

# Control of Mitochondrial Dynamics by Fas-induced Caspase-8 Activation in Hippocampal Neurons

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Cells undergo apoptosis mainly via two pathways—the mitochondrial pathway and the cytosolic pathway. It has been well documented that activation of the mitochondrial pathway promotes mitochondrial fragmentation and inhibition of mitochondrial fragmentation partly represses cell death. However, the mitochondrial events following activation of the cytosolic pathway are less understood. In this study, we treated Fas-activating antibody and found mitochondrial fragmentation without cell death in hippocampal primary neurons and HT-22 cell lines. Fas antibody treatment, in fact, promoted rapid activation of caspase-8, while executioner caspase-3 activation was not observed. Furthermore, blockage of caspase-8 efficiently prevented Fas antibody-induced mitochondrial fragmentation. These results suggest that the cytosolic pathway induced by death receptor activation promotes caspase-8-dependent mitochondrial fission.

**Key words:** Fas, Mitochondrial dynamics, Hippocampal neurons, Apoptosis

## INTRODUCTION

Mitochondria play essential roles in the survival of cells via generation of adenosine triphosphate, calcium storage, and regulation of apoptotic signaling [1, 2]. To maintain cellular functions, mitochondria continually change their shape between a small, fragmented form and an elongated, tubular form through fission and fusion processes [3]. Elongation of mitochondrial morphology by suppression of fission or induction of fusion is observed under conditions requiring high energy, such as starvation. On the other hand, fragmentation of mitochondria is often associated with lower energy demand of the cells after high glucose treatment, suggesting that mitochondrial morphology is

tightly controlled by metabolic status of the cells [4].

In addition to metabolic status, it is well known that mitochondrial fragmentation is closely associated with apoptosis [5]. During the activation of apoptosis, mitochondrial outer membrane pore (MOMP) formation is required, as the release of mitochondrial cytochrome c to the cytosol is essential for the apoptosome formation and subsequent activation of executioner caspase-3 [6]. Interestingly, MOMP-forming Bcl-2 family molecules such as Bax and Bak oligomerize at the mitochondrial fission foci where they form complexes with fission-promoting protein machinery [7]. Furthermore, the blockage of the mitochondrial fission complex partly prevents caspase-3 activation and apoptosis of the cells [8]. Therefore, it appears that mitochondrial fission is required for mitochondria-dependent apoptosis.

Cell death can also be caused by the activation of death receptor in the mitochondria-independent manner [9]. For instance, Fas ligand promotes their receptor trimerization, and it interacts with Fas-associated death domain (FADD). Procaspase-8 translocates to the Fas-FADD complex, and it triggers activation of initiator

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caspase-8, which in turn directly activates caspase-3 without formation of mitochondria-dependent apoptosomes [10]. A crosstalk of these two major apoptotic pathways has been also identified. Stimulation of death receptors triggers activation (cleavage) of BH3-only protein Bid to tBid via caspase-8-dependent proteolysis. As tBid inactivates anti-apoptotic Bcl-2 protein, it ultimately promotes MOMP and apoptosome formation [10].

Although it is well known that activation of the mitochondrial apoptosis pathway induces fragmentation of mitochondria, less is known whether death receptor-dependent signaling influences mitochondrial morphology. In this study, we found that Fas activation in hippocampal primary neurons or HT-22 cells induced mitochondrial fragmentation without induction of apoptosis. These results suggest that Fas may play roles in the control of mitochondrial dynamics other than apoptosis induction in neurons.

## MATERIALS AND METHODS

### *Hippocampal neuron and HT22 culture, gene transfection and reagents*

Primary hippocampal neurons were obtained from rat embryos (embryonic day 17, E17), according to previously reported culture method [11]. Pregnant female rats were sacrificed under Isoflurane anesthesia to minimize suffering. The hippocampal regions were separated from rat embryos, trypsinized, and dissociated into single neurons. The neurons were plated onto glass coverslips coated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA) at  $1 \times 10^5$  cells/cm<sup>2</sup>, and maintained in 5% CO<sub>2</sub> at 37°C with neurobasal media (Gibco, NY, USA) containing 2% B27 supplement (Gibco, NY, USA), 200 mM GlutaMax (Invitrogen, Carlsbad, CA, USA), and 1% penicillin/streptomycin (Gibco, NY, USA). Fas signaling was induced in cultured neurons and HT22 cells with a Fas-activating antibody (500 ng/ml, Millipore, Billerica, MA, USA) in serum-free medium with or without 25 μM Z-IETD (Sigma-Aldrich, St. Louis, MO, USA). For gene transfection of dsRed-mito, mitochondrial marker (Clontech) into cultured neurons, the Calphos mammalian transfection kit (Clontech) was used according to the manufacturer's protocol. Transfection into the cultured neurons was performed 2 days prior to experimental observations on DIV10. Hippocampal cell line HT-22 cells were maintained in 5% CO<sub>2</sub> at 37°C. The experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Korea University Institutional Animal Care and Use Committee. The protocol was approved by the Committee on the Ethics of Animal Experiments

