Apoptotic cell death and CPP32-like activation induced by thapsigargin and their prevention by nerve growth factor in PC12 cells

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Abstract

Thapsigargin, an endoplasmic reticular Ca\(^{2+}\)-ATPase inhibitor, induced apoptotic cell death (chromatin condensation and DNA fragmentation) accompanied by the activation of CPP32-like protease, a member of the interleukin-1β converting enzyme protease (ICE) family, but not the activation of ICE-like protease. Nerve growth factor (NGF) completely inhibited the cell death and CPP32-like activation induced by thapsigargin while Ac-Asp–Glu–Val–Asp-CHO, an inhibitor of CPP32-like protease, reduced the cell death. PD98059, a specific inhibitor of Map kinase kinase, did not reduce the protective effect of NGF on thapsigargin-induced cell death. These results suggest that calcium ion-induced apoptotic cell death was mediated by CPP32-like, but not ICE-like, protease and was regulated by a neurotrophic factor possibly, through the Map kinase cascade independent pathway. © 1998 Elsevier Science B.V.

Keywords: Thapsigargin; Calcium; Apoptosis; Nerve growth factor; CPP32; PC12 cell

1. Introduction

Calcium ion is essential for cellular functions including signal transduction [1]. However, uncontrolled calcium stress has been linked causally to a variety of neurodegenerative diseases, including ischemia, excitotoxicity and Alzheimer’s disease [2–5]. N-Methyl-D-aspartate (NMDA), an agonist of an NMDA subtype of glutamate receptor, induced a marked calcium influx immediately after treatment of neurons such as of the spinal cord or cortex [3,6]. β-Amyloid protein was reported to increase calcium accumulation [7]. To know the mechanisms of calcium stress-induced cell death, we examined the effect of thapsigargin, a specific inhibitor of endoplasmic reticular Ca\(^{2+}\)-ATPase [8,9], on the rat pheochromocytoma PC12 cell line which shows a sympathetic neuron-like phenotype in response to nerve growth factor (NGF) [10]. Thapsigargin, which increases \([\text{Ca}^{2+}]_i\), has been used as an important experimental tool in a large number of \(\text{Ca}^{2+}\)-dependent intracellular functions, including control of cellular growth [11,12].

The best characterized neurotrophic molecule is the NGF, which supports the development and survival of peripheral sympathetic and sensory neurons [13]. It also has been shown in in vitro experiments
that NGF is essential for the development and maintenance of septal cholinergic neurons [14,15]. The differentiation of neurons by neurotrophins such as NGF is mediated by the activation of the Map kinase cascade through several steps including Ras and Raf-1 kinase [16–18].

Genetic studies in the roundworm Caenorhabditis elegans identified the ced3 gene, whose functioning is required for all of the developmentally programmed death in the hermaphrodite [19]. CED3 is a cysteine protease that cleaves protein at Asp–X peptide bonds. Recent evidence has implicated the CED/ICE-related protease CPP32 in the effector pathway in non-neuronal cells [20,21]. In the present study, we reported that thapsigargin induced apoptotic cell death and CPP32-like activation in PC12 cells and that NGF or a peptide inhibitor of CPP32-like protease inhibited it.

2. Materials and methods

2.1. Materials

7S NGF from mouse submaxillary glands was purchased from Sigma (St. Louis, USA), Hoechst 33258 (bis-benzimide) from Molecular Probes (Oregon, USA), Ac-Asp–Glu–Val–Asp–4-methyl-coumaryl-7-amide (Ac-DEVD-MCA), Ac-Tyr–Val–Ala–Asp-coumaryl-7-amide (Ac-YVAD-MCA), Ac-DEVD-CHO tetrapeptide aldehyde and 7-amino-4-methyl-coumarin AMC from Peptide Institute (Osaka, Japan), and PD98059 from BIOMOL Research Laboratories (PA, USA). Indo-1 and its acetoxymethyl ester and other chemicals were purchased from Wako Pure Chemical (Osaka, Japan).

