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RESEARCH ARTICLE

Lysosomal chymotrypsin B potentiates apoptosis via cleavage of Bid

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Abstract We have reported that chymotrypsin B (CtrB) is not just a digestive enzyme but is also stored in lysosomes. Herein, we demonstrated a broad distribution of CtrB and explored the involvement of CtrB in apoptosis. Exposure of RH-35 cells to H_2O_2 or palmitate induced the redistribution of lysosomal CtrB into the cytoplasm as a result of lysosomal membrane permeabilization (LMP). Suppression of CtrB significantly blocked apoptosis, while overexpression of CtrB sensitized apoptosis markedly. CtrB could cleave Bid under neutral conditions. In RH-35 cells with Bid silenced, apoptosis induced by CtrB protein was attenuated, suggesting that CtrB mediates apoptosis of RH-35 cells mainly through processing Bid. Our data also suggest that LMP occurs earlier than mitochondrial outer membrane permeabilization; Bid activation initiated by caspase-8 might be reinforced by CtrB in consequence of LMP, which causes a positive feedback loop leading to the accumulation of tBid, and results in lysosome- and mitochondrion-dependent apoptosis.

Keywords Chymotrypsin B · Lysosome · Mitochondrion · Apoptosis · Bid

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Introduction

Apoptosis is a fundamental physiological process in mammals in which cells die by activating a suicide mechanism. Mitochondria are a major checkpoint of apoptotic regulation. They serve as sensors and amplifiers of cellular damage. Following mitochondrial outer membrane permeabilization (MOMP), mitochondria release a number of factors such as cytochrome *c*, Smac/DIABLO, apoptosis inducing factor (AIF), and endonuclease G, which are critically involved in cell death signaling. Mitochondria are also centrally involved in the activation of caspases, a family of otherwise dormant cysteine proteases that cleave a subset of cellular proteins. The activation of caspases can be achieved through two major mechanisms: (1) the extrinsic pathway, characterized by death receptor-mediated recruitment and activation of apical caspase-8, and (2) the intrinsic pathway, characterized by assembly of cytosolic (APAF-1) and mitochondrial (cytochrome *c*) factors.

Recently accumulating evidence has indicated that, in addition to mitochondria, lysosomes are tightly linked with apoptotic signaling [1, 2]. Several lysosomal acid-dependent proteases known as cathepsins (e.g., cathepsin B, D, L, etc.) have been implicated in apoptosis induction following their relocalization to the cytosol as a result of moderate lysosomal rupture, or lysosome membrane permeabilization (LMP) [3]. LMP is an early event that occurs

hours prior to MOMP, resulting in mitochondria-dependent apoptosis [4, 5]. These results suggest that a lysosomal–mitochondrial pathway is involved in the mediation of apoptotic cell death.

It was generally accepted that one of the important molecules that bridges the LMP and MOMP is Bid. Studies in cell-free systems suggested that Bid could be cleaved by multiple lysosomal cathepsins, and that truncated Bid (tBid) induces subsequent rapid cytochrome *c* release from mitochondria. This was later confirmed in several cellular models [6, 7]. However, the physiological functions of cathepsins have been controversial because the optimum pH for their enzymatic activities is acidic and they are usually autolyzed or denatured under the physiological conditions of the cytosol (pH 7.4) [8]. Thus, there might be other proteases, such as proteases with optimum pH at 7.4, that mediate apoptosis [9–11].

During our study of Bid-mediated MOMP [12, 13], an unknown caspase-8-like activity capable of converting Bid into tBid at neutral pH was detected in highly purified rat liver lysosomal extracts, which was eventually identified as chymotrypsin B (CtrB). The serine protease chymotrypsin B has been long recognized as a digestive enzyme exclusively secreted by the pancreas. However, our data indicated that it is also expressed and resides intracellularly in the lysosomes of hepatocytes, and can function intracellularly as a pro-apoptotic protease in apoptosis mediated by the extrinsic pathway [14].

Although our previous studies support the involvement of lysosomal chymotrypsin B in the extrinsic pathway-mediated apoptosis, the general applicability of these findings remains to be investigated. The distribution of chymotrypsin B in different tissues and its role in apoptotic regulation by the intrinsic pathway also needs to be determined. We hereby provide evidence indicating that in addition to the pancreas, chymotrypsin B is widely expressed in different rat tissues including cerebrum, cerebellum, heart, lung, liver, kidney, and spleen in the form of both activated enzyme and zymogen (chymotrypsinogen B). Upon exposure to different apoptotic stimuli such as oxidative stress (H_2O_2) or free fatty acids (palmitate), lysosomal chymotrypsin B is released into the cytosol of RH-35 hepatoma cells as a result of LMP via a caspase-8- and Bid-dependent mechanism. LMP was found to be an upstream event of MOMP. A low concentration of tBid is sufficient to initiate LMP. Chymotrypsin B, but not cathepsin B or cathepsin D, significantly cleaves Bid at cytosolic conditions (pH 7.4), thereby presumably initiating a positive feedback leading to the accumulation of cellular tBid, which eventually results in apoptosis. Thus, our findings further demonstrate that lysosomal chymotrypsin B is an important molecule that mediates apoptosis via a Bid-dependent mechanism.

Materials and methods

Expression analysis of chymotrypsin B in various tissues of rats

Sprague–Dawley rats were killed and the cerebrum, cerebellum, heart, lung, liver, kidney, spleen, and pancreas were carefully dissected out. For analysis of chymotrypsin B mRNA, total RNA was isolated from each tissue by using TRIzol® (Invitrogen, Shanghai, China) and converted into cDNA with M-MLV Reverse transcriptase (Promega, Shanghai, China). The mRNA expression patterns of chymotrypsin B in different tissues were investigated using real-time qPCR with a MyIQ System (Bio-Rad, Shanghai, China) by methods we described previously [14]. Samples and standards were run once in triplicate, and the qPCR was repeated three times. Data were collected with MyIQ system software, version 1.0. The PCR products were verified by DNA sequencing.

For analysis of chymotrypsin B protein, 50 mg wet mass of tissues were sonicated in 400 µl 2× sodium dodecyl sulfate (SDS)-sample buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 4% SDS, 2% 2-mercaptoethanol and protease inhibitors). Solublized samples (20 µl for cerebrum, cerebellum, heart, lung, liver, kidney, and spleen, 0.2 µl for pancreas) were subjected to immunoblot analysis of chymotrypsin B protein expression with an anti-chymotrypsin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunoblot analysis was repeated three times.

For analysis of intracellular localization of chymotrypsin B in hepatocytes, lysosomes and mitochondria were purified from rat livers as we described previously [14], and were subjected to immunoblot analysis of chymotrypsin B (antibody from Santa Cruz Biotechnology), cathepsin D (a lysosomal marker; antibody from Santa Cruz Biotechnology), α-subunit of FoF_1-ATPase (a mitochondrial marker; antibody by home-made [14]), or GAPDH (a cytosolic marker; antibody from Santa Cruz Biotechnology).

The localization of lysosomal chymotrypsin B was further assayed by immunofluorescence microscopy. Primary rat hepatocytes were grown on glass coverslips and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) containing 0.1 M PIPES, 1 mM EGTA, and 3 mM $MgSO_4$. Cells were permeabilized with 0.0125% (w/v) CHAPS in PBS at 37°C for 10 min and blocked for 1 h at room temperature with PBS containing 5% goat serum albumin, 5% glycerol, and 0.04% sodium azide. After incubation with primary antibodies against chymotrypsin B (home-made) or cathepsin D (Santa Cruz Biotechnology; a lysosomal marker) in blocking buffer at 4°C overnight, cells were washed three times with PBS, incubated with FITC-conjugated or Cy3-conjugated second antibodies

(Sigma) in blocking buffer for 1 h at 37°C. Cells were imaged with an Olympus FV500 laser scanning confocal microscope.

Cell culture and exposure to H_2O_2 or palmitate

Rat hepatoma RH-35 cells were cultured as previously reported [14]. Cells were challenged with apoptotic stimuli by treatment with 100 μM of H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA) or 300 μM of sodium palmitate (Sigma) for indicated intervals. In some experiments, cells were cultured in the presence or absence of N-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK; a specific inhibitor of Chymotrypsin B; Sigma), IETD-CHO (caspase-8-selective inhibitor; Biomol, Plymouth Meeting, PA, USA) or ammonium chloride (inhibitor of lysosomal acidification, Sigma) before H_2O_2 or palmitate exposure.

Redistribution of mitochondrial cytochrome *c* and lysosomal chymotrypsin B

The redistribution of mitochondrial cytochrome *c* and lysosomal chymotrypsin B were detected by immunoblot. RH-35 cells were exposed to 100 μM of H_2O_2 or 300 μM of sodium palmitate for indicated intervals. For analysis of mitochondrial cytochrome *c*, cells were treated with 0.025% digitonin and the mitochondrial fractions were obtained after 5 min centrifuge at 10,000 × *g* [15]. For analysis of lysosomal chymotrypsin B, lysosomal fractions were isolated from cells with the Lysosome Enrichment Kit (Pierce, Rockford, IL, USA). Cytosolic fractions were also collected for the detection of redistributed cytochrome *c* or chymotrypsin B. Mitochondrial fractions, lysosomal fractions, and cytosolic fractions were subjected to immunoblot analysis of cytochrome *c* or chymotrypsin B with antibodies against cytochrome *c* (BD Pharmingen, San Jose, CA, USA) or chymotrypsin B (Santa Cruz Biotechnology).

