Published in final edited form as:

Transl Stroke Res. 2018 June ; 9(3): 215–222. doi:10.1007/s12975-017-0572-0.

Dysfunction of Membrane Trafficking Leads to Ischemia-Reperfusion Injury after Transient Cerebral ischemia

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Abstract

Neurons requires an extraordinarily high level of membrane trafficking activities because of enriched axonal terminals and dendritic branches. For that reason, defects in the membrane trafficking pathway are a hallmark of most, and may be all, neurodegenerative disorders. A major cellular membrane trafficking pathway is the Golgi apparatus (Golgi hereafter) – late endosome - lysosome axis for supplying lysosomal enzymes. This pathway is regulated by N-ethylmaleimide sensitive factor (NSF) ATPase. This review article is to discuss a novel hypothesis that brain ischemia inactivates NSF ATPase, resulting in a cascade of events of disruption of the Golgi – endosome - lysosome pathway, release of cathepsin B (CTSB), and induction of mitochondrial outer membrane permeabilization (MOMP) during the postischemic phase. This hypothesis is supported by recent studies demonstrating that NSF is trapped into inactive protein aggregates in neurons destined to die after brain ischemia. Consequently, Golgi, transport vesicles (TVs), and late endosomes (LEs) are accumulated and damaged, which is followed by CTSB release from these damaged structures. Moderate release of CTSB cleaves Bax-like BH3 protein (Bid) to become active truncated Bid (tBid). Active tBid is then translocated to the mitochondrial outer membrane, resulting in oligomerization of BCL2 Associated X Protein (Bax) forming the mitochondrial outer membrane pores, and releasing mitochondrial intramembranous proteins. Extensive CTSB release, however, can digest cellular proteins indiscriminately to induce cell death. Based on these new observations, we propose a novel hypothesis, i.e., brain ischemia leads to NSF inactivation, resulting in a massive buildup of damaged Golgi, TVs and LEs, fatal release of CTSB, induction of MOMP, and eventually brain ischemia-reperfusion injury.

Keywords

Brain ischemia-reperfusion injury; Membrane trafficking; NSF; Cathepsin B; Golgi; Transport vesicle; Late endosome; Lysosome; mitochondrial outer membrane permeabilization

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Compliance with Ethical Standards:

Conflict of Interest: Dong Yuan declares no conflict of interest. Chunli Liu and Bingren Hu are husband and wife

Ethical approval: This article does not contain any studies with human subjects. All the experimental procedures involving using animals were approved by the Animal Use and Care Committee in the University of Maryland School of Medicine.

Introduction

Transient cerebral brain ischemia or cardiac arrest leads to delayed neuronal death that occurs after 3 days of reperfusion. During the delayed period, all neurons appear perfectly normal under the light microscope [1–6]. Under electron microscopy (*EM*), however, massive buildup of damaged Golgi, transport vesicles (*TVs*), and late endosome (*LE*) takes place over time in neurons destined to die after transient cerebral or global ischemia [7–10].

Membrane trafficking

In a typical cell, the entire surface membrane turns over every hour via endocytosis and exocytosis [11]. The membrane trafficking activities of endocytic, exocytic, organelle delivery of newly synthesized proteins, and autophagy are mediated either via transport vesicles (*TVs*) or membranous organelles. In eukaryotic cells, membranous or secretory proteins are mostly synthesized in the endoplasmic reticulum (ER)-associated polyribosomes. After synthesis, they are translocated to the ER lumen and then transported into Golgi apparatus before being delivered to the destined or targeted organelles or cell membrane (Fig. 1A). At the destination, the delivery transport vesicle or organelle membrane fuses with the target organelle or cell membrane to assemble or deliver the cargo (Fig. 1A). Neurons have enriched axonal terminals and dendritic branches with extraordinarily large surface area, and thus require an extremely high level of endocytic, exocytic, the protein delivery, and autophagic membrane trafficking activities (Fig. 1A). This may be why defects in the membrane trafficking pathway preferentially affect neurons and are a hallmark of most, and maybe all, neurodegenerative disorders [12].