2.2. Assessment of cellular injury

PC12 cells were obtained from a RIKEN cell bank (Ibaragi, Japan) and subcultured in 48-well plates or 35 mm dishes coated with poly-D-lysine at a density of about 2.5 × 10^5 cells per well or 1 × 10^6 cells per dish in a DMEM medium supplemented with 5% precolostrum newborn calf serum and 5% horse serum for 1–2 days before the experiment. Cells were washed twice with DMEM and then 0.5 ml of DMEM was added. Unless specified in Section 3, no serum was added to DMEM because the serum itself has a relatively high lactate dehydrogenase (LDH) activity (about 30% of the value of “No addition”). LDH activity of the serum itself was subtracted from each value. Thapsigargin was added to cells with or without 7S NGF and other reagents. Cells were then maintained under 95% air/5% CO, for 18 h at 37°C. Morphological changes of cells were checked throughout the course of the experiment by a phase-contrast microscopy; cell viability was checked by trypan blue staining. Cell injury was assessed by measurement of the activity of LDH released into the medium for 18 h after the treatment.

2.3. Analysis of DNA fragmentation

Low molecular weight DNA was isolated from 3 × 10^6 cells as described by Hockenberg et al. [22]. Agarose (1.2%) gel electrophoresis of DNA was performed in a 40 mM Tris–HCl buffer (pH 8.1) containing 2 mM EDTA. After electrophoresis for 60 min, gels were treated with RNase (10 μg/ml) for 3 h at 37°C and then stained with ethidium bromide (0.5 μg/ml) for 15 min at room temperature.

2.4. Nuclear staining and morphology

Cells were fixed with a 10% formalin neutral buffer solution (pH 7.4) for 5 min at room temperature. Cells were washed with distilled water and then stained with 8 μg/ml H33258 for 5 min. After a 5 min washing with distilled water, nuclear morphology was observed under a fluorescence microscope (Olympus, IX70 model).

2.5. Measurement of thapsigargin-induced calcium accumulation in cells

Calcium accumulation in PC12 cells was monitored by Ca^{2+}-sensitive fluorescent dye, Indo-1, using ACAS 570 (Meridian) as described previously [3].

2.6. Activity of CPP32 and ICE protease

Cells were treated with 2 or 10 nM thapsigargin in the presence or absence of NGF for 18 h. Cells were washed with phosphate buffered-saline (PBS) and suspended in 50 mM Tris–HCl (pH 7.4), 1 mM EDTA
and 10 mM EGTA. Cells were treated with 10 μM digitonin for 10 min. Lysates were then obtained by centrifugation at 20,000 × g for 5 min, and cleared lysates containing 50–100 μg protein were incubated with 50 μM of enzyme substrate Ac-DEVD-MCA and Ac-YVAD-MCA for CPP32 and ICE, respectively, at 37°C for 5–10 min. Levels of AMC were measured using a spectrofluorometer (Hitachi 850, Japan) with excitation at 380 nm and emission at 460 nm, and the activity was expressed as pmol AMC release/min/mg protein.

3. Results and discussion

Thapsigargin (20 nM) increased LDH release from PC12 cells regardless of the presence of calf serum (Fig. 1(A)). Treatment of PC12 cells with thapsigargin (10–20 nM) increased LDH release from the cells depending on the concentration of thapsigargin (Fig. 1(B)). The loss of cell viability was also confirmed by phase-contrast microscopy (Fig. 2) and trypan blue exclusion (data not shown). Cells treated with 10 nM thapsigargin shrank by phase-contrast observation and showed chromatin condensation and nuclear fragmentation detected by staining with H33258 (Fig. 2) and DNA fragmentation (data not shown). Thapsigargin increased the number of apoptotic cells (Table 1). A small number of cells were damaged by serum deprivation through apoptosis depending on experimental conditions (Fig. 1 and Table 1). As reported previously [23], serum withdrawal induces apoptotic cell death in PC12 cells. Thapsigargin probably induces apoptosis of PC12 cells by a different mechanism from that of serum deprivation because thapsigargin induced apoptotic cell death in the presence of serum (Fig. 1(A) and Table 1).

The neuron-like processes increased in length on culturing the cells with 100 ng/ml NGF for 18 h (Fig. 2). NGF completely inhibited LDH release from the cells, chromatin condensation and nuclear fragmentation, as induced by treatment of the cells with thapsigargin for 18 h (Figs. 1 and 2 and Table 1). Thapsigargin produced a sustained increase in [Ca^{2+}], in the presence of extracellular Ca^{2+} (Fig. 3), while thapsigargin produced a transient increase in [Ca^{2+}], in the absence of extracellular Ca^{2+} (data not shown). The calcium accumulation by 10 nM thapsigargin was not significantly inhibited by treatment of the cells with NGF, indicating that NGF did not modify the Ca^{2+} concentration in the cells (Fig. 3). PC12 cells treated with NGF exhibit characteristics of sympathetic neurons including the extension of neurites in need of the new protein synthesis [10]. We then
Fig. 2. Effect of NGF on morphology of the cytotoxicity produced by thapsigargin. Cells were treated with thapsigargin in the presence or absence of NGF. (A) Phase-contrast photomicrographs, (a) Control, (b) 10 nM thapsigargin, (c) 100 ng/ml NGF, (d) 10 nM thapsigargin + 100 ng/ml NGF. (B) Nuclear morphology of cells stained with H33258, (a) Control, (b) 10 nM thapsigargin, (c) 100 ng/ml NGF, (d) 10 nM thapsigargin + 100 ng/ml NGF. See Section 2 for further details of the method.