Acridine orange staining

Lysosomal permeabilization was determined by staining with acridine orange (AO; Invitrogen) [16, 17]. AO is a cell-permeable metachromatic lysosomotropic fluorophore that gives rise to red fluorescence at high and green fluorescence at low concentrations. AO accumulates by proton trapping in intact lysosomes due to the fact that it becomes positively charged in the acidic lysosomal milieu. Following lysosomal membrane destabilization, acridine orange is released from lysosomes into the cytosol where it emits enhanced green fluorescence that can be monitored by fluorescence microscopy. RH-35 cells were exposed to H_2O_2 for 1 h or palmitate for 30 h, and loaded with AO (5 μg/ml) for 15 min. Images of AO-emitted red (lysosomal) and green (nuclear and cytosolic) fluorescence were viewed by an inverted laser scanning confocal microscope (model FV500; Olympus, Tokyo, Japan).

RNA interference

The vectors pSUPER.retro.neo and pSUPER.retro.puro (OligoEngine, Seattle, WA, USA) were used for expression of shRNA in RH-35 cells. For silencing of chymotrypsin B expression, a sequence corresponding to nucleotides 421–439 of rat chymotrypsin B gene was selected. This sequence was inserted into the pSUPER.retro.neo backbone after digestion with *Bgl*II and *Hin*dIII. This vector was referred to as pSUPER-Ctrb. A control vector, pSUPER-LacZ, with no significant homology to rat chymotrypsin B gene was used as a non-silencing control. For silencing of Bid expression, a sequence corresponding to nucleotides 502–521 of rat Bid gene was selected. This sequence was inserted into the pSUPER.retro.puro backbone after digestion with *Bgl*II and *Hin*dIII, giving a vector referred to as pSUPER-Bid. The empty vector pSUPER.retro.puro served as a non-silencing control.

The vectors pSUPER-Ctrb, pSUPER-LacZ, pSUPER-Bid, and pSUPER.retro.puro were transformed into One Shot TOP10 competent cells (Invitrogen), amplified, and purified by using Qiagen Endofree Plasmid DNA Maxi kit (Qiagen, Shanghai, China).

For silencing of chymotrypsin B expression, RH-35 cells (5×10^6) were pelleted and resuspended in 100 μl of Amaxa nucleofection buffer (solution V; Amaxa Biosystems, Cologne, Germany) with 3 μg of the pSUPER-Ctrb vector (or pSUPER-LacZ as a non-silencing control). This suspension was transferred to a sterile cuvette and treated using program T-030 on an Amaxa Nucleofector II device. After recovery for 30 min in complete DMEM medium, the cells were plated. Cells were used for experiments 48 h after transfection.

The Bid siRNA RH-35 cell line was generated as follows. pSUPER-Bid plasmid containing the Bid-targeting sequence was transfected into RH-35 cells using a standard Lipofectamine^{Plus} (Invitrogen) method. Stably transfected RH-35 cells were selected in DMEM containing 1,200 μg/ml puromycin, and individual colonies were subcloned.

Construction and expression of myc-tagged chymotrypsin B

The expression plasmid for chymotrypsin B was constructed as previously described [14] and cloned into pcDNA3.1, producing a plasmid pcDNA3.1-Ctrb encoding myc-tagged chymotrypsin B. RH-35 cells resuspended in nucleofection buffer solution V were transfected with pcDNA3.1-Ctrb plasmid by the Amaxa Nucleofector II

device coupled with program T-030. Cells were used for experiments 48 h after transfection. The transfection efficiency was estimated with a GFP-tagged chymotrypsin B plasmid as reported previously [14].

Intracellular delivery of chymotrypsin B, cathepsin B and cathepsin D

Recombinant chymotrypsin B [14], cathepsin B (Sigma) or cathepsin D (Sigma) was delivered to RH-35 cells using the BioPORTER protein transfection reagent (Gene Therapy Systems; San Diego, CA, USA) as we reported previously [14]. Briefly, RH-35 cells stably transfected with Bid-silencing pSUPER-Bid vector or non-silencing pSUPER.retro.puro control vector were grown in 35-mm dishes and incubated with BioPORTER pre-mixed with chymotrypsin B, cathepsin B or cathepsin D, respectively. Cells were incubated for an additional 12 h and then subjected to flow cytometry analysis of apoptosis.

Assessment of apoptosis

To assay nuclear morphology for apoptosis (i.e., chromatin condensation and nuclear fragmentation), cells were incubated in medium containing the nuclear binding dye Hoechst 33342 (25 μM; Sigma) for 5 min at room temperature, and the images were captured by a Nikon TE2000 fluorescence microscope (Tokyo, Japan) using excitation and emission filters of 380 and 430 nm, respectively. Apoptotic cells were also quantified by flow cytometry with a FACSCalibur flow cytometer and the percentage of hypodiploid (apoptotic) cells was analyzed by BD Cell Quest software [18].

Induction of lysosomal membrane permeabilization and mitochondrial outer membrane permeabilization by tBid

Lysosomes and mitochondria were purified from rat livers as we described previously [14]. Bid was cleaved by caspase-8 to form tBid [12]. Lysosomes or mitochondria were incubated with indicated concentrations of tBid for 30 min, respectively, followed by centrifugation at 18,000 × *g* for 10 min. The supernatant was subjected to immunoblot analysis to assess the release of lysosomal cathepsin D or mitochondrial cytochrome *c*. The percentage of lysosomal cathepsin D or mitochondrial cytochrome *c* released into the supernatant was used as an index of LMP or MOMP, respectively.

Cleavage of Bid by chymotrypsin B in cell-free systems

Recombinant Bid was expressed and purified as previously described [14]. Five micrograms of Bid was incubated with chymotrypsin B, cathepsin B, or cathepsin D at neutral (pH 7.4) or acidic pH (pH 4.5 for cathepsin D, pH 5.5 for cathepsin B and chymotrypsin B) at 37°C for 30 min. The cleavage of Bid was analyzed after SDS-polyacrylamide gel electrophoresis (PAGE).

The cleavage of Bid by chymotrypsin B, cathepsin B, or cathepsin D in cytosolic circumstances was also mimicked. Five micrograms of Bid was incubated with chymotrypsin B, cathepsin B, or cathepsin D in the presence of liver cytosolic components (containing 10 μg protein) at 37°C for 30 min. The cleavage of Bid was monitored by immunoblot analysis with an anti-Bid antibody (Santa Cruz Biotechnology).

The effects of lysosomal chymotrypsin B on Bid cleavage were further investigated in isolated intact rat liver lysosomes. Lysosomes were incubated with tBid (0.4 μM) and Bid (2 μM) in the absence or presence of TPCK at 37°C for 30 min. The tBid accumulation in the supernatant was measured by immunoblot analysis with an anti-Bid antibody (Santa Cruz Biotechnology).

Immunoblot analysis

Samples were resolved by 10% SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membrane, and blotted with antibodies against chymotrypsin (Santa Cruz), cytochrome *c* (BD Pharmingen), caspase-3 (Cell Signaling Technology, Danvers, MA, USA), poly(ADP-ribose)polymerase (PARP; Cell Signaling Technology), caspase-8 (Santa Cruz), Bid (Santa Cruz), cathepsin D (Santa Cruz), myc (Cell Signaling Technology), α-subunit of FoF_1-ATPase (home-made), GAPDH (Santa Cruz Biotechnology), *β*-actin (Santa Cruz) or *β*-tubulin (Santa Cruz). They were then incubated with appropriate peroxidase-conjugated secondary antibodies (Santa Cruz), and visualized using a chemiluminescent substrate (ECL; GE Amersham Pharmacia, Beijing, China) with Kodak X-OMAT film (Rochester, NY, USA).

Data analysis

All data are expressed as the mean ± SD unless otherwise indicated. Differences between groups were compared by analysis of variance followed by a post hoc Bonferroni test to correct for multiple comparisons. Differences were considered to be statistically significant at $p < 0.05$.

Results

Broad tissue distribution of chymotrypsin B

We reported previously that chymotrypsin B is not just a digestive enzyme secreted by the pancreas, but also a

component of rat liver [14]. Herein, we examined the expression profiles of chymotrypsin B in different rat tissues. Chymotrypsin B mRNA was quantified by real-time PCR, and the PCR products were verified by DNA sequencing. Results of qPCR revealed that chymotrypsin B mRNA is expressed ubiquitously in eight rat tissues we examined (cerebrum, cerebellum, heart, lung, liver, kidney, spleen, and pancreas) (Fig. 1a), with a comparatively higher level of expression in the pancreas.