of the Korea University (Permit Number: KUIACUC20110304-2). Staurosporin (STS) and Z-IETD were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### *Immunocytochemistry and measurement of mitochondrial fragmentation*

Cells were fixed with 4% paraformaldehyde, and washed with PBS several times. To prevent non-specific binding of antibody, samples were incubated with blocking solution containing 3% bovine serum albumin (Santa Cruz, CA, USA) and 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in PBS, pH 7.4. Primary antibodies were incubated for 2 hours at room temperature. Primary antibodies used here were as follows: Fas (1:500, Millipore, Billerica, MA, USA), FasL (1:500, Santa Cruz, CA, USA), and Cytochrome c (1:500, BD bioscience, Bedford, MA). After washing with PBS, Donkey anti-rabbit Alexa488 (1:500, Jackson ImmunoResearch) or Donkey anti-mouse cy3 (1:500, Jackson ImmunoResearch) secondary antibodies were applied for 30 minutes at room temperature. Hoechst 33342 (1:3000, Invitrogen, Carlsbad, CA, USA) was used for counter staining of nuclei. Images were acquired by confocal microscopy (Cal Zeiss, Thornwood, NY, USA). For quantification, the percentage of cells with fragmented mitochondria.

### *Immunoblots*

Hippocampal neurons were harvested with RIPA lysis buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% Triton X-100, protease and phosphatase inhibitor cocktail (Roche Applied science)). After cell lysis, lysates were pelleted by centrifugation at 10,000 g for 20 minutes, 4°C. Protein contents at supernatants were quantified using BCA (bicinchoninic acid, Pierce, Rockford, IL, USA) protein assay kit according to manufacturer's instruction. After quantification, samples were mixed with 2X SDS laemmli buffer (120 mM Tris-HCl, pH6.8, 4% sodium dodecyl sulfate, 20% Glycerol), and then heated for 5 minutes at 100°C. Proteins (20 μg) were separated on a 10% SDS-polyacrylamide (PAGE) gel and transferred onto a polyvinylidene difluoride (PVDF). Immunoblotting was performed following antibodies: anti-Cleaved Caspase 8 (1:1000, Millipore, Billerica, MA, USA), anti-Cleaved Caspase 3 (1:1000, Cell signaling Technology, Danver, MA, USA), anti-pAkt (1:1000, Santa Cruz, CA, USA), anti-pErk (1:1000, Santa Cruz, CA, USA), anti-Actin (1:1000, Sigma-Aldrich, St. Louis, MO, USA).

### *Statistical Analysis*

Quantitative data were analyzed using the unpaired Student's t

test.

**RESULTS**

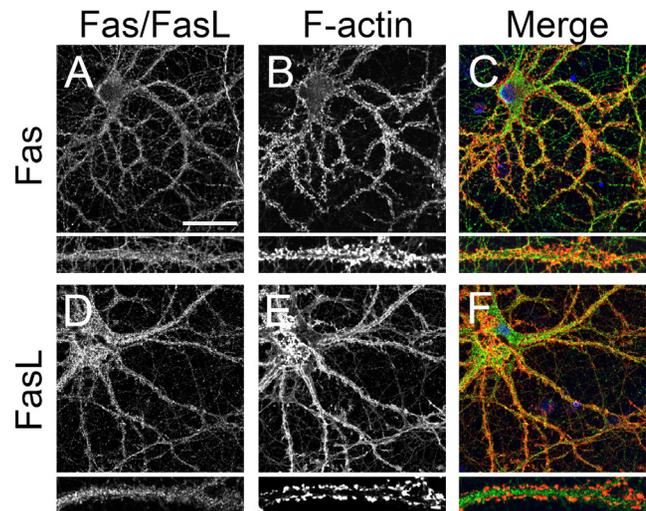
**Expression of Fas and Fas ligand (FasL) in cultured neurons**

First, we examined the expression and distribution of Fas and FasL in cultured mature hippocampal neurons (Fig. 1). Both Fas and FasL were expressed in the cell bodies and neuronal processes. High magnification images of dendrites show distribution of Fas and FasL in dendritic shafts, but much less in synapses. Post-synaptic structure of the dendritic spine was visualized by F-actin labeling.