Membrane trafficking requires membrane-to-membrane fusion either between transport vesicles and organelles, or between two organelles, encompassing the following 4 major types: (1) to deliver organelle proteins; (2) exocytic or secretory pathway, (3) the endocytic pathway; and (4) the autophagy pathway (Fig. 1A). Among membrane trafficking structures, the late endosome (*LE*), representing the stomach of the cell [13,14], acts as a central hub receiving: (i) newly synthesized lysosomal proteins (e.g., cathepsins) from Golgi-derived transport vesicles (*TVs*), and (ii) degradative cargo from both the endocytic and autophagic pathways (Fig. 1A). *LE* (luminal pH=5.5) then fuses with lysosome to become a more acidic (pH<5) hybrid endolysosome or *EL* (=secondary lysosome) for the execution of degradation [13,14]. After digesting cargo, *EL* becomes a lysosome (*L*) that contains evenly distributed content. The lysosome is then recycled to fuse with *LE* again for the next round of digestive process (Fig. 1, A and B) [15].

As shown in Fig. 1B, the core elements of the membrane trafficking machinery are: (i) **NSF** (N-ethyl-maleimide sensitive factor ATPase); (ii) **SNAP** (soluble NSF attachment protein), and (iii) **SNAREs** (soluble NSF attachment protein receptors). NSF is the sole ATPase for regenerating active SNAREs after membrane-to-membrane fusion. SNAP is an adaptor for connecting NSF to SNAREs and also for stimulating the NSF ATPase activity. SNAREs are fusion proteins and divided into vesicle SNAREs (v-SNAREs) and target membrane SNAREs (t-SNAREs). Interaction between v- and t-SNAREs brings two cellular membranous organelles together, also known as SNAREs tethering, before membrane-to-

membrane fusion (Fig. 1B). After membrane-to-membrane fusion, SNAREs form inactive complexes and must be regenerated by NSF ATPase for the next round of fusion (Fig. 1B). During this process, cytosolic free NSF ATPase interacts with SNAREs via an adaptor protein SNAP and hydrolyzes adenosine triphosphate (ATP) to dissociate the inactive SNARE complex into active SNAREs (Fig. 1B) [16]. To date, there is only a single form of NSF ATPase in most organisms except in *Drosophila* that expresses dNSF-1 and dNSF-2 [17]. For that reason, NSF deficiency will bring all neuronal membrane trafficking activities to a halt (Fig. 1B). In comparison, SNAREs are both redundant and abundant [11,16]. Three sets of SNAREs are relatively organelle-specific, including: (i) presynaptic SNAREs (e.g., syntaxin-1 and synaptobrevin); (ii) trans-Golgi network SNAREs [e.g., STX16 and Vti1a]; and (iii) late endosome or LE SNAREs (e.g., Vti1b) [15,16]. The SNARE organelle specificity may be utilized to investigate specific Golgi-to-LE, and LE-to-lysosome fusion events and organelle damage after brain ischemia [18].

Depletion of active NSF ATPase owing to its inactive deposition in neurons after brain ischemia