We then assayed the protein levels of chymotrypsin B in rat tissues by immunoblot with a commercially available polyclonal antibody from Santa Cruz Biotechnology. Figure 1b shows a representative immunoblot. Similar to the mRNA expression profile of chymotrypsin B, chymotrypsin B protein was also found in all eight tissues we examined (cerebrum, cerebellum, heart, lung, liver, kidney, spleen, and pancreas). As a digestive enzyme secreted by the pancreas, the chymotrypsin B is abundant in the pancreas tissues. Even though the amount of pancreas sample loaded in immunoblot assays was just 1% of other tissue samples, it still gave a strong chymotrypsin B band, predominantly in its zymogen form (~28 kDa). In non-pancreas tissues, chymotrypsin B protein was relatively higher in heart, lung, and liver, and mainly in its activated enzyme form (~26 kDa). This result suggests that the small amount of active chymotrypsin B found in tissues other than pancreas may exhibit different functions independent from digestion.

We have purified chymotrypsin B from liver lysosomes [14], suggesting that chymotrypsin B is cached in rat liver lysosomes. This was further verified by the following experiments. We first isolated the lysosomes, mitochondria, and the cytosolic fractions from rat liver, and examined the subcellular localization of chymotrypsin B by immunoblots. As shown in Fig. 1c, chymotrypsin B could be detected in lysosomes, but not in other organelles such as mitochondria. In addition, by immunofluorescence, we found that chymotrypsin B is co-localized with cathepsin D (a marker enzyme of lysosomes) in primary rat hepatocytes (Fig. 1d), showing that chymotrypsin B is a component of lysosomes.

Involvement of chymotrypsin B in apoptosis induced by H_2O_2 or palmitate

Since a relatively higher level of chymotrypsin B was found in rat liver tissue and mainly located in lysosomes, we used RH-35 rat hepatoma cells as a model to further investigate the role of lysosomal chymotrypsin B in cell apoptosis. Exposure of RH-35 cells to 100 μM H_2O_2 or 300 μM palmitate significantly increased the percentage of apoptotic nuclei in cultures of RH-35 cells, as characterized by morphological criteria including chromatin condensation and nuclei fragmentation. Figure 2a shows the typical images of apoptotic nuclei upon H_2O_2 or palmitate treatment. Apoptotic cell death was quantified by flow cytometry analysis of DNA, and the results are shown in Fig. 2b. Caspase-3 activation was involved in H_2O_2- or palmitate-induced apoptosis, as confirmed by immunoblot analysis (Fig. 2c). In addition, during H_2O_2- or palmitate-induced apoptosis, cytochrome *c* was released from the mitochondria (Fig. 2d), suggesting that these two typical pathological apoptotic stimuli trigger apoptosis via intrinsic pathways.

We also found that chymotrypsin B was also redistributed from the lysosomes to cytosol as a consequence of H_2O_2 or palmitate exposure (Fig. 2d). Using AO as a lysosomotropic fluorophore, we further confirmed found that LMP occurred (Fig. 2e). Interestingly, when comparing the time-course of the release of cytochrome *c* (a result of MOMP) and chymotrypsin B (a result of LMP) into the cytosol during H_2O_2- or palmitate-induced apoptosis (Fig. 2d), we found that LMP was an early event that occurred at least 60 min prior to the MOMP. To determine the involvement of released chymotrypsin B in apoptosis, the activity of chymotrypsin B was inhibited with TPCK, a specific inhibitor of chymotrypsin B, prior to H_2O_2 or palmitate exposure. TPCK pretreatment significantly prevented H_2O_2- or palmitate-induced release of mitochondrial cytochrome *c* (Fig. 3a) and attenuated apoptosis in RH-35 cells (Fig. 3b, c), implying that chymotrypsin B might be a mediator of H_2O_2- and palmitate-induced intrinsic apoptotic pathway activation. Thus, we used H_2O_2 as the proapoptotic stimulus to further study the mechanism underlying chymotrypsin B-mediated apoptosis.

RH-35 cells were transiently transfected for overexpression of recombinant chymotrypsin B or transfected with shRNAs to silence endogenous chymotrypsin B expression using Amaxa Nucleofector System with a ~50% transfection efficiency (data not shown). The overexpression of a Myc-tagged chymotrypsin B was confirmed by immunoblot analysis using the anti-chymotrypsin B antibody as well as anti-Myc antibody (Fig. 3d). Overexpression of Myc-tagged chymotrypsin B increased H_2O_2-induced apoptotic cells from ~40.2 to ~58.9% (Fig. 3d; $p < 0.01$). Since the transfection efficiency was only about 50%, the effect of overexpressed chymotrypsin B in the mediation of apoptosis may be underestimated. We also found that silencing the endogenous chymotrypsin B expression with pSUPER-Ctrb expressing a chymotrypsin B-specific shRNA decreased H_2O_2-induced apoptosis from ~27.7 to ~14.8% (Fig. 3e, $p < 0.01$), consistent with the partial reduction of H_2O_2-induced apoptotic cells by the chymotrypsin B inhibitor TPCK (Fig. 3c).

