Activation of caspase-3-like protease by digitonin-treated lysosomes

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Abstract Apoptosis, a naturally occurring programmed cell death or cell 'suicide', has been paid much attention as one of the critical mechanisms for morphogenesis and tissue remodeling. Activation of cysteine aspartases (caspases) is one of the critical steps leading to apoptosis. Although a mitochondria-mediated pathway has been postulated to be one of the activation mechanism of caspase-3, another subcellular compartment might be involved in the activation of the enzyme. The present study shows that the supernatant fraction of digitonin-treated lysosomes strongly activates Ac-DEVD-CHO inhibitable caspase-3-like protease. Activation of caspase-3-like protease by digitonin-treated lysosomal fractions was specifically suppressed by leupeptin and E-64, inhibitors of cysteine protease. These results indicate that leakage of lysosomal cysteine protease(s) into the cytosolic compartment might be involved in the activation of caspase-3-like protease.

Key words: Apoptosis; Digitonin treatment; Caspase-3; Cathepsin; Lysosomal protease; Protease inhibitor

1. Introduction

Apoptosis or programmed cell death plays important roles in the regulation of tissue development and homeostasis [1,2]. In the last few years, many of the molecules that participate in the biochemical pathway that mediates the highly ordered process of apoptosis have been identified. A family of cysteine proteases, the 'caspases', are at the heart of this pathway [3,4]. Among them, caspase-3 (DEVD-specific CPP32) functions at a late step of the protease cascade [5], and cleaves 'death substrates', such as poly-ADP ribose polymerase (PARP) and inhibitor of caspase-activated DNase (ICAD) [5,6].

Recent studies also revealed the involvement of mitochondria in the activation pathway of caspase-3 [7,8]. Opening of the permeability transition pore (PTP) of mitochondria increases membrane permeability for low molecular weight compounds, decreases the membrane potential, induces large amplitude swelling, and releases cytochrome c (Cyt. c) [9]. In the presence of dATP, caspase-3 is activated by an apoptotic protease activating factor (Apaf) complex consisting of Apaf-1 (human homolog of CED-4 protein), Apaf-2 (Cyt. c) and Apaf-3 (pro-caspase 9) [9–11].

Recent studies also suggested that oxidative stress plays a critical role in the mechanism of apoptosis [12–14]. Changes in mitochondrial functions leading to energy depletion, increase in cytosolic Ca^{2+} level, thiol oxidation, lipid peroxidation and DNA damage are also involved in the mechanism of apoptosis [15–18]. In this context, hydrogen peroxide-induced cellular damage is often enhanced by the presence of a catalytically active form of intracellular iron, leading to damage of lysosomal membranes and the release of their enzymes into the cytosol [19–22]. Thus a mild increase in the permeability of lysosomal membranes might possibly induce cellular changes leading to apoptosis while extensive damage of the lysosomal membranes might result in cellular swelling and necrosis [19,20]. To test the possible involvement of lysosomal enzymes in the mechanism of apoptosis, the effects of digitonin, an agent that disrupts membranes by reacting with cholesterol [23], on the release of lysosomal enzymes and the activation of caspases were investigated. The present work showed that digitonin perturbed the lysosomal membrane and that the released lysosomal enzymes activated caspase-3-like protease.

2. Materials and methods

2.1. Chemicals

Fluorogenic tetrapeptide substrates (Ac-YVAD-MCA, Ac-DEVD-MCA), inhibitors of the caspase family (z-VAD-fmk, Ac-DEVD-CHO) and inhibitor of cathepsin B (CA-074 Me) were obtained from the Peptide Institute (Osaka, Japan). Various protease inhibitors were obtained from Sigma Co. (St. Louis, MO, USA). All other chemicals were of analytical grade and obtained from Nacalai Tesque (Kyoto, Japan). Digitonin was dissolved in ethanol and used at final ethanol concentrations lower than 0.5%.

2.2. Isolation of mitochondria and cytosolic fractions

After fasting overnight, liver mitochondria were isolated from Wistar rats weighing 200–250 g by the method of Hogeboom [24]. A cytosolic fraction of rat liver (S100) was obtained from a post-mitochondrial fraction of liver homogenate by centrifugation at $105\,000 \times g$ for 60 min. The samples were kept at −80°C until use.