Only cytoplasmic free NSF hexamer ATPase is the active form for dissociating the inactive SNARE complex, whereas deposition of NSF into a Triton-insoluble fraction results in NSF inactivation [17]. This is consistent with the confocal microscopic results showing that NSF is depleted from the cytoplasm during the period of 0.5 – 72 h of reperfusion only from CA1 neurons destined to die after transient cerebral ischemia [18,19]. Depletion of the cytosolic ATPase can be viewed by confocal microscopy. Fig. 2 shows confocal microscopic images of CA1 neurons labeled with NSF antibody. Brain sections were obtained from a sham-operated control rat and rat subjected to 15 min of ischemia followed by 24 h of reperfusion. NSF is located in the peri-nuclear and dendritic trunk (Sham, arrowheads), as well as in the neuropil (Sham, arrows) of sham-operated control CA1 neurons. NSF is mostly depleted from the CA1 neuronal perinuclear region and dendritic trunk (24h, arrowheads), but remains relatively at the sham control level in the CA1 neuropil (24h, arrows) at 24 h of reperfusion after transient cerebral ischemia. In contrast, NSF is less affected in surviving neurons such as those in the DG after the same period of transient cerebral ischemia [18,19]. Depletion of cytoplasmic NSF is owing to deposition of NSF into inactive protein aggregates, as demonstrated by the sucrose gradient centrifugation study [18]. The NSF-containing aggregates form very dense structures *in vivo* and cannot be accessed by NSF antibody during immunostaining of brain sections [18–20]. As mentioned, NSF is the sole ATPase to regenerate active SNAREs for controlling membrane trafficking [15–17]. Intracellular NSF deficiency can result in severe neuronal damage and eventually neuronal death [15–19].

Ultrastructural characterization of buildup of Golgi fragments, and transport vesicles (TVs), and late endosomes (LEs) after brain ischemia

Golgi and endosomal membranes are among the most dynamic cellular structures with the highest concentration of SNAREs, thus requiring the highest amount of NSF to regenerate active SNAREs [21]. In contrast, other membranes, such as the ER membranes, may have a

relatively lower concentration of SNAREs, thus requiring a lower level of NSF for regenerating active SNAREs [21]. For that reason, inactivation of NSF affects mainly the Golgi – LE – lysosome axis. In cultured cells or cell free systems, inactivation of either NSF or the key SNAREs leads to massive buildup of *Golgi fragments, TVs and enlarged LEs* [21,22]. Expression of an activity-deficient mutant of syntaxin 11 (an endosomal SNARE protein) inhibits the late endosome to lysosome fusion in macrophages, resulting in an accumulation of enlarged LEs [22].

Could inactivation of NSF also lead to buildup of Golgi fragments, and *TVs*, and *enlarged LEs* after brain ischemia? EM studies demonstrate that this is the case. Massive accumulation of protein aggregate-associated *Golgi* fragments, *TVs*, and enlarged *LEs* is the most prominent ultrastructural change in neurons destined to die after brain ischemia [7–10]. As shown in Fig. 3, under the EM, most prominent changes in neocortical and CA1 neurons destined to undergo neuronal death after transient cerebral ischemia are massive accumulation of protein aggregate-associated Golgi fragments, TVs, and enlarged LEs. A sham-operated control CA1 neuron, stained with the osmium-uranium-lead, has normal rough endoplasmic reticulum (ER), polyribosome rosettes (small arrows), Golgi (G), mitochondria (M), endolysosomes (EL, also known as secondary lysosome), and LE (large arrows) (Fig. 3a). However, the intact Golgi structures are difficult to find in CA1 and some neocortical neurons after transient cerebral ischemia, because they are completely fragmented (Fig. 3b, Gf). Meanwhile, massive buildup of TVs (Fig. 3b, arrowheads) and enlarged LEs (Fig. 3b, large arrows), as well as monoribosomes (Fig. 3b, small arrows), were observed in postischemic neurons. The Golgi fragments (Gfs), TVs and LEs are often associated with protein aggregates (Fig. 3, Stars). The LE structures in the insets of Figs. 3a and 3b can be better viewed with a higher magnification of EM images shown in Figs. 3c and 3d. EL from the sham-operated control neuron have intact lipid membranes and contain partially digested cargo (Fig. 3c, EL) while LE contains multi-vesicular structures (Fig. 3c, arrow). In comparison, the LE structures from the postischemic neuron are enlarged, containing opaque vesicle structures, and have multiple membranous breaks or damage (Fig. 3d, arrowheads). As described above, accumulation of damaged Golgi fragments, TVs and LEs in neurons observed by EM may be a result of NSF deficiency after transient cerebral or global ischemia.