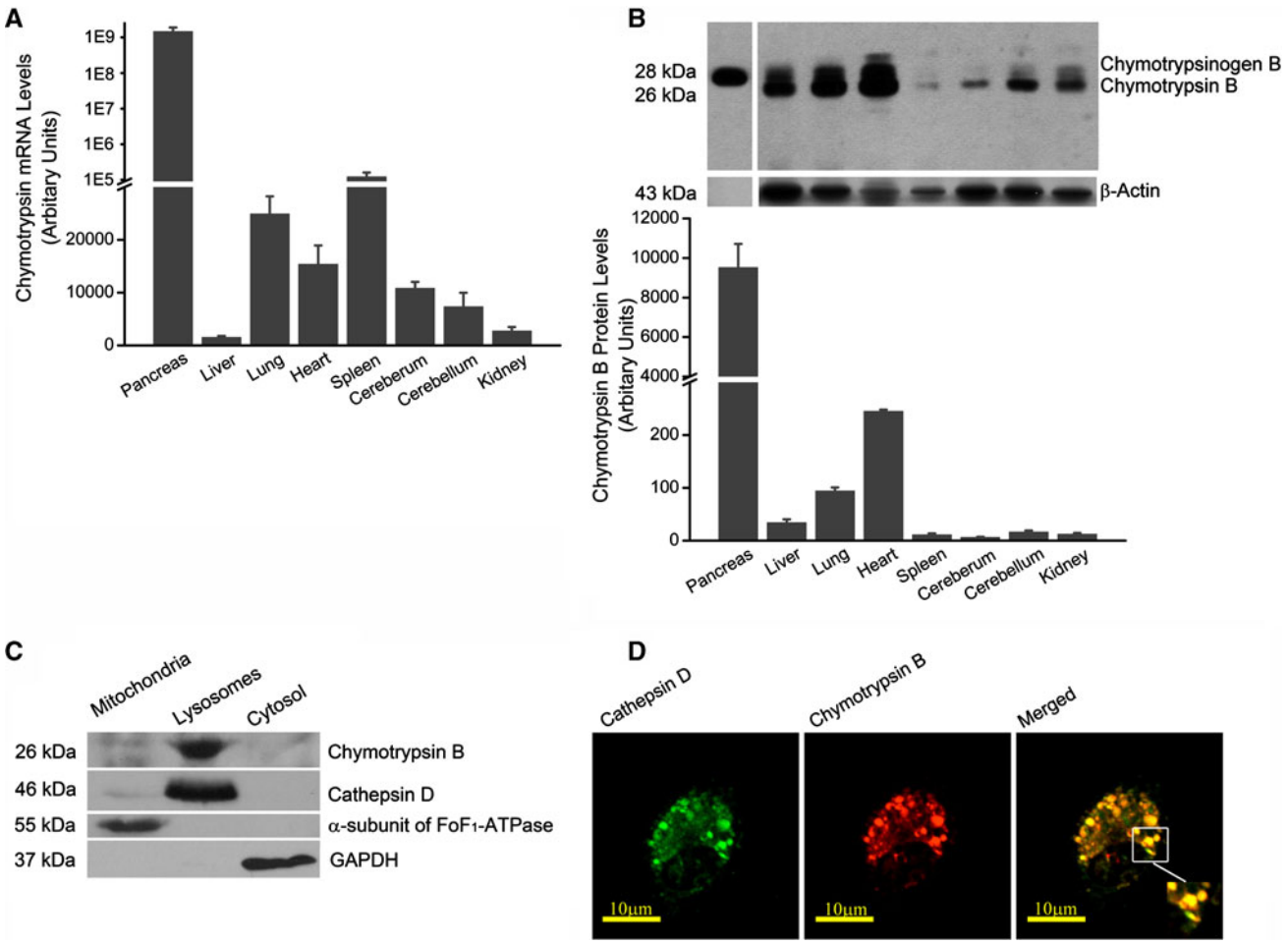


Fig. 1 Expression of chymotrypsin B in different rat tissues. **a** Detection of chymotrypsin B mRNA by quantitative real-time PCR. Total RNA isolated from rat cerebrum, cerebellum, heart, lung, liver, kidney, spleen and pancreas was subjected to real-time qPCR analysis. The data shown are means ± SD of three experiments. **b** Detection of chymotrypsin B protein by immunoblot. Fifty milligrams wet mass of rat cerebrum, cerebellum, heart, lung, liver, kidney, spleen and pancreas were sonicated in 400 μl 2× SDS-sample buffer. Solublized samples (20 μl for cerebrum, cerebellum, heart, lung, liver, kidney and spleen, 0.2 μl for pancreas) were subjected to immunoblot analysis of chymotrypsin B protein expression. Chymotrypsin B is expressed ubiquitously in cerebrum, cerebellum, heart, lung, liver, kidney, spleen and pancreas. The intensity of the chymotrypsin B bands was quantified and is shown as means ± SD of three experiments. Noted that the amount of pancreas sample loaded on SDS-PAGE was only 1% of other tissue samples, thus the amount of β-actin in the pancreas sample was too low to be detected. **c** Intracellular localization of chymotrypsin B. Lysosomes, mitochondria, and cytosolic fractions were prepared from rat livers and were subjected to immunoblot analysis of chymotrypsin B, cathepsin D (a lysosomal marker), α-subunit of FoF_1-ATPase (a mitochondrial marker), or GAPDH (a cytosolic marker). Chymotrypsin B could only be detected in the lysosomal fractions. **d** Chymotrypsin B localized in lysosomes. Permeabilized primary rat hepatocytes were incubated with anti-chymotrypsin B antibody and anti-cathepsin D antibody, followed by incubation with Cy3-labeled and FITC-labeled secondary antibodies. Fluorescence images were recorded with an Olympus Fluo-View FV500 laser scanning confocal microscope. The merged red fluorescence of chymotrypsin B and the green fluorescence of cathepsin D (lysosomal marker) yielding a yellowish fluorescence indicated the lysosomal localization of chymotrypsin B in rat hepatocytes. *Scale Bar* 10 μm

Chymotrypsin B-mediated Bid cleavage emerges as a key connection between LMP and MOMP

The time-course of the release of cytochrome *c* and chymotrypsin B during apoptosis suggested that LMP was an early event that occurred at least 60 min prior to the MOMP (Fig. 2d). To ascertain whether the MOMP is down-stream of LMP, RH-35 cells were pretreated with lysosome inhibitor NH_4Cl before H_2O_2 exposure. Cytochrome *c* was absent in the cytosol of NH_4Cl-pretreated RH-35 cells as assayed by immunoblot (Fig. 4a). Moreover, H_2O_2-induced apoptosis was prevented by NH_4Cl pretreatment as determined by both nuclear staining and flow cytometry analysis (Fig. 4b). These data suggest that LMP is upstream of MOMP during H_2O_2-induced apoptosis. Our finding is consistent with other reports showing mitochondrial-dependent apoptosis mediated by lysosomes [17].

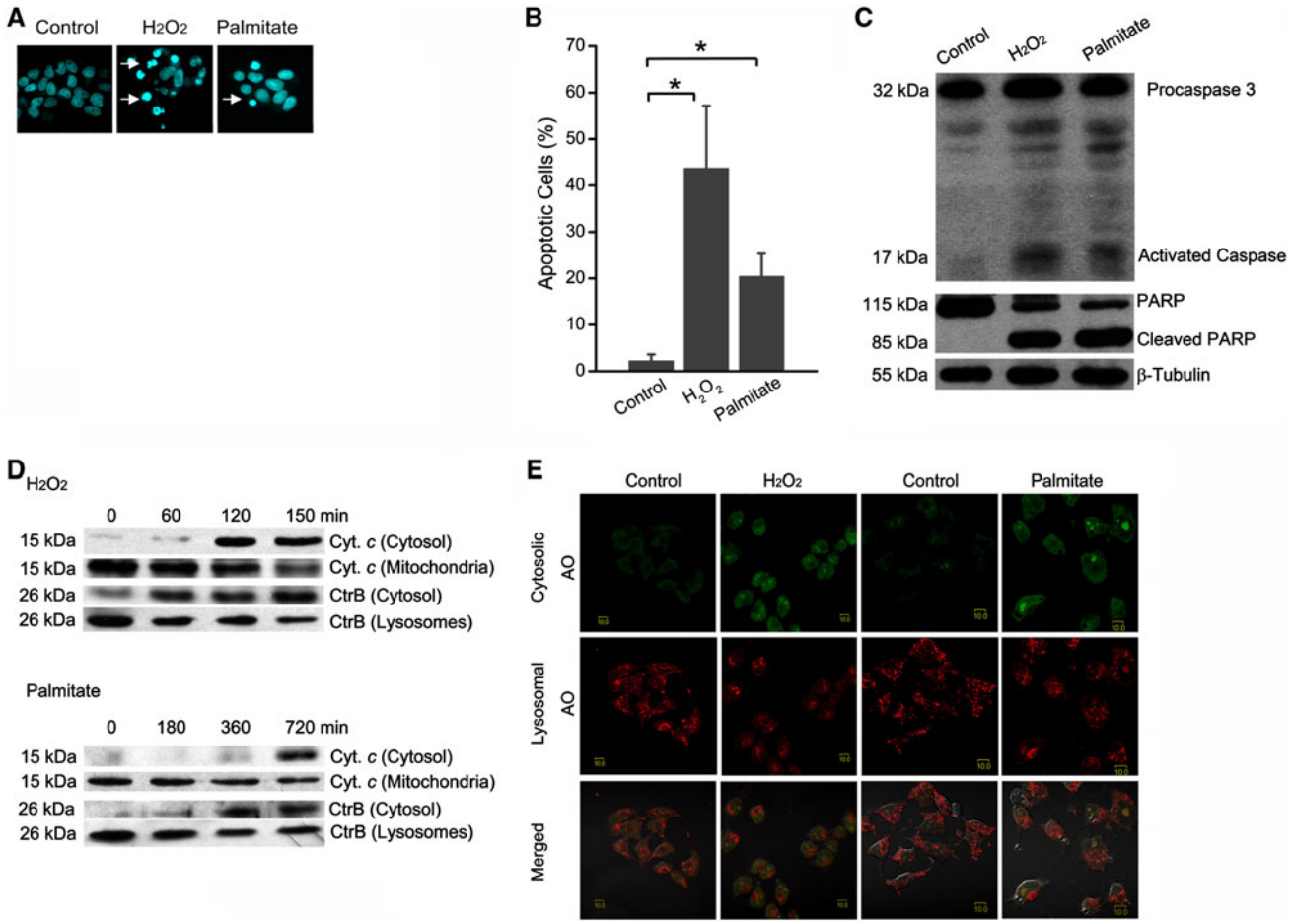


Fig. 2 Hydrogen peroxide-induced and palmitate-induced apoptosis in RH-35 cells was accompanied by the redistribution of mitochondrial cytochrome *c* and lysosomal chymotrypsin B. RH-35 cells were exposed to 100 μM of H_2O_2 for 12 h or 300 μM of palmitate for 36 h. Cells were examined for apoptosis. **a** Observation of apoptotic nuclei. After staining with Hoechst 33342, the nuclear morphology was observed with a Nikon TE2000 fluorescence microscope. H_2O_2 or palmitate exposure significantly increased the percentage of apoptotic nuclei, as indicated by the *white arrows*. **b** Quantitative analysis of apoptosis. The percentage of apoptotic cells were analyzed with a FACSCalibur flow cytometer after being staining with propidium iodide. H_2O_2 or palmitate exposure increased the percentage of apoptotic cells significantly. $*p < 0.05$. **c** H_2O_2 or palmitate exposure activated caspase-3. The activation of caspase-3 and the cleavage of PARP were analyzed by Western blot. H_2O_2 or palmitate exposure activated caspase-3 and caused the cleavage of its substrate, PARP. **d** H_2O_2 or palmitate exposure caused a time-dependent redistribution of mitochondrial cytochrome *c* and lysosomal chymotrypsin B. RH-35 cells were exposed to 100 μM H_2O_2 or 300 μM palmitate for indicated intervals, and the mitochondrial fractions, lysosomal fractions, and cytosolic fractions were isolated. The redistribution of mitochondrial cytochrome *c* and lysosomal chymotrypsin B into the cytosol was detected by immunoblot. H_2O_2 or palmitate exposure triggered the redistribution of cytochrome *c* and chymotrypsin B time-dependently. **e** H_2O_2 or palmitate exposure caused the redistribution of lysosomal acridine orange. RH-35 cells were exposed to H_2O_2 for 1 h or palmitate for 30 h, and loaded with acridine orange (AO; 5 μg/ml) for 15 min. Images of AO-induced red (lysosomal) and green (nuclear and cytosolic) fluorescence were viewed with an Olympus FV500 microscope. Exposure of RH-35 cells to H_2O_2 or palmitate induced an increase in the cytosolic AO fluorescence (green fluorescence), and a decrease in the lysosomal AO fluorescence (red fluorescence), suggesting the occurrence of lysosomal membrane permeabilization (LMP)

Upon LMP, lysosomal chymotrypsin B and other proteases (e.g., cathepsins) are released into the cytosol, where they might cleave other molecules to initiate signaling cascades leading to MOMP and apoptosis. Since Bid is generally accepted as an important molecule that bridges the LMP and MOMP, we compared the ability of chymotrypsin B and cathepsins to process Bid in cytosolic circumstances. Recombinant Bid was incubated with chymotrypsin B, cathepsin B, or cathepsin D in the presence of cytosolic extracts. Notably, only chymotrypsin B cleaved Bid to form tBid (Fig. 4c), whereas neither cathepsin B nor cathepsin D showed obvious Bid cleavage activity under cytosolic circumstances.