2.3. Purification of lysosomes

Rat liver lysosomes were isolated by a single two-phase partition on dextran/polyethylene glycol by the method of Osada et al. [25]. Briefly, a crude mitochondrial fraction was washed twice with 0.25 M sucrose at 4°C and resuspended in a solution containing 0.32 M

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Abbreviations: AMC, 7-amino-4-methyl-coumarin; Apaf, apoptotic protease activating factor; ICAD, inhibitor of caspase-activated DNase; caspase, cysteine protease cleaving after aspartic acid; Cyt. c, cytochrome c; ICE, interleukin-1β-converting enzyme; NAGA, *N*-acetyl-β-D-glucosaminidase; PARP, poly-ADP ribose polymerase; PMSF, phenylmethylsulfonyl fluoride; PTP, permeability transition pore; S100, cytosolic fraction of rat liver; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone

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sorbitol, 0.1 mM EDTA and 5 mM ammonium phosphoric acid buffer (pH 7.8) (SEAPB). The mixture was centrifuged at 19 000×*g* for 20 min. The pellet was resuspended in SEAPB and superimposed on a 6.6% dextran T500/6.6% polyethylene glycol 40000 two-phase mixture containing SEAPB. After centrifugation at 600×*g* for 2 min, the upper and lower phases, corresponding to lysosomal and mitochondrial fractions, respectively, were collected. The two fractions were centrifuged at 19 000×*g* for 30 min and washed with 0.25 M sucrose. Highly purified lysosomes were also isolated from the liver of the rat after administration of dextran one day before isolation by the method of Arai et al. using a percoll density gradient centrifugation [26].

2.4. Treatment of mitochondrial and lysosomal fractions by digitonin and activation of caspase-3-like protease

Supernatants of crude mitochondrial and lysosomal fractions were obtained by centrifugation at 20 000×*g* for 10 min at 4°C after treatment of each fraction with 40 μM digitonin on ice for 10 min. To determine whether the supernatant of digitonin-treated mitochondrial and lysosomal fractions activate caspase-3-like protease, these supernatants were incubated with S100 at 37°C for 120 min. In some experiments, various inhibitors were added to the reaction mixture.

2.5. Assay for enzymes

Activities of arylsulphatase and acid phosphatase were measured by the method of Bergmeyer et al. [27]. *N*-acetyl-β-D-glucosaminidase (NAGA) activity was assayed as described by Tarentino and Maley [28]. After incubation with the supernatants of crude mitochondrial or lysosomal fractions, caspase activity of S100 was assayed in 20 mM HEPES buffer, pH 7.5, containing 0.1 M NaCl and 5 mM DTT at 37°C for 60 min as described previously [29] using a 10 μM solution of either Ac-YVAD-MCA (for caspase-1) or Ac-DEVD-MCA (for caspase-3). Protein concentrations were determined by the method of Lowry et al. [30] using bovine serum albumin as a standard.

3. Results and discussion

3.1. Activation of caspase-3-like protease by a crude mitochondrial fraction

To test the activation of caspase-1- and 3-like proteases, a supernatant of the digitonin-treated crude mitochondrial fraction was incubated with rat liver cytosol (S100) for 120 min at 37°C. Caspase-3- but not caspase-1-like protease in S100 was strongly activated by the supernatant (Fig. 1). This activity was inhibited by Ac-DEVD-CHO, a specific inhibitor of caspase-3. The activation of caspase-3-like protease was dependent on the concentration of digitonin used for the treatment of the crude mitochondrial fraction at 4°C for 10 min (Fig. 2A); maximum activation was observed at a digitonin concentration of 80 μM. The activation of caspase-3-like protease was dependent on the time of incubation with the supernatant of crude mitochondrial fraction (Fig. 2B). Caspase-3-like protease was also activated by incubating cytosol with a supernatant fraction of a freeze/thawed crude mitochondrial fraction (data not shown). These results suggested that some factors that activate caspase-3-like protease were released from the crude mitochondrial fraction by digitonin treatment.

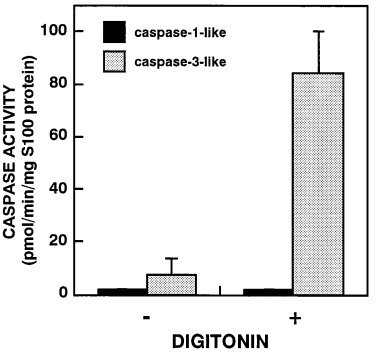

Fig. 1. Effect of a supernatant of a digitonin-treated mitochondrial fraction on the activation of caspase-3-like protease. The mitochondrial supernatant was obtained by centrifugation at 20 000×*g* for 10 min at 4°C after treatment of the crude mitochondrial fraction (1.5 mg protein/ml) with or without 40 μM digitonin on ice for 10 min. To activate caspase-3-like protease, 100 μl of S100 (1 mg protein/ml) was incubated with the 200 μl supernatant of crude mitochondrial fraction at 37°C for 120 min. After incubation, the mixture was incubated with 10 μM solution of fluorogenic peptide substrate, Ac-YVAD-MCA (for caspase-1) or Ac-DEVD-MCA (for caspase-3) in 20 mM HEPES medium containing 0.1 M NaCl, 5 mM DTT at 37°C for 60 min. Data are the means ± S.D. from three separate experiments.