Cathepsin B (CTSB)

In comparison with other cathepsins, cathepsin B (*CTSB*) shows the highest level of expression in neurons [23,24]. CTSB has three forms (Figs. 4 and 5): (i) 46kDa proCTSB located in Golgi/TVs, (ii) 33kDa CTSB in LE, and (iii) 24/25kDa CTSB in EL and lysosomes. After synthesis from the ER, the 46kDa proCTSB moves to Golgi where it is further modified, and then packed into TVs that move toward and fuse with LE via NSF-mediated machinery (Fig. 5). In the acidic environment of the LE's lumen (~pH 5.5), the proregion of 46kDa proCTSB is cleaved to become a mature **33kDa** LE CTSB (Fig. 5). The LE then fuses with lysosome (mediated also by the NSF machinery) to become endolysosome (EL=secondary lysosome) (Fig. 5). In the more acidic luminal environment of the EL's (pH<5) and lysosomal (~pH 4.5) lumen, the **33kDa** LE CTSB is further cleaved

into a **24/25 kDa** EL/lysosomal CTSB [24–27] (Figs. 4 and 5). **46kDa** Golgi proCTSB, **33kDa** LE CTSB and **24/25 kDa** lysosomal CTSB all have proteolytic activities [24].

Neuronal release of 33 kDa LE CTSB after brain ischemia

Inhibition of NSF ATPase leads to a halt of the CTSB delivery from Golgi to LE, and then from LE to lysosome (Fig. 5), thus trapping CTSB inside Golgi, TV and LE structures, resulting in enlargement of these structures [28–33]. It has been shown that Golgi, LE, and lysosome structures have the highest level of NSF, SNAP and SNAREs to accommodate for the higher membrane trafficking activities [21,22]. Therefore, inactivation of NSF ATPase after transient cerebral ischemia may interfere mainly with the Golgi-to-LE and LE-to-lysosome membrane fusion, resulting in accumulation of 33kDa LE CTSB and concomitant reduction of 24/25 kDa lysosomal CTSB [18]. In addition, the 46kDa proCTSB can autocleave its own propeptide in Golgi and TVs to become a 33kDa CTSB [25,26], particularly in the postischemic acidosis condition [5,18], and thus may also contribute to the accumulation of 33 kDa CTSB and concomitant reduction of 46kDa Golgi CTSB.

This has been observed in a recent study showing that 33kDa CTSB is significantly increased, while 46 kDa Golgi and 24/25kDa lysosomal CTSB are correspondingly decreased in the cytosolic and intracellular microsomal membrane fraction prepared from the brain tissue samples after transient cerebral ischemia [18]. Therefore, 33kDa CTSB may be released not only from massively enlarged LEs, but also from Golgi and TVs (Fig. 5). However, CTSB may not be released from ELs or lysosomes, because EL/lysosomal CTSB is 24/25kDa. Release of 33kDa CTSB (that is an active protease) into the cytoplasm can further damage Golgi and LE, resulting in amplified release of 33kDa CTSB into the cytoplasm (Fig. 5).

In consistent with the biochemical analysis described above, confocal microscopic study further shows that CTSB antibody stains only small dots in non-ischemic sham-operated control CA1 neurons. In contrast, both the size and number of CTSB-immunostained structures are dramatically increased in CA1 neurons after transient cerebral ischemia [18]. Furthermore, CTSB-immunostained punctate pattern in sham-operated control CA1 neurons become diffused or evenly distributed throughout the cytoplasm of CA1 pyramidal neurons after transient cerebral ischemia [18]. These results suggest that CTSB may be released from the structures of Golgi fragments, TVs, and LEs (see Fig. 5).

Why CTSB is an important cathepsin after brain ischemia?