We further investigated the protease activities of chymotrypsin B, cathepsin B, or cathepsin D on Bid at acidic (pH 4.5 for cathepsin D, pH 5.5 for cathepsin B and chymotrypsin B) or neutral (pH 7.4) conditions. Consistent with previous reports [6], both cathepsin B and cathepsin D

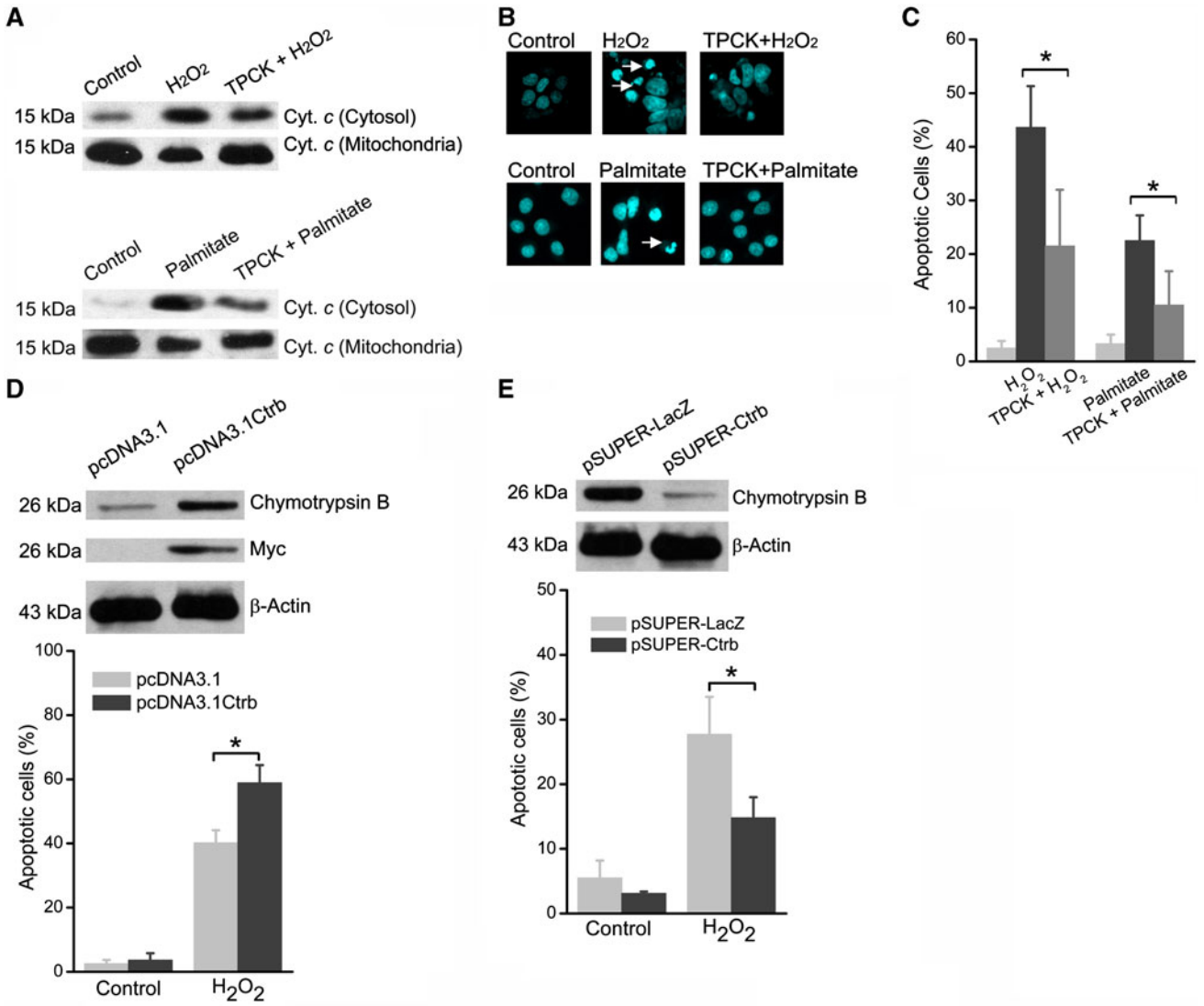


Fig. 3 Inhibition of chymotrypsin B prevented apoptosis in RH-35 cells. **a** TPCK blocked cytochrome *c* release from mitochondria. RH-35 cells were pretreated with 20 μM TPCK (a specific inhibitor of chymotrypsin B) and then exposed to 100 μM H_2O_2 for 150 min or 300 μM palmitate for 720 min. The localization of cytochrome *c* was detected by immunoblot. In RH-35 cells pretreated with TPCK, which inhibited the activity of chymotrypsin B, the H_2O_2- or palmitate-induced redistribution of mitochondrial cytochrome *c* into the cytosol was blocked. **b** TPCK prevented the formation of apoptotic nuclei. RH-35 cells were pretreated with 20 μM TPCK and then exposed to 100 μM H_2O_2 for 12 h or 300 μM palmitate for 36 h. H_2O_2 or palmitate exposure caused the formation of apoptotic nuclei as indicated by the *white arrows*, and TPCK pretreatment prevented the formation of apoptotic nuclei. **c** TPCK inhibited apoptosis. RH-35 cells were pretreated with 20 μM TPCK and then exposed to 100 μM H_2O_2 for 12 h or 300 μM palmitate for 36 h. The percentage of apoptosis was analyzed by flow cytometry. TPCK significantly inhibits H_2O_2- or palmitate-induced apoptosis. $^*p < 0.05$. **d** Overexpression of chymotrypsin B potentiated H_2O_2-induced apoptosis. RH-35 cells were transfected with pcDNA3.1-CtrB or pcDNA3.1 for 72 h and then exposed to 100 μM H_2O_2 for 12 h. The chymotrypsin B expression was analyzed by immunoblot, and the percentage of apoptosis was determined by flow cytometry. Overexpression of chymotrypsin B significantly increased the apoptosis induced by H_2O_2. $^*p < 0.01$. **e** Silencing chymotrypsin B expression decreased H_2O_2-induced apoptosis. RH-35 cells were transfected with pSUPER-Ctrb or pSUPER-LacZ for 72 h, and then exposed to 100 μM H_2O_2 for 12 h. Chymotrypsin B expression was analyzed by immunoblot, and the percentage of apoptosis was assayed for by flow cytometry. Silencing chymotrypsin B expression significantly decreased the apoptosis induced by H_2O_2. $^*p < 0.01$

cleaved (or degraded) Bid effectively at acidic pH (Fig. 4d). However, neither cathepsin B nor cathepsin D cleaved Bid to form tBid at neutral pH. On the contrary, chymotrypsin B cleaved Bid rapidly at both acidic and neutral pH conditions. In addition, Bid cleaved by chymotrypsin B at neutral pH triggered the release of cytochrome *c* from isolated mitochondria (Fig. 4e).

To further explore the mechanism of chymotrypsin B-dependent apoptosis in RH-35 cells, recombinant chymotrypsin B was directly delivered into RH-35 control

cells or cells with Bid-silenced. The silencing of Bid expression in RH-35 cells was achieved by transfecting cells with pSUPER-Bid plasmid expressing a Bid-specific shRNA, and cells were selected with puromycin. Transfection with pSUPER plasmids and selection with puromycin only caused a slight increase in the basal apoptotic percentage (~5.5 vs. ~3.1% of nontransfected cells). Intracellular delivery of chymotrypsin B, cathepsin B, or cathepsin D proteins with BioPORTER reagents triggers a marked increase in apoptosis (Fig. 4f). Silencing of Bid expression significantly attenuated the chymotrypsin B-induced apoptosis. In contrast, Bid silencing had no significant effect on cathepsin B- or cathepsin D-induced apoptosis of RH-35 cells. These observations suggest that chymotrypsin B might induce apoptosis via mechanisms related with Bid processing whereas cathepsins B and D might induce apoptosis via Bid-independent mechanisms.

tBid-induced chymotrypsin B release potentiates tBid-dependent MOMP

It was reported that caspase-8 and Bid were necessary mediators of LMP in TNF-α-induced apoptosis in rat hepatoma cells [19]. To explore whether caspase-8 and Bid are also involved in LMP in oxidative stressed RH-35 cells, the time course of caspase-8 activation and Bid cleavage was examined after H_2O_2 exposure. As shown in Fig. 5a, exposure of RH-35 cells to 100 μM H_2O_2 caused a rapid activation of caspase-8, which was deactivated soon. Following caspase-8 activation, intracellular tBid level increased time-dependently. Inhibition of caspase-8 with its specific inhibitor IETD-CHO deterred the redistribution of lysosomal chymotrypsin B in H_2O_2-treated RH-35 cells (Fig. 5b), suggesting that the activity of caspase-8 is necessary for H_2O_2-induced lysosomal permeabilization. Furthermore, Bid silencing by its shRNA attenuated H_2O_2-induced redistribution of lysosomal chymotrypsin B in a time-dependent manner (Fig. 5b), suggesting that Bid is also required for lysosomal permeabilization and chymotrypsin B release in H_2O_2-treated RH-35 cells.