3.2. Inhibition of caspase-3-like protease activation by various protease inhibitors

A crude mitochondrial fraction has been known to be contaminated with lysosomes. Because lysosomal membranes contain cholesterol, digitonin might permeabilize those membranes. In fact, activities of lysosomal enzymes are detected in a supernatant of a crude mitochondrial fraction by treatment with digitonin (Table 1). Activation of caspase-3-like protease by the supernatant was inhibited by leupeptin and E-64, inhibitors of cysteine protease, but not by other inhibitors such as PMSF, aprotinin and pepstatin A (Fig. 3). CA-074 Me [31], a membrane permeable specific inhibitor of cathepsin B which is one of the major cysteine proteases in lysosome [32], also had no inhibitory effects on caspase-3-like protease activation, indicating that cathepsin B is not involved in this activation. Since caspase-3-like protease activity, once activated by the supernatant, was not inhibited by leupeptin and E-64, these inhibitors had no ability to inhibit caspase-3-like activity. In contrast, caspase-3-like activity was inhibited by Ac-DEVD-CHO, z-VAD-fmk and TPCK even if these inhibitors were added after activation of caspase-3-like protease by the super-

Table 1
Activity of lysosomal enzymes in the supernatant of crude mitochondrial fraction, upper and lower phases of the two-phase partition of mitochondrial fraction, and highly purified lysosomal fraction after the treatment with digitonin

	Enzyme activity (μmol/min/ml extract)		
	Acid phosphatase	Arylsulphatase	NAGA
Crude mitochondrial fraction	10.62 ± 0.64	0.33 ± 0.10	30.86 ± 3.85
Two-phase partition			
Lower phase (mitochondrial fraction)	2.06 ± 0.38	0.15 ± 0.06	12.43 ± 1.92
Upper phase (crude lysosomal fraction)	34.81 ± 14.45	0.84 ± 0.16	107.20 ± 43.33
Highly purified lysosomes	94.77 ± 26.20	3.92 ± 1.28	192.55 ± 13.03

Each fraction was treated with 40 μM digitonin. NAGA, *N*-acetyl-β-D-glucosaminidase; data are the means ± S.D. from three separate experiments.

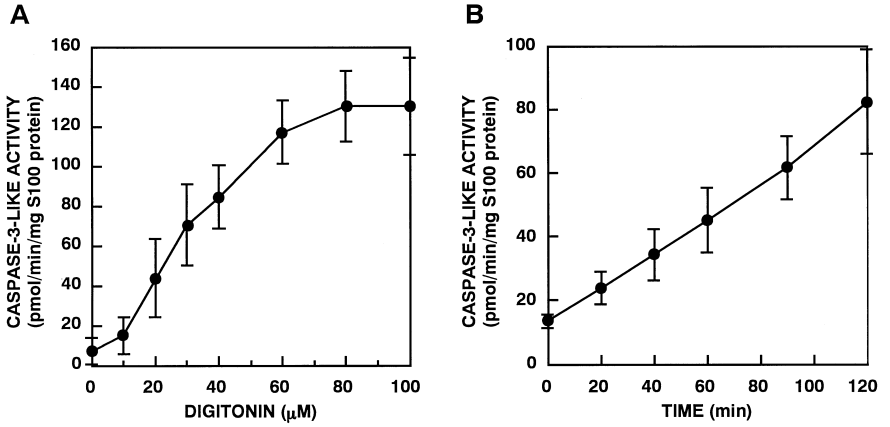

Fig. 2. Effect of digitonin concentration and incubation time on the activation of caspase-3-like protease by the supernatant of a digitonin-treated crude mitochondrial fraction. Experimental conditions were as described in Fig. 1. A: Dose-dependent curve of digitonin for the treatment of crude mitochondrial fraction. The crude mitochondrial fraction was treated with various concentrations of digitonin for 10 min on ice. B: Time-dependent curve for caspase-3-like protease activation by a digitonin-treated crude mitochondrial supernatant. S100 was incubated with the supernatant of a digitonin (40 μM)-treated crude mitochondrial supernatant for the indicated time at 37°C. Data are the means ± S.D. from three separate experiments.

natant, indicating that these inhibitors directly inhibited caspase-3-like activity (Fig. 3).

