by an ELISA system specific for mouse cytochrome c (R&D Systems, Minneapolis, Minn., USA). Homogenates were prepared from an equivalent lesioned area (striatum) in both strains. Results are expressed as ng cytochrome c/mg protein. Values obtained from the non-ischemic hemisphere were subtracted from the values for the ischemic hemisphere in order to obtain a measure for cytochrome c release specific to ischemia. The cytosolic origin of the samples was proven by the fact that cytochrome oxidase, a mitochondrial protein, was not detected in these brain fractions (data not shown).

Statistical Analysis

Data are presented as mean ± SEM. All statistical analyses were made with the SigmaStat 2.0 software package (Jandel Scientific, Erkrath, Germany). For comparison between WT and $BID^{-/-}$ groups the Mann-Whitney Rank Sum test was used. $p < 0.05$ was considered statistically significant.

Results

Expression of BID in the Brain

We characterized BID expression by demonstrating its presence in brain. A 22-kDa band corresponding to BID was observed in murine whole brain homogenates (n = 2). The 22-kDa band was absent in homogenates from $BID^{-/-}$ mice (n = 2; fig. 1).

Activation of BID after Cerebral Ischemia

In wild type mice, we demonstrated by Western Blot analysis that BID was cleaved to its 15-kDa active fragment (tBID) 2 h following 2 h of middle cerebral artery occlusion (fig. 2).

Protection in $BID^{-/-}$ Mice after Focal Cerebral Ischemia

In order to investigate if BID mediates ischemic cell death, $BID^{-/-}$ and wild-type littermate mice were subjected to 30 min of middle cerebral artery occlusion and 48 h of reperfusion. Blood flow distal to the occlusion dropped below 20% in the two groups and returned to

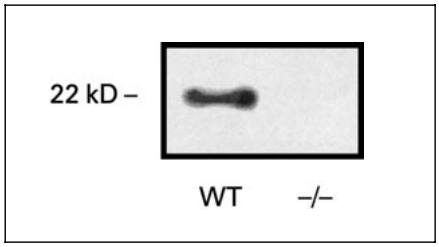

Fig. 1. BID is expressed in mouse brain. Mouse (C57B/6) brain homogenate was probed for BID with a rabbit polyclonal antibody (Western blotting). A 22-kDa band was detected which corresponds to recombinant mouse BID [12]. This band was not detected in brain homogenate from BID$^{-/-}$ mice (n = 3). From [21].

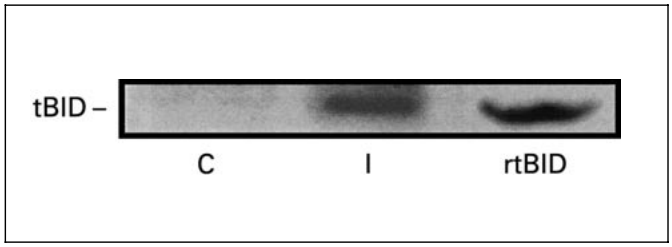

Fig. 2. BID cleavage after focal ischemia. Two hours after 2 h of MCAo, brain homogenates from the ischemic (I) and contralateral hemisphere were subjected to immunoprecipitation with an anti-tBID antibody followed by Western blot analysis. tBID obtained by incubation of recombinant BID with recombinant active caspase 8 was used as positive control (tBID) (n = 2). From [21].

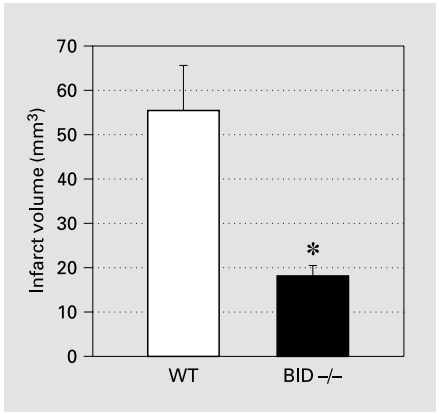

Fig. 3. Ischemic damage is reduced in BID$^{-/-}$ mice. Infarct volume was reduced by 67% in BID$^{-/-}$ mice as compared to wild type mice (n = 8 per group; * $p < 0.01$) after 30 min middle cerebral artery occlusion and 48 h reperfusion. From [21].

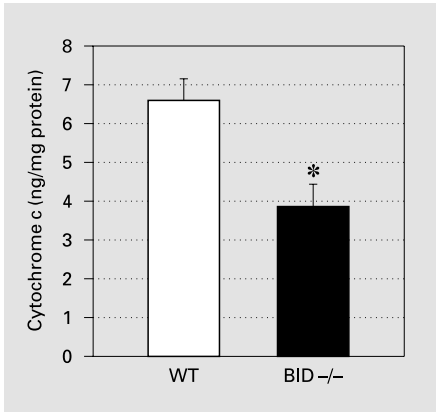

Fig. 4. Cytochrome c release after focal ischemia. Following ischemia, 41% less cytochrome c was measured by ELISA in the cytosol of BID$^{-/-}$ mice (n = 4) as compared to wild type littermates (n = 4; * $p < 0.03$). From [21].

pre-occlusion levels after recirculation. The mean infarct volume was 67% smaller in BID$^{-/-}$ mice (55.5 ± 29.0 mm^3 and 18.3 ± 6.4 mm^3 in wild type and mutant, respectively; n = 8 per group; $p < 0.002$; fig. 3). In wild type animals, the lateral striatum and the adjacent cerebral cortex showed extensive ischemic injury, while the cortex was spared in BID$^{-/-}$ mice. Animals had only slight neurological deficits 24 and 48 h after ischemia (0.4 ± 0.2) and there was no difference between groups. Following ischemia, brain edema was minimal and did not differ between groups: the volumes of the ischemic hemispheres were 102 ± 3% and 104 ± 5% of the contralateral hemispheres in wild type and mutants, respectively.