Table 1
Thapsigargin-induced apoptotic cell death

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<th>Apoptotic cells (%)</th>
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<td>Serum − Serum</td>
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<td>Thapsigargin</td>
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Cells were treated with 20 nM thapsigargin in the presence or absence of NGF. Nuclear staining with H33258 was done as described in Section 2. The number of apoptotic cells was counted by the observation of condensed or fragmented nuclei. Each value represents the ratio of apoptotic cells ± S.E.M of three sister cultures (50–100 cells).

* P < 0.01 (vs. thapsigargin only).

Fig. 3. Effect of NGF on thapsigargin-induced calcium ion accumulation. Cells were cultured for 1 day in the presence (●) or absence (○) of 100 ng/ml NGF. Thapsigargin (10 nM) was added at arrow. Each value represents the mean ± S.E.M. for 20 cells. See Section 2 for further details of the method.
Fig. 4. Protective effect of NGF on thapsigargin-induced cell injury in the presence of cycloheximide. (A) Phase-contrast photomicrographs of cells treated with thapsigargin for 18 h. (a) no addition, (b) thapsigargin only (10 nM), (c) NGF (100 ng/ml) + thapsigargin, (d) NGF only, (e) NGF + cycloheximide (1 μg/ml), (f) cycloheximide + NGF + thapsigargin. (B) LDH release from cells treated with thapsigargin. Each value represents the mean ± S.E.M. of three sister cultures, "*" P < 0.01 (vs. thapsigargin only).
examined whether the new protein synthesis is necessary for the NGF-dependent survival. Cycloheximide, an inhibitor of protein synthesis, did not reduce the protective effect of NGF on thapsigargin-induced cell death; cycloheximide did inhibit NGF-induced neurite outgrowth (Fig. 4). Using neurite outgrowth in PC12 cells as a measure of differentiation, studies have implicated the MAP kinase pathway in NGF signaling. We used the specific inhibitor (PD98059) of Map kinase kinase 24, which blocks the differentiation of PC12 cells induced by NGF, to examine if the protective effect of NGF on thapsigargin-induced apoptosis is dependent on the MAP kinase pathway. As shown in Fig. 5, PD98059 did not reduce the protective effect of NGF on thapsigargin-induced LDH release, while NGF-dependent neurite outgrowth was reduced (data not shown).

Recently, it is reported that interleukin-1β converting enzyme (ICE)-like cysteine protease family is essential for apoptotic cell death induced by several stimuli including Fas ligand and TNF [20,21]. Therefore, we measured the activities of CPP32-like and ICE-like protease using Ac-DEVD-MCA and Ac-YVAD-MCA as the enzyme substrates, respectively, after treatment of the cells with thapsigargin for 18 h. Thapsigargin increased the CPP32-like activity, but not the ICE-like activity (Fig. 6A and B). NGF inhibited the thapsigargin-induced activation of CPP32 depending on the concentration of NGF (Fig. 7). Ac-DEVD-CHO tetrapeptide aldehyde inhibitor (100–200 μM) of CPP32 attenuated apoptotic cell death and CPP32 activation induced by 10 nM thapsigargin (LDH release (Mean ± S.E.M., n = 3); No addition 27.7 ± 1.9%, thapsigargin 53.3 ± 2.1, 50 μM Ac-DEVD-CHO + thapsigargin 45.2 ± 2.2%, 100 μM Ac-DEVD-CHO + thapsigargin 39.5 ± 0.84%, 200 μM AC-DEVD-CHO + thapsigargin 34.3 ± 1.9%, * P < 0.05 vs. thapsigargin only).