3.3. *Caspase-3-like protease activation by digitonin-treated lysosomes*

To test the possible involvement of lysosomal enzymes in the activation of caspase-3-like protease, we isolated a crude lysosomal fraction from a rat liver homogenate using a single two-phase partition on dextran/polyethylene glycol [25] and a highly purified lysosome fraction using a percoll density gradient centrifugation method [26]. Caspase-3-like protease was strongly activated by the supernatant of a digitonin-treated crude lysosomal fraction (upper phase) but not by a mitochondrial fraction (lower phase) (Fig. 4). In accordance with the fact, much higher activities of lysosomal enzymes were detected in the supernatant of upper phase than those of lower phase (Table 1). Furthermore, the ability of the supernatant from purified lysosomes to activate caspase-3-like protease was stronger than that from a crude lysosomal fraction (upper phase) in parallel with the higher activities of lysosomal enzymes (Fig. 4 and Table 1).

Thus, the present work demonstrated for the first time that the enzyme released from digitonin-treated lysosomes activated caspase-3-like protease and that the activation was specifically inhibited by inhibitors of cysteine protease. Since a specific inhibitor against cathepsin B (CA-074 Me) did not inhibit the activation, other lysosomal cysteine proteases such as cathepsin L, cathepsin S, etc. [32] might be involved in this activation.

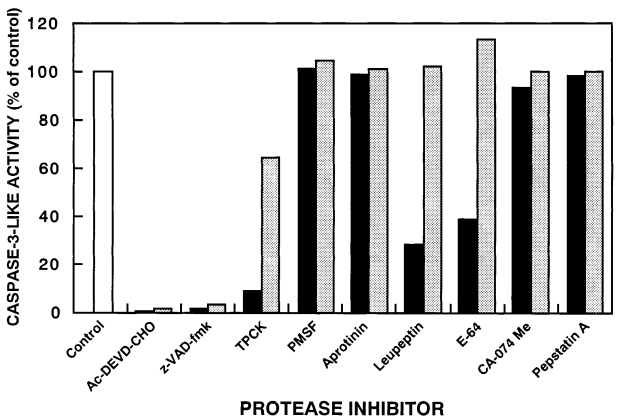

Fig. 3. Effect of protease inhibitors on the activation of caspase-3-like protease by a supernatant of a digitonin-treated crude mitochondrial fraction. Experimental conditions were as described in Fig. 1. Caspase-3-like protease in S100 was activated by the digitonin-treated supernatant of crude mitochondrial fraction for 120 min at 37°C. Inhibitors were added to the reaction mixture before or after activation of caspase-3-like protease. Then caspase-3-like activity was measured in the presence of substrate. Concentrations of z-VAD-fmk, Ac-DEVD-CHO, PMSF, TPCK, leupeptin, aprotinin, E-64, CA-074 Me and pepstatin A were 30 μM, 30 μM, 0.1 μM, 30 μM, 10 μg/ml, 10 μg/ml, 10 μM, 10 μM and 10 μg/ml, respectively. White bar, control without inhibitors; shaded bars, inhibitors were added after activation of caspase-3-like protease in S100 by the supernatant; black bars, inhibitors were added before activation of caspase-3-like protease in S100 by the supernatant. Data were expressed by % of control.

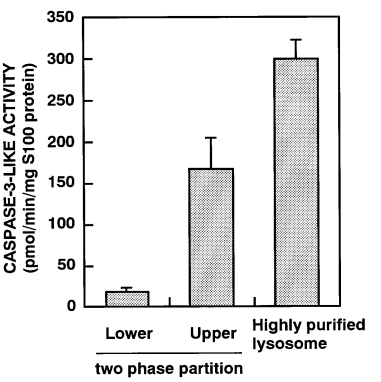

Fig. 4. Activation of caspase-3-like protease by digitonin-treated crude and purified lysosomal fractions. Assay system for caspase-3-like protease activation was as described in Fig. 1. A crude lysosomal fraction (upper phase) was separated from mitochondrial fraction (lower phase) by a single two-phase partition on dextrane/polyethylene glycol [25]. Highly purified lysosomes were isolated using a percoll density gradient centrifugation method [26]. Each fraction (1.5 mg protein/ml) was treated with 40 μM digitonin and the supernatants (200 μl) were incubated with S100 (100 μl). Data are the means ± S.D. from three separate experiments.

Some proteases and endonucleases in lysosomes have been reported to induce apoptosis of cells in the tadpole tail [33]. Apoptosis of cultured fibroblasts and hepatocytes is also induced by the photo-oxidative disruption of lysosomal membranes [34] and by cathepsin B [35], respectively. Inositol-3-phosphate (IP3) increased the intracellular concentration of calcium ions and induced apoptosis in neuronal cells of monkeys through lysosomal enzymes activated by calpain [36] by a leupeptin inhibitable mechanism [37]. Taken together, a moderate release of enzymes from lysosomes into the cytosol might activate caspase-3 and induce apoptosis of cells.

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