There are at least 15 cathepsins distributed in various organs. The cathepsins most commonly implicated in cell death are **CTSB** and **cathepsin D (CTSD)** [34,35]. Among cathepsins, CTSB is the most abundant cathepsin in neurons [22,23]. Our recent study shows that CTSB is dramatically increased in the cytoplasm of CA1 neurons after global brain ischemia while CTSD is unchanged (data not shown), suggesting that CTSB may be a major cathepsin released into the cytoplasm of neurons destined to die after transient cerebral ischemia.

Could CTSB inhibitors offer neuroprotection against brain ischemia-reperfusion injury?

Of relevance, at cytosolic neutral pH ($\text{pH} \approx 7.4$), CTSB exhibits proteolytic activity whereas most other cathepsins have minimal activities [13]. Furthermore, although not yet studied in mature brain ischemia models, CTSB knockout (KO) in mice protects neonatal hippocampal neurons from hypoxic-ischemic injury [36]. Therefore, CTSB KO in mice might also protect adult mature neurons from ischemia-reperfusion injury.

Previous studies show that the chemical agents CA-074me and E64d might offer neuroprotection by inhibiting CTSB after brain ischemia [37,38]. However, these inhibitors are not ideal therapeutic agents for the following reasons. First, treatment of cells with CA-074me or E-64d increases both lysosomal CTSB half-life and concentration by >3-fold, and thus may postpone CTSB release and result in more severe brain injury [39,40]. Second, E-64d is not a CTSB-specific inhibitor and recent studies indicate that CA-074me is not CTSB-specific either, despite previous claims [41–43]. Third, the inhibitory effects of CA-074me and E64d are pH-dependent; they are strong inhibitors at pH4.5 in the lysosomal luminal acidic environment, but ineffective inhibitors for released CTSB at the cytosolic neutral pH [44]. Because cytosolic CTSB is harmful while lysosomal CTSB is required for degradation of toxic aggregates and damaged subcellular organelles, the ideal inhibitors will be strong inhibitors for released CTSB at neutral pH, and weak inhibitors for lysosomal acidic luminal CTSB. Such inhibitors are not currently available.

CTSB Release induces mitochondrial outer membrane permeabilization (MOMP)

Previous studies show that moderate CTSB release induces MOMP by activation of the tBid – Bax or Bak pathway, resulting in cell death, as shown in Fig. 6 [45,46]. Bak is translationally repressed in mature neurons and thus its protein is not expressed under either normal or stress conditions [47], whereas both Bid and Bax are expressed in mature neurons [48]. The full-length Bid protein does not expose its mitochondrial anchoring segment and is inactive in the cytosol of healthy cells. Moderate cytoplasmic release of CTSB cleaves the Bid autorepression domain to produce active truncated Bid (tBid), resulting in tBid translocation to the mitochondrial outer membrane. tBid then induces oligomerization of Bax to form mitochondrial outer membrane pores (Fig. 6). As a result, MOMP is induced, leading to cell death (Fig. 6).

MOMP has been detected in several paradigms of neuronal death and is commonly considered as the ‘point of no return’ [45]. A distinctive sign of MOMP is cytoplasmic release of proteins from the intermembrane space (between the inner and outer mitochondrial membranes) such as cytochrome C (CytC), apoptosis inducing factor (AIF), endonuclease G (EndoG), and Htra2 [49–52] (Fig. 7). CTSB-dependent Bid cleavage, as well as tBid and Bax translocation to mitochondrial outer membrane have been observed in *in vivo* and cell culture studies [53–56]. Bcl2 and Bcl-xL may also be involved in MOMP

[53–56]. Extensive CTSB release, however, can digest proteins indiscriminately to directly induce cell death [46].