Since caspase-8 and Bid are well known mediators of MOMP, it is also important to determine the relationship between LMP and MOMP, and the role of Bid, caspase-8 and lysosomal chymotrypsin B acting in concert to mediate them. In H_2O_2-treated RH-35 cells, LMP is an early event that occurred at least 60 min prior to MOMP (Fig. 2d). At an early time point when LMP occurred (60 min after H_2O_2 exposure), the intracellular tBid level was much lower than that at the later time when MOMP appeared (120 min after H_2O_2 exposure; Fig. 5a). Thus we speculated that low concentrations of tBid could be sufficient to induce LMP, while MOMP might require a higher concentration of tBid. To determine the dose dependence of tBid to induce LMP or MOMP, isolated rat liver lysosomes or mitochondria were incubated with various concentrations of tBid separately. Indices of LMP and MOMP were obtained by monitoring the percentage of the lysosomal cathepsin D or mitochondrial cytochrome *c*, respectively, that was released into the supernatant. As shown in Fig. 5c, incubation of lysosomes with 0.2 μM tBid already resulted in LMP. In contrast, MOMP did not occur not until the tBid level was increased to 1 μM or higher (Fig. 5d). As expected, Bid did not induce LMP or MOMP.

We further investigated whether tBid-induced release of lysosomal chymotrypsin B could cleave Bid to tBid. Isolated rat liver lysosomes were incubated with 0.4 μM of tBid and 2 μM of Bid, and the tBid levels in the supernatant were determined. 0.4 μM of tBid is sufficient to trigger LMP, which caused the release of lysosomal proteases into the supernatant, where they cleaved Bid to form tBid and caused a significant increase in the tBid level (Fig. 5e). TPCK, the specific inhibitor of chymotrypsin B, significantly blocked the increase in tBid level, suggesting that chymotrypsin B is the key lysosomal protease processing Bid (Fig. 5e). Altogether, our results demonstrate that a lower concentration of tBid can trigger LMP to release chymotrypsin B, which cleaves cytosolic Bid to tBid, with tBid accumulation then leading to MOMP and mitochondrial release of cytochrome *c*, which eventually causes cell apoptosis.

Discussion

We recently reported that chymotrypsin B is cached in rat liver lysosomes [14]. Here we profiled the expression patterns of chymotrypsin B and found that chymotrypsin B is present in various tissues including cerebrum, cerebellum, heart, lung, liver, kidney, spleen, and pancreas. Interestingly, we found that that chymotrypsin B protein exists predominantly in the zymogen form (chymotrypsinogen B; ~28 kDa) in pancreas, but exists mainly in its activated form (~26 kDa) in other tissues such as heart, liver, and lung. This suggests that the active chymotrypsin B found in those tissues has other functions not related to digestion.

Indeed, our previous studies suggested that lysosomal chymotrypsin B is involved in extrinsic pathway-mediated apoptosis [14]. However, the general applicability of those findings was uncertain. We therefore examined the putative role and mechanisms of chymotrypsin B in apoptosis triggered by different stimuli. Our data indicate that transient overexpression of recombinant chymotrypsin B significantly increased H_2O_2-induced apoptotic cells from ~40.2 to ~58.9%, which may be an underestimate of the true effect since the transfection efficiency was only about

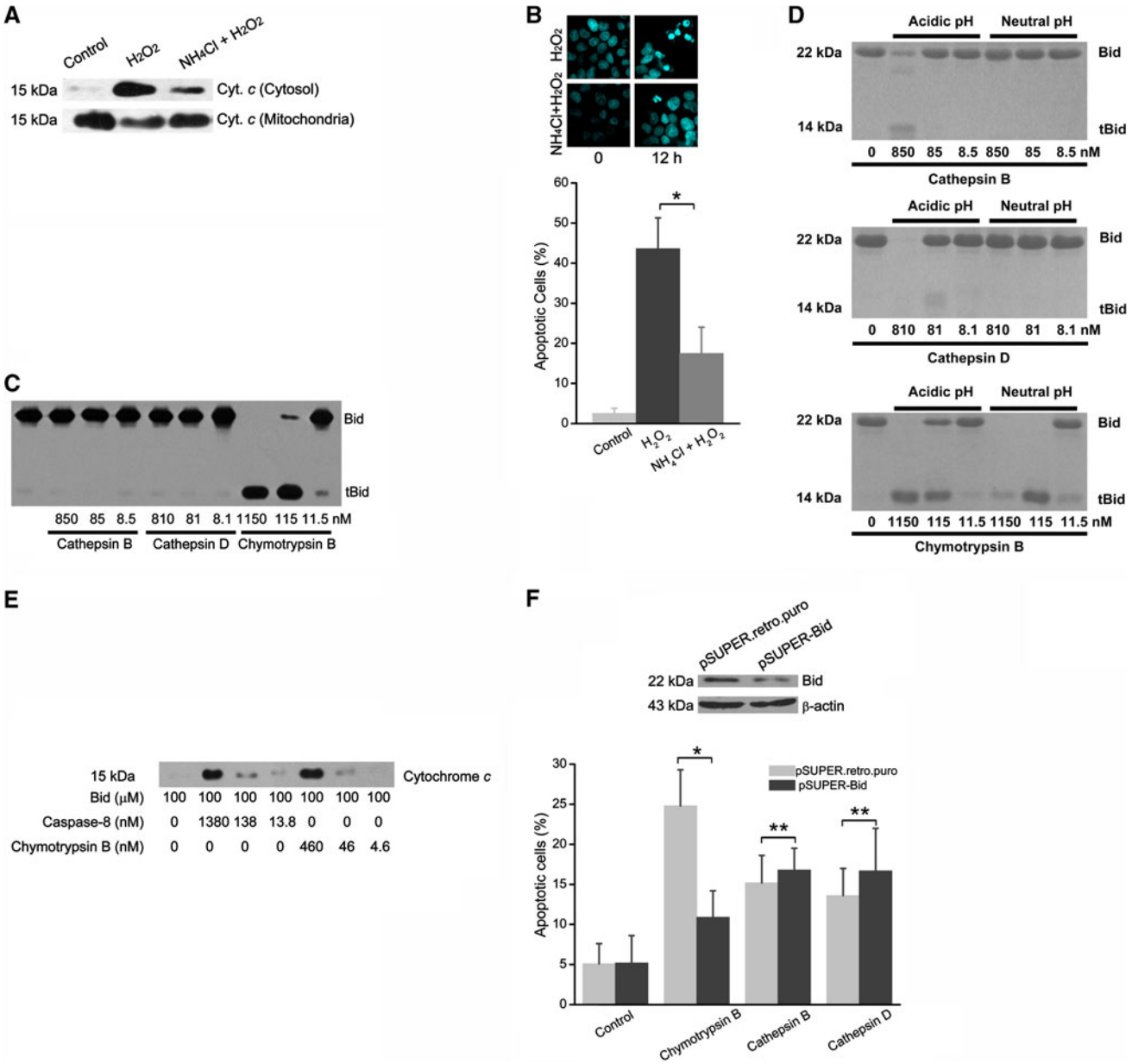
50%. Interestingly, silencing the endogenous chymotrypsin B expression showed a modest but statistically significant reduction on H_2O_2-induced apoptosis, suggesting that endogenous chymotrypsin B is involved in cell apoptosis. The partial attenuation of cell apoptosis by chymotrypsin B shRNA may be due in part to the complexity of lysosomal proteases. Upon lysosomal permeabilization, chymotrypsin B is one among dozens of lysosomal proteases that were released into the cytosol, where they initiate a cascade leading to the execution of apoptosis. Since chymotrypsin B is one of the important proteases mediating apoptosis, but not the only protease, it seems reasonable that silencing chymotrypsin B could only partially attenuate apoptosis. Furthermore, we also found that TPCK, a specific inhibitor of chymotrypsin B, significantly prevented H_2O_2- or palmitate-induced release of mitochondrial cytochrome *c* and attenuated apoptosis in cells. These data suggested that chymotrypsin B appears to play an instrumental role in apoptosis irrespective of whether it is triggered by extrinsic mediators (such as TNF-α) or intrinsic mediators (such as H_2O_2 or palmitate).

Lysosomes are emerging as an additional point of entry into apoptosis, and the permeabilization of lysosomes with subsequent release of lysosomal proteases into the cytosol is a crucial step in apoptosis in different cell types [20, 21]. In the present studies, we investigated the underlying mechanisms leading to LMP and LMP-dependent apoptosis. In RH-35 hepatoma cells, H_2O_2 or palmitate induced LMP rapidly as evidenced by the redistribution of lysosomal AO or chymotrypsin B. By comparing the time-courses of H_2O_2-induced LMP and MOMP, we found that LMP occurred at least 60 min prior to MOMP. Moreover, pretreatment of cells with NH_4Cl, a reagent that inhibits the acidification of lysosomes and LMP, suppressed the release of mitochondrial cytochrome *c* and apoptosis, suggesting that LMP is an upstream event of MOMP.