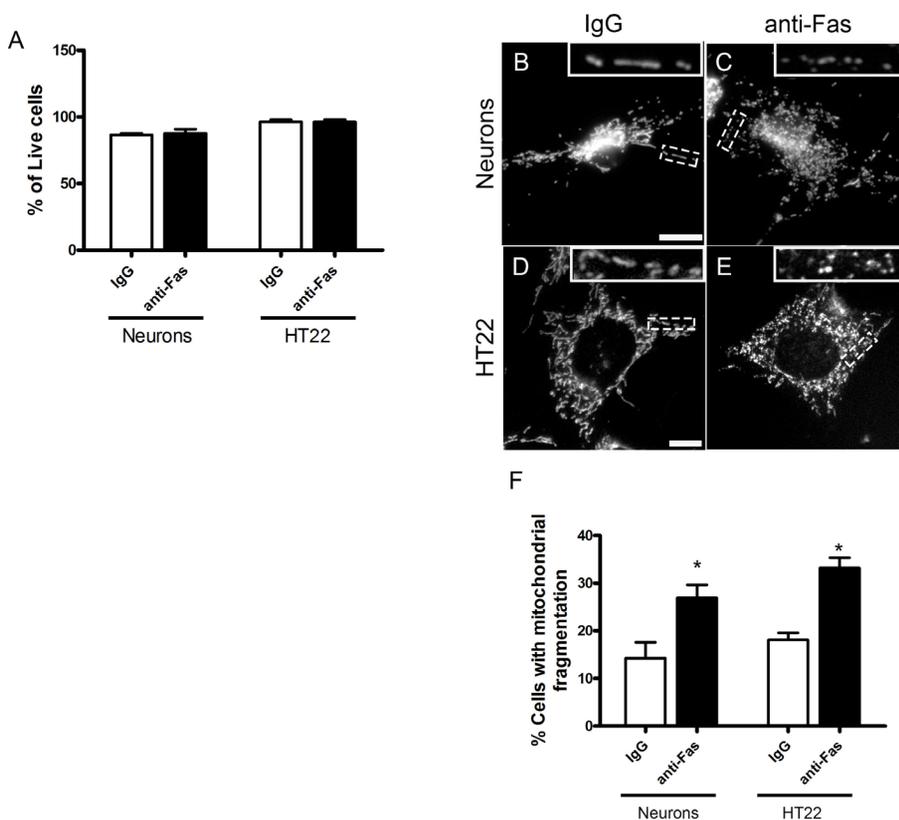
**Effects of Fas activation on the mitochondrial morphology in neurons**

To test whether Fas activation promotes death receptor-mediated neuronal death, we treated Fas-activating antibody for 24 hours on cultured hippocampal neurons or the neuronal cell line, HT-22. Viability of neurons was assessed by their nuclear pyknosis [12]. Interestingly, Fas activation did not alter the viability of the cultured hippocampal neurons and HT-22 cells (Fig. 2A). On the other hand, treatment of Fas antibody for 6 hours promoted mitochondrial fragmentation (Fig.

2B~F). Mitochondrial morphology was visualized by either transfection of mitochondrial marker, DsRed-mito (neurons), or immunolabeling of mitochondrial protein cytochrome C (HT-22 cells). Typically, neurons showed elongated mitochondrial



**Fig. 1.** Immunocytochemical analysis of localization of Fas (A~C) and FasL (D~F) in cultured hippocampal neurons (DIV14). Fas and Fas ligand were double labeled with marker for filamentous actin (F-actin). Nuclei were counter-stained with Hoechst33342 (blue). Scale bar=40 μm for A~F.



**Fig. 2.** Effects of Fas activation on the survival and mitochondrial fragmentation in hippocampal neurons. (A) Quantitative data of cells with normal nuclear morphology.  $p < 0.05$  in Students' t-test comparison ( $n = 3$  in each group). Cultured neurons and HT22 cells were treated with IgG (500 ng/ml) Fas-activating antibody (500 ng/ml) for 24 hours. (B~E) Mitochondrial morphology after Fas activation for 6 hours. (F) Quantitative data of cell with mitochondrial fragmentation.  $p < 0.05$  in Students' t-test comparison ( $n = 3$  in each group). Scale bar=40 μm for B~C; 20 μm for D~E.

morphology, but a small subset of neurons exhibited rather fragmented mitochondria. By the treatment of Fas antibody, a substantially larger proportion of cells exhibited mitochondrial fragmentation. Similarly, mitochondrial fragmentation was also monitored after Fas antibody treatment in HT-22 cells (Fig. 2F).