Several recent studies indicate that a vacuole-like structure between mitochondrial inner and outer membranes is a distinctive morphological sign of MOMP [54,55]. This was observed by an immunogold EM study showing that mitochondrial outer membrane was associated with clusters of immunogold-labeled ubiquitinated proteins (Fig. 7, 24h, arrows), suggesting that neuronal mitochondrial outer membrane proteins were damaged and thus ubiquitinated at 24 h of reperfusion when CTSB was released after brain ischemia [24]. Many mitochondrial inner and outer membranes are separated to form vacuole-like structures, as observed in brain sections in vivo with the EM three-dimensional reconstruction technique (Fig. 7B, 24h, yellow color, arrows), suggesting damage to the mitochondrial outer membrane and induction of MOMP may occur in neurons displaying CTSB release after brain ischemia. Western blotting further shows the increased protein ubiquitination in the isolated mitochondrial fraction, indicating that mitochondrial proteins are indeed damaged after brain ischemia (Fig. 7C, 24h).

Concluding Remarks

It is well established that neuronal death after cardiac arrest or transient cerebral ischemia occurs in a delayed fashion selectively in some population of neurons such as hippocampal CA1 and neocortical pyramidal neurons [1–6]. The search for mechanisms underlying neuronal ischemia-reperfusion injury has been extensive, but the underlying mechanisms remain incompletely understood. Basic on the latest studies, this review article proposes a novel hypothesis that brain ischemia inactivates NSF ATPase, resulting in a cascade of events of disrupting the Golgi – endosome - lysosome pathway. Consequently, CTSB is released from damaged Golgi fragments, TVs and LEs into the cytoplasm, resulting in induction of MOMP. MOMP is commonly considered as cell damage that has reached a ‘point of no return’ [45]. Our latest studies also show that depletion of active NSF also occurs in neocortical neurons after 20 min of transient cerebral ischemia [18]. Therefore, the NSF inactivation-induced damaging events have emerged as a novel and common mechanism of brain ischemia-reperfusion injury. However, the details of the cascade of events resulting in fatal CTSB release and induction of MOMP after brain ischemia remain to be studied. Understanding the cascade of events of membrane trafficking damage, CTSB release, and induction of MOMP will provide novel insights into the mechanisms of brain ischemia-reperfusion injury and identify new therapeutic targets for its treatment.

Acknowledgments

Funding: This work was supported by National Institutes of Health (NIH) grants: NS36810, NS40407, and NS097875; by Veteran Affairs Merit grant: I01BX001696; and by American Heart Association 0940042N-5 to B.R.H.

Common Abbreviations

NSF	N-ethylmaleimide sensitive factor ATPase
E329Q	a NSF deficient mutant

SNAREs	soluble NSF attachment protein receptors
SNAP	soluble NSF attachment protein
CTSB	cathepsin B
CTSD	cathepsin D
TVs	transport vesicles
LE	late endosome
EL	endolysosome
L	lysosome
MOMP	mitochondrial outer membrane permeabilization
Bax	BCL2 Associated X Protein
Bid	Bax-like BH3 protein
CytC	cytochrome C
AIF	apoptosis inducing factor
EndoG	endonuclease G
Htra2	mitochondrial protease
IRI	ischemia-reperfusion injury
Tg	transgenic
DG	dentate gyrus
EM	electron microscopy

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Fig. 1.

A: Cellular membrane trafficking encompasses 4 major types: (1) secretory or exocytic route, (2) endocytic pathway; (3) newly synthesized biomolecules delivery; and (4) autophagy pathway (Fig. 1A). ER = endoplasmic reticulum. V = transport vesicle. LE = late endosome. EE = early endosome. EL = endolysosome. AP = autophagosome. L = lysosome.

B: Example of LE and lysosomal membrane fusion in the inset of A. Membrane fusion is mediated by active SNAREs tethering to bring two membranes together. After membrane fusion, SNAREs form inactive complexes and must be regenerated by NSF ATPase for the next round of fusion. During this process, NSF ATPase binds to inactive SNAREs via a SNAP adaptor to hydrolyze adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and inorganic phosphate for providing the energy. Brain ischemia leads to deposition NSF into inactive protein aggregates, thus stopping all membrane trafficking activities in postischemic neurons.