Cytochrome C Release in BID$^{-/-}$ Mice after Focal Cerebral Ischemia

Next we investigated whether BID was part of apoptotic signaling pathways in vivo by comparing the levels of cytosolic cytochrome c in homogenates of ischemic brains from wild type and BID$^{-/-}$ mice subjected to 2 h of MCAo. A significant reduction (–41%) was found in the mutant brain by an ELISA method (3.9 ± 1.1 vs. 6.6 ± 1.1 ng/mg protein for mutant and wild type, respectively, n = 4; $p < 0.03$; fig. 4). As an important precondition to interpreting these experiments, we verified that there were no pre-morbid differences between wild type and BID$^{-/-}$ groups in brain volume, vascular anatomy, resting

absolute blood flow (209 ± 24 vs. 220 ± 17 ml/100 g/min in wild type and mutants, respectively), or overt behavior.

Phenotypes of BID$^{-/-}$ Mice

Macroscopically we observed no differences in brain size or weight, or differences in histology (HE and cresyl violet) between mutants and wild type strains. There were no differences in numbers of neurons when other sites like superior cervical ganglia and facial nerve nucleus were counted (Korsmeyer, pers. commun.). The BID$^{-/-}$ mice show no other obvious abnormal neuronal phenotypes and the development of neuronal lineages is apparently normal [16].

Discussion

We established that BID is a critical mediator of ischemic cell death within the central nervous system. We observed reduced ischemic brain injury in mice with deletion of the BID gene (fig. 3). We also found BID cleavage in ischemic brain after ischemia (fig. 2). Treatment with caspase inhibitors attenuated cell death and tissue injury after in vivo and in vitro ischemia [21, 22]. Hence, BID as well as caspases are critical cell death mediators of ischemic brain injury.

In non-neuronal cells, caspase activation can occur via a sequence of events known as type II death receptor signaling [25], in which stimulation of death receptors activates caspase 8. Caspase 8 in turn cleaves BID to tBID. After migration to the mitochondrial membrane and interaction with Bax tBID causes cytochrome c release from mitochondria, which results in the activation of caspase 3 and other caspases. Our data show that the molecular pathway for ischemic cell death in neurons resembles the type II pathway. Caspase 8 is constitutively expressed in brain and focal ischemia in vivo triggers activation of caspase 8 [5]. We have evidence that caspase 8 cleaved BID to tBID from brain homogenates [21]. Taken altogether, it is likely that caspase 8 mediates BID cleavage in the CNS as it is the cysteine protease with the strongest BID-cleaving activity [12].

Regarding cytochrome c release, BID acts upstream of both cytochrome c release and caspase-3 activation in non-neuronal cells [12]. In the CNS, we found attenuated cytochrome c release in ischemic striatum from BID$^{-/-}$ mice following middle cerebral artery occlusion (fig. 4) and less caspase 3 activation after oxygen glucose deprivation in BID$^{-/-}$ cortical neurons [21]. In addition, BID deletion reduces cytochrome c release, and protects against fas-mediated liver injury [16] and against ischemia-induced CNS injury in vivo as shown in our present study.

Despite strong evidence linking BID to pore formation in the outer mitochondrial membrane, and to cytosolic release of cytochrome c, cytochrome c release was not completely abrogated in cells and tissues of BID$^{-/-}$ mice (fig. 4). Release was apparently not caused by rupture of mitochondrial membranes as we did not detect cytochrome oxidase, a mitochondrial protein, in cytosolic fractions at the same time (not shown). Our data suggest that BID is not the sole mechanism for cytochrome c release after cerebral ischemia. Other mechanisms and molecules may contribute to the release process such as unique mechanisms related to Bax, Bad, Bcl-2, Bcl-X_L, oxygen radicals, MnSOD, or opening of the mitochondrial permeability transition [26–29]. Furthermore, BCL-2, and BCL-w modulate cytochrome c release and, for example, an increase in their expression could protect tissues from injury during ischemia [30, 31]. BID and other mechanisms may also effect survival in non-neuronal cell types (e.g., astrocytes, endothelial cells, microglia) which were spared in ischemic BID$^{-/-}$ mouse brain. Further work is necessary to clarify this point. Nevertheless, the results generated using BID$^{-/-}$ mice convincingly demonstrate a prominent role for BID in acute CNS injury. Future strategies using gene therapy including dominant-negative or anti-sense approach to eliminate BID may be utilized to determine if they may help reduce brain injury after cerebral ischemia.

In summary, BID promotes cell death in the brain following focal cerebral ischemia. Because BID is strategically located upstream of mitochondria and caspase 3 processing [32], BID presents an attractive therapeutic target for central nervous system diseases in which apoptotic cell death is prominent.

Acknowledgments

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