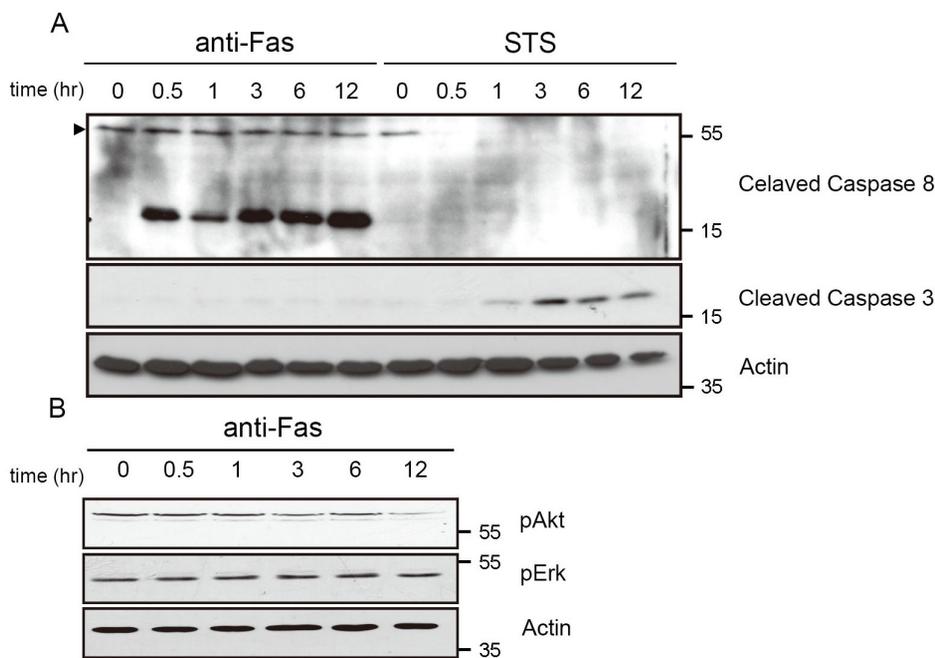
**Activation of death receptor signaling without caspase-3 activation**

Because we observed no cell death, but mitochondrial fragmentation in neurons, we wondered whether death receptor signaling is activated (Fig. 3). It is known that Fas activation promotes caspase-8 cleavage [13-16]. As expected, we found that caspase-8 cleavage was rapidly induced by Fas antibody treatment as early as 30 minutes after treatment. However, we failed to detect

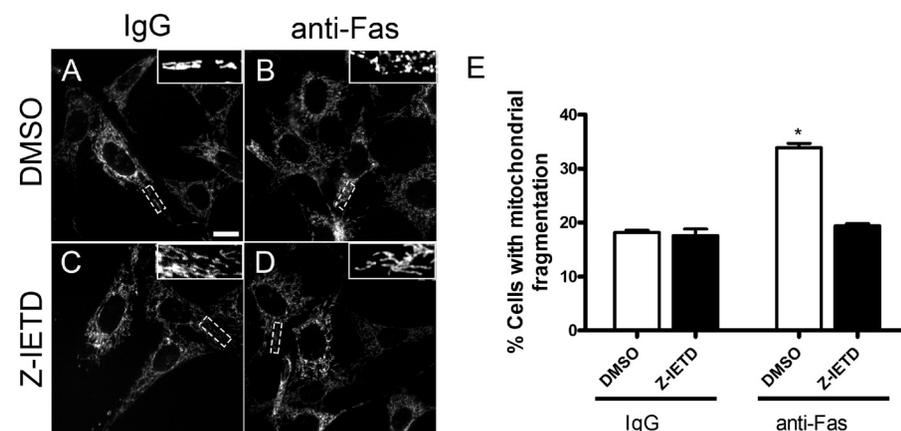
caspase-3 activation at all time points examined. Conversely, treatment of staurosporine (STS), which is a potent activator of the mitochondrial apoptosis pathway, strongly promoted caspase-3 activation without caspase-8 cleavage (Fig. 3A). Erk or Akt pathways can also be activated upon Fas activation in some cells [17, 18]. However, we failed to detect signs of the activation of these signaling pathways, as their phosphorylation status was unaltered (Fig. 3B).