Fig. 2.

Confocal microscopic images of CA1 neurons labeled with NSF antibody. Brain sections were obtained from a sham-operated control rat and rat subjected to 15 min of ischemia followed by 24 h of reperfusion. NSF is located in the peri-nuclear and dendritic trunk (Sham, arrowheads), as well as in the neuropil (Sham, arrows) of sham-operated control CA1 neurons. NSF is mostly depleted from the CA1 neuronal perinuclear region and dendritic trunk (24h, arrowheads), but remains relatively at the sham control level in the CA1 neuropil (24h, arrows) at 24 h of reperfusion after transient cerebral ischemia.

Fig. 3.

EM micrographs of hippocampal CA1 neurons stained with uranium-lead. CA1 tissue sections were obtained from rats subjected to non-ischemic sham surgery or 20 min of ischemia followed by 24 h of reperfusion. **a:** A sham neuron shows normal rough endoplasmic reticulum (ER), ribosomal rosettes (small arrows), mitochondria (M), Golgi apparatus (G), late endosome (large arrow), and endolysosome (EL, also known as secondary lysosome). **b:** A 24 h reperfusion neuron shows accumulation of Golgi fragments (Gf), transport vesicles (arrowheads), enlarged late endosome (large arrows), monoribosomes (small arrows), and ribosomal aggregates (stars). **c and d:** Higher magnifications of the insets of “a and b” show that ELs and a LE (arrow) from the sham control neuron has intact lipid membranes, whereas an enlarged LE from the postischemic neuron has a number of membrane break damage (arrowheads). Scale bar = 0.5 μ m.

Fig. 4.

CTSB has three active forms: (i) 46 kDa Golgi CTSB located in Golgi/TVs, (ii) 33 kDa CTSB in late endosome (LE), and (iii) 24/25 kDa lysosome CTSB in endolysosome (EL) and lysosome (L).

Fig. 5.

Inactivation of NSF ATPase after brain ischemia leads to a cascade of events of massive buildup of Golgi fragments, TVs and enlarged LEs, fatal release of 33 kDa CTSB, and brain ischemia-reperfusion injury.

Fig. 6.

CTSB release induces mitochondrial outer membrane permeabilization (MOMP): Released CTSB converts Bid to active truncated Bid (tBid), and leads tBid and Bax mitochondrial (M) translocation and Bax oligomerization. Bax oligomerization forms mitochondrial outer membrane pore, resulting in MOMP. Consequently, mitochondrial intermembrane space proteins are released into the cytoplasm, eventually resulting in neuronal death after brain ischemia. CytC = cytochrome C. AIF = apoptosis inducing factor. EndoG = endonuclease G. HtrA2/Omi = a proapoptotic mitochondrial serine protease. SMAC/Diablo = Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI.

Fig. 7.

A: Ubiquitin immunogold EM micrographs of mitochondrion (M). EM micrographs of CA1 neurons from a sham-operated non-ischemic control rat and a rat subjected to 15 min ischemia followed by 24h of reperfusion. Ubiquitin immunogold labels mitochondrion and ubiquitinated protein aggregates at 24h of reperfusion (arrows). **B:** EM three-dimensional reconstruction of CA1 neuronal mitochondria in brain sections from sham-operated control and 24h reperused rats. The red indicates the mitochondrial outer membrane. The yellow (arrows) represents the vacuole structures formed between the inner- and outer-mitochondrial membranes after brain ischemia. Scale bars in A and B = 0.5 μm. **C:** Western blotting of ubiquitin in the samples of detergent-salt insoluble mitochondrial pellets from sham-operated rats and rats subjected to 15 min of ischemia followed by 24 h of reperfusion. The molecular size is shown on the left.