As a result of LMP, a large number of lysosomal proteases are released into the cytosol. It has been reported that lysosome-dependent apoptosis is mainly mediated by papain-like cathepsins since cathepsin-specific inhibitors prevented apoptosis induced by lysosomal-damaging agents in some types of cells [6, 7]. Cathepsin B and cathepsin D are two of the most abundant and well-studied cathepsins that have been reported to be involved in apoptotic regulation via cleaving Bid. However, the function of cathepsins in apoptotic induction is still a matter of controversy because the optimal pH of cathepsins is acidic (lysosomal pH) rather than neutral pH (cytosolic pH). Most cathepsins might lose their activity rapidly upon release into the neutral cytosol [2, 8, 22]. In certain cell types, cathepsin-specific inhibitors failed to prevent mitochondrial membrane permeability (MOMP) induced by lysosomal-damaging agents [23, 24], and cells lacking the expression of cathepsins B, D, L, or S were as sensitive as wild-type control cells to hydroxychloroquine-induced apoptosis [24]. These inconsistencies might be ascribed to the different cell types and pro-apoptotic stimuli used.

Since chymotrypsin B is a novel member of the known lysosomal proteases and is capable of cleaving Bid, we compared the Bid-processing functions of chymotrypsin B and cathepsins B and D. Consistent with our previous report, chymotrypsin B cleaved Bid rapidly in cytosolic circumstances (pH 7.4) and induced the release of cytochrome *c* from mitochondria in a cell-free system. However, we did not find the effective cleavage of Bid by either cathepsin B or cathepsin D in cytosolic

Fig. 4 Chymotrypsin B cleaved Bid rapidly and induced the release of cytochrome *c* from mitochondria. **a** Ammonium chloride inhibited cytochrome *c* release from mitochondria. RH-35 cells were pretreated with 1 mM NH_4Cl (an inhibitor of lysosomal acidification) and then exposed to 100 μM H_2O_2 for the indicated intervals. The localization of cytochrome *c* was detected by immunoblot. In RH-35 cells pretreated with NH_4Cl, the H_2O_2-induced redistribution of mitochondrial cytochrome *c* into the cytosol was inhibited. **b** Ammonium chloride prevented apoptosis. RH-35 cells were pretreated with 1 mM NH_4Cl and then exposed to 100 μM H_2O_2 for 12 h. The nuclear morphology was observed, and the percentage of apoptotic cells was analyzed by flow cytometry. NH_4Cl pretreatment caused a significant decrease in apoptosis ($*p < 0.05$). **c** Cleavage of Bid by chymotrypsin B, cathepsin B, and cathepsin D during cytosolic circumstances. Five micrograms of recombinant Bid was incubated with the indicated concentrations of chymotrypsin B, cathepsin B and cathepsin D for 1 h under circumstances mimicking the cytosol. The cleavage of Bid was assayed by immunoblot. Chymotrypsin B cleaved Bid during cytosolic conditions in a dose-dependent manner. **d** Cleavage of Bid by chymotrypsin B, cathepsin B, and cathepsin D at neutral or acidic pH conditions. Five micrograms of recombinant Bid was incubated with different concentrations of chymotrypsin B, cathepsin B, and cathepsin D for 1 h and the cleavage of Bid was assessed by SDS-PAGE. Each of these proteases show Bid-cleaving (or Bid-degrading) activity at acidic pH. However, neither cathepsin B nor cathepsin D showed Bid-cleaving activity at neutral pH. On the contrary, chymotrypsin B showed potent Bid-cleaving activity at neutral pH. **e** Induction of cytochrome *c* release from mitochondria by chymotrypsin B-cleaved Bid. Bid was incubated with the indicated concentrations of caspase-8 or chymotrypsin B at 37°C for 1 h, and mixed with an aliquot of rat liver mitochondria equal to 50 μg of protein at 30°C for 30 min. The release of cytochrome *c* into the supernatants was detected by immunoblot. Chymotrypsin B-cleaved Bid induced the release of mitochondrial cytochrome *c* rapidly. **f** Intracellular delivery of chymotrypsin B, cathepsin B, and cathepsin D induced apoptosis in Bid-silencing cells. pSUPER-Bid plasmid containing the Bid targeting sequence and pSUPER.retro.puro as the non-silencing control were transfected into RH-35 cells, and stable transfected cell lines were generated. The expression of Bid was assayed by immunoblot. Chymotrypsin B, cathepsin B, and cathepsin D were delivered intracellularly to the stable Bid-silencing cell line or non-silencing control cell line by BioPORTER, and apoptosis was assayed by flow cytometry. Intracellular delivery of chymotrypsin B, cathepsin B, and cathepsin D induced apoptosis in RH-35 cells. Silencing Bid expression significantly attenuated chymotrypsin B-induced apoptosis. However, silencing of Bid expression shows no significant effects on cathepsin B- or cathepsin D-induced apoptosis. $*p < 0.05$; $**p > 0.05$



circumstances (pH 7.4) as reported elsewhere [6]. Bid-degrading activity could not be observed at pH 7.4 unless the concentrations of cathepsin B or cathepsin D were very high (~1 mM; data not shown). These data suggest that chymotrypsin B, but not cathepsin B or cathepsin D, accounts for most of the processing of Bid after LMP in RH-35 cells.

To further examine the role of Bid in lysosomal protease-dependent apoptosis, we generated RH-35 cell lines stably expressing a Bid-targeting shRNA. Silencing Bid expression effectively attenuated the apoptosis induced by intracellular delivery of recombinant chymotrypsin B, implying that Bid is the major effector molecule in chymotrypsin B-dependent apoptosis. In contrast, silencing Bid expression showed no effects on apoptosis induced by intracellular delivery of cathepsin B or cathepsin D. Therefore, Bid activation by lysosomal chymotrypsin B should be an important event in apoptosis of RH-35 hepatoma cells.

The mechanism underlying chymotrypsin B-dependent cleavage of Bid remains to be determined. In our previous report, we identified F67 of rat Bid (and also murine Bid) as the rat chymotrypsin B-specific cleavage site of rat Bid [14]. However, this cleavage site is absent in human Bid. Our recent studies indicate that human chymotrypsin B cleaved human Bid rapidly, and the cleaved human Bid could trigger the rapid redistribution of mitochondrial cytochrome *c* (Cao et al., unpublished results). Thus,