Ca2+ ionophores were used to induce apoptosis in several cells such as thymocyte [25], PC12 cells [26] and cortical cells [27]. However, the existence of various non-specific effects of Ca2+ ionophores has raised the question whether effects other than the increase in [Ca2+], could be involved in the initiation of apoptosis [28–30]. Therefore, we used the selective endoplasmic reticular Ca2+-ATPase inhibitor thapsigargin [8,9] in the present study to increase the [Ca2+]. The mechanism of thapsigargin-mediated increase in [Ca2+] involves the initial release of Ca2+ from intracellular, non-mitochondrial Ca2+ pools, which in turn promote an influx of extracellular Ca2+ [9]. Thapsigargin induced cell death accompanied by chromatin condensation and nuclear fragmentation (Fig. 2), suggesting the apoptotic cell death. Recently, several reports showed that thapsigargin could induce apoptotic cell death in other cell types such as thymocytes, lymphoma cells or hematopoietic cells [31–34].

Apoptotic cell death and CPP32-like activation induced by thapsigargin were attenuated by the presence of NGF (Figs. 1, 6 and 7 and Table 1). The mechanism whereby NGF protects thapsigargin-induced apoptosis is unknown at present. The protective effect of NGF is not dependent on the new protein synthesis because cycloheximide did not reduce the protective effect of NGF on thapsigargin-induced cell death (Fig. 4). Cycloheximide, however, did inhibit neurite outgrowth (Fig. 4A). Moreover, cycloheximide itself did not inhibit the thapsigargin-induced apoptosis (data not shown), suggesting that the machinery for apoptosis already existed in the cells. NGF binds to and activates the tyrosine kinase.
of TrkA, the high affinity NGF receptor. Phosphotyrosyl motifs on TrkA bind Shc proteins, which bind and activate Grb/mSOS, thereby activating Ras. Ras activation results in activation of the protein kinases such as raf-1 followed by activation of Map kinase and Map kinase [16–18]. As shown in Fig. 5, PD98059, a specific inhibitor of MAP kinase kinase, did not inhibit the protective effect of NGF, suggesting that the Map kinase cascade is not necessary for survival. This agrees with results of recent reports [35,36]. An alternative signal transduction pathway different from Map kinase cascade after ras and/or raf activation by NGF maybe involved in the maintenance of cells. NGF may regulate the BCL-2 family such as BAD, which is phosphorylated by raf-1 kinase and then regulate the release of factors such as cytochrome c from mitochondria, necessary for apoptosis of some kinds of cells [37–39].

The apoptotic cell death was accompanied by the activation of CPP32-like protease, but not ICE-like, and the inhibitory peptide (Ac-DEVD-CHO) of CPP32-like protease reduced the thapsigargin-induced cell death (Fig. 7). The possibility that other ICE family proteases are involved in thapsigargin-induced apoptosis cannot be ruled out, because the specificity of the peptide substrate may not be so strict [40]. Recently, it has been reported [20,21,41] that CPP32 is a key enzyme relating to the downstream of events on apoptosis induced by several stimuli including FAS antigen. Also, Enari et al. [20] reported that sequential activation of ICE-like and CPP32-like proteases occurred during Fas-mediated

Fig. 6. Effect of NGF on thapsigargin-induced CPP32-like and ICE-like protease activities. Cells were incubated with 2 or 10 nM thapsigargin in the presence (dotted bar) or absence (open bar) of 100 ng/ml NGF for 18 h. Activities of CPP32-like (A) and ICE-like (B) protease were measured as described in Section 2. Each value represents the mean ± S.E.M. of three sister cultures. * P < 0.05, ** P < 0.01 (vs. no addition).

Fig. 7. Effect of NGF on thapsigargin-induced LDH release and CPP32-like protease activity. Cells were incubated with 10 nM thapsigargin in the presence or absence of 10–100 ng/ml NGF or Ac-DEVD-CHO tetrapeptide aldehyde inhibitor (200 μM) for 18h. LDH release (open bars) and CPP32-like protease activity (dotted bars) were measured as described in Section 2. Each value represents the mean ± S.E.M. of three sister culture. * P < 0.01 (vs. thapsigargin only).
apoptosis. On the other hand, Kuida et al. reported [42] that apoptosis was decreased in the brain in CPP32-deficient mice. Interestingly, Fas-mediated apoptosis was normal in thymocyte and hepatocyte of the CPP32-deficient mice. The mechanism whereby calcium activates CPP32-like protease is unknown at present. Reactive oxygen species may mediate the activation of CPP32-like protease because an antioxidant such as TMPO, a spin trapper, has attenuated thapsigargin-induced cell death [our unpublished data].

In conclusion, thapsigargin activates CPP32-like protease followed by apoptotic cell death, while NGF inhibits the activation of CPP32-like protease and cell death.

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References