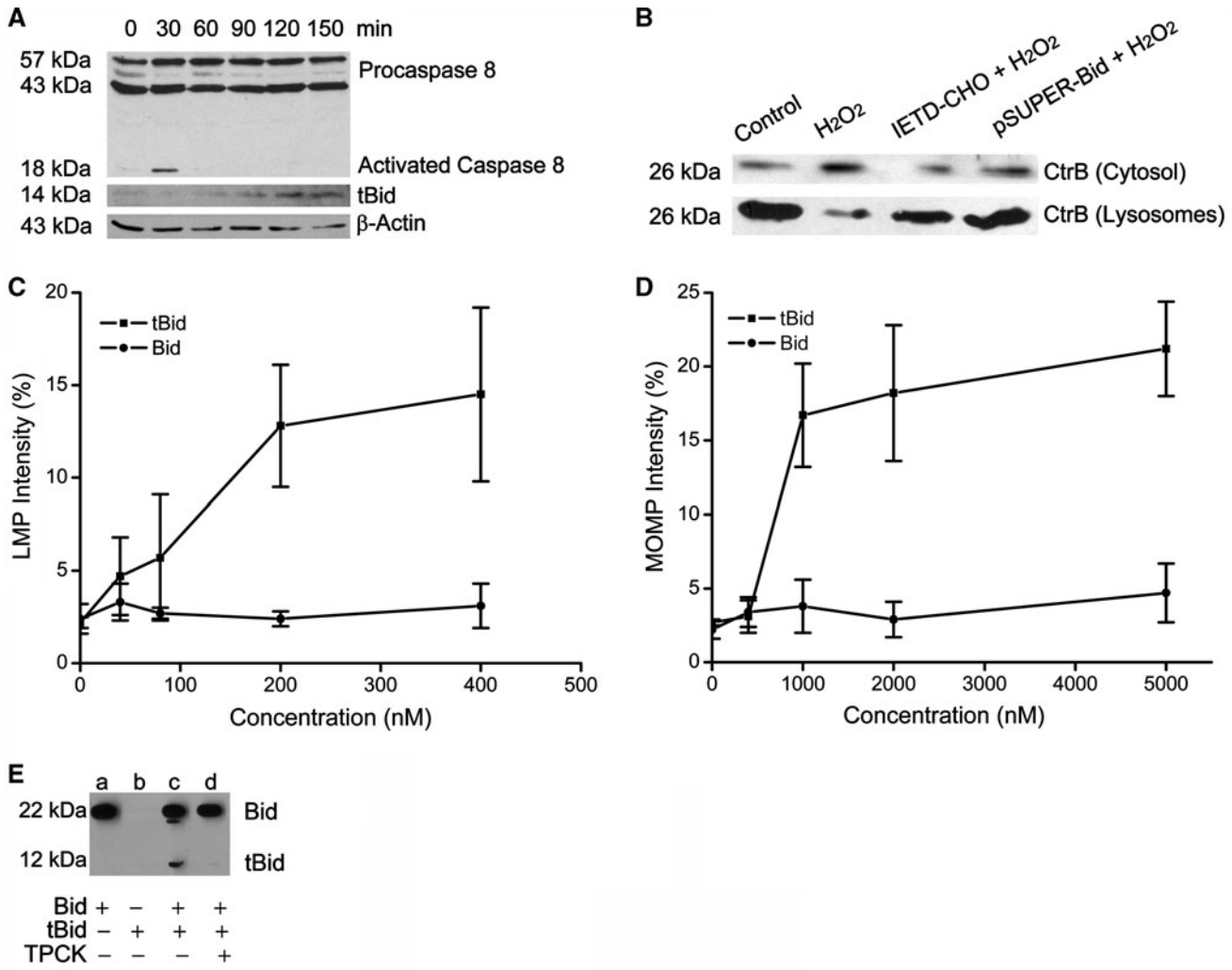


Fig. 5 Relationship between tBid level and triggering of LMP and MOMP. **a** Caspase-8 was activated in RH-35 cells exposed to H_2O_2. RH-35 cells were exposed to H_2O_2 for the indicated intervals. The activation of caspase-8 and the cleavage of Bid were assayed by immunoblot. Caspase-8 was activated rapidly after H_2O_2 exposure, and the cellular tBid levels increased time-dependently. **b** Caspase-8 and Bid were necessary for hydrogen peroxide-induced lysosomal permeabilization. Cells were exposed to H_2O_2 for the indicated intervals and the redistribution of lysosomal chymotrypsin B was analyzed by immunoblot. In RH-35 cells incubated with IETD-CHO (caspase-8 inhibitor) or in Bid-silencing cells, the H_2O_2-induced redistribution of lysosomal chymotrypsin B was inhibited. **c** tBid induced LMP in a dose-dependent manner. Rat liver lysosomes equal to 50 μg of protein was incubated with tBid at 30°C for 30 min. After incubation, the reaction mixture was pelleted by centrifugation for 5 min at 18,000 × *g*. The supernatants and the pellets were collected and were analyzed for cathepsin D content by immunoblot. The percentage of cathepsin D released into the supernatants was used as an index of LMP. **d** tBid induced MOMP in a dose-dependent manner. Rat liver mitochondria equal to 50 μg of protein were incubated with tBid at 30°C for 30 min. After incubation, the reaction mixture was pelleted by centrifugation for 5 min at 12,000 × *g*. The supernatants and the pellets were collected and were analyzed for cytochrome *c* content by immunoblot. The percentage of cytochrome *c* released into the supernatants was used as an index of MOMP. **e** tBid triggered chymotrypsin B-dependent Bid cleavage in isolated rat liver lysosomes. An aliquot of rat liver lysosomes equal to 50 μg of protein was incubated with 2 μM Bid or 2 μM Bid and 0.4 μM of tBid in a final volume of 50-μl at 30°C for 30 min. After incubation, the reaction mixture was pelleted by centrifugation for 5 min at 18,000 × *g*. The supernatant was analyzed by immunoblot. Detectable tBid (seen in *lane c*) derived from added full length Bid (added at 2 μM in *lanes a*, *c*, *d*) was observed only when trace amounts of tBid was also added (b, c, 0.4 μM not detectable at this exposure) but was not observed in the presence of TPCK (**d**)

chymotrypsin B should also mediate apoptosis in cells with human origins by processing Bid. However, in order to fully understand the mechanism underlying chymotrypsin B-dependent cleavage of Bid, further studies are necessary to identify the cleavage site of human Bid.

During extrinsic pathway-mediated apoptosis, LMP was mediated by caspase-8/Bid [19, 25]. Interestingly, we found that caspase-8 is rapidly activated in RH-35 cells following H_2O_2 exposure. We then asked if caspase-8/Bid also mediated LMP during H_2O_2-induced apoptosis.

Notably, inhibition of caspase-8 by IETD-CHO markedly reduced the H_2O_2-induced lysosomal release of chymotrypsin B. In addition, silencing Bid expression effectively inhibited the redistribution of lysosomal chymotrypsin B. These findings demonstrated that caspase-8/Bid are critical in triggering LMP during H_2O_2-induced, intrinsic pathway-mediated apoptosis.

We noted that at the time point when LMP occurred (60 min after H_2O_2 exposure), the cellular tBid level was much lower in comparison with that at a later time when MOMP appeared (90 min after H_2O_2 exposure). This raised the possibility that LMP is more sensitive to tBid than MOMP. This hypothesis was tested by a study in a cell-free system where different concentrations of tBid were incubated with lysosomes or mitochondria. We observed that concentrations as low as 0.2 μM tBid could induce LMP but not MOMP, which did not occur unless the tBid level was increased to 1 μM or above. Since chymotrypsin B released from lysosomes can cleave cytosolic Bid to tBid, we hypothesized that chymotrypsin B-mediated Bid cleavage might be a key bridge between LMP and MOMP. Exposure of RH-35 cells to H_2O_2 activates caspase-8 rapidly, which cleaves Bid to tBid in levels sufficient to elicit LMP and the release of chymotrypsin B, which can further cleave more Bid. This chymotrypsin B-dependent processing of Bid and the consequent accumulation of tBid was observed in cell-free systems using isolated rat liver lysosomes. Elevated levels of tBid could also further trigger LMP through this positive feed-back pattern. When the cellular tBid accumulates to a sufficient concentration, MOMP occurs with the mitochondrial release of cytochrome *c* (Fig. 6).

As is well documented, Bcl-2 family members (tBid, Bak, Bax) have permeabilizing properties for mitochondrial membranes. It was also reported recently that these proteins may have similar destabilization properties for lysosomal membranes. However, the precise mechanisms remain unknown [19, 25]. We have investigated the ability of tBid to bind and permeabilize liposomes via pore formation in the lipid membranes [12]. We found such ability is dependent on the phospholipid components of liposomes. In the present study, we noted that lower tBid concentration was sufficient to induce LMP in comparison with MOMP. Thus, one of the major challenges for future investigation is to define the molecular details of LMP and how the mechanism of LMP differs from that of MOMP.

In summary, we report here a chymotrypsin B-mediated lysosomal pathway that leads to the amplification of mitochondria-dependent apoptotic signaling. Although a digestive enzyme secreted by pancreas, chymotrypsin B is also widely expressed in different tissues (cerebrum, cerebellum, heart, lung, liver, kidney, and spleen). In rat hepatocytes, chymotrypsin B is subcellularly localized in lysosomes and released into the cytosol as a result of LMP. Bid activation by caspase-8 initiates LMP, which is further reinforced by lysosomal chymotrypsin B-dependent Bid processing, which results in MOMP and, finally, apoptosis. Chymotrypsin B, but not cathepsin B and cathepsin D, plays a major role in apoptosis through a Bid-dependent mitochondrial pathway in stressed RH-35 hepatoma cells. In addition, we found that a lower level of tBid is sufficient to trigger LMP than that required to trigger MOMP. This study provides a basis for further study of the molecular mechanisms of LMP and MOMP.

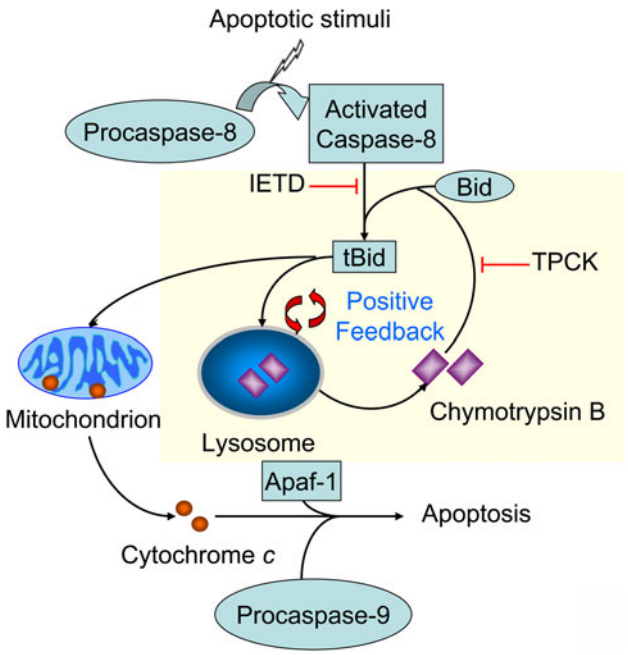


Fig. 6 Hypothesis of involvement of lysosomal chymotrypsin B in the mitochondrial apoptotic pathway. Upon exposure to apoptotic stimuli, lysosomal chymotrypsin B was released into the cytosol of RH-35 hepatoma cells as a result of LMP via a caspase-8- and Bid-dependent mechanism. Chymotrypsin B causes significant cleavage of Bid under cytosolic conditions (pH 7.4), thereby presumably initiating a positive feedback that results in the accumulation of cellular tBid, which eventually leads to MOMP, and induced the execution of apoptosis

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