**Prevention of Fas-induced mitochondrial fragmentation by caspase-8 inhibition**

To test whether caspase-8 activation is required for the Fas-induced mitochondrial fragmentation, we co-treated caspase-8 specific inhibitor, Z-IETD, with Fas antibody (Fig. 4). Interestingly,



**Fig. 3.** Time course changes of signal activations after Fas antibody (500 ng/ml) or staurosporin (STS, 1  $\mu$ M) treatments. Samples (20  $\mu$ g) were loaded each lane in SDS-PAGE, and immunoblotting was performed with specific antibodies: (A) Cleaved caspase-8 (p18), Cleaved caspase-3, Actin (as loading control). (B) pAkt, pErk, Actin (as loading control). Arrowhead indicates a non-specific band.



**Fig. 4.** The effects of Z-IETD treatment in Fas-induced mitochondrial fragmentation (A~D). Image of immunocytochemistry of mitochondria. HT-22 cells were pre-treated with DMSO or Z-IETD (25  $\mu$ M) for 1 hour, after pretreatment cells were incubated with IgG (500 ng/ml) anti-Fas (500 ng/ml) for 6 hours (E) Quantitative data of cell with mitochondrial fragmentation.  $p < 0.05$  in Student's t-test comparison ( $n = 3$  in each group). Scale bar = 30  $\mu$ m for A~D.

Z-IETD completely prevented Fas-induced mitochondrial fragmentation, suggesting that mitochondrial fragmentation is dependent on caspase-8 activation.

## DISCUSSION

In this study, we for the first time demonstrated that Fas-mediated death receptor signaling triggered caspase-8-dependent mitochondrial fragmentation, although it did not promote apoptosis. Fas and FasL are widely expressed in many organs, and their expressions appear to be regulated during development. For example, expressions of two proteins gradually increases during the embryonic developmental period and down-regulated at the postnatal stage [19]. We also found that both Fas and FasL proteins are distributed in mature hippocampal neurons, enriched in the cell body and dendritic shaft.

It is known that Fas activation *per se* can trigger T-cell death [20]. However, some cells are not sensitive to Fas activation [21, 22]. For example, HeLa cells require co-treatment of Fas ligand and translation inhibitor, cycloheximide (CHX), to promote apoptosis [23, 24]. Although the precise mechanisms remain unclear, this co-treatment initiates Bax-dependent mitochondrial apoptosis [23], suggesting that Fas activation alone is insufficient to trigger apoptosis in these cell types [24, 25]. In neurons, it has been reported that Fas-mediated mechanisms are involved in ischemia- or ethanol-induced neuronal death [26, 27]. These conditions, in fact, promote multiple responses including microglial activations and astroglial reactions, which would trigger the release of many cytokines in addition to the FasL [28, 29]. Therefore, although the blockade of Fas signaling would suppress these types of neuronal death [30, 31], Fas activation might not be sufficient to induce neuronal death. In addition, it is known that Fas is involved in the pathological motoneuron death, but the genetic knockout of Fas or FasL failed to modify developmental programmed cell death of motoneurons [32]. In this respect, it appears that Fas activation plays a limited role in the control of neuronal death.

It is also known that death receptor pathways participate in many biological processes in addition to cell death [33]. Activation of TNF- $\alpha$  signaling, which is mediated by death receptors, increases surface expression of AMPARs, and enhances synaptic activity via the activation of PI3K signaling [34, 35]. Because TNF receptors and Fas share strong structural homologies, it is plausible that Fas activation also mediates the non-apoptotic events in neurons. However, we failed to detect the phosphorylation of Akt or Erk proteins, suggesting that Fas did not activate these downstream signalings in neurons. In immature neurons, Fas signaling is involved in the neuronal branch formation process,

which requires binding FADD to receptors. Activation of caspase-8 is not essential in this phenomenon, but involvement of MAPK signaling is also not clearly identified for the branch-promoting activity [18]. Collectively, it appears that Fas activation triggers multiple signaling events to promote context-dependent neuronal responses.

In this study, we found that Fas activation induced rapid cleavage of caspase-8 in mature hippocampal neurons. Although we did not explore detailed signaling events, blockade of caspase-8 activity prevented Fas-dependent mitochondrial fragmentation, suggesting that caspase-8 activation is required for the mitochondrial fragmentation. In canonical Fas signaling, caspase-8 activation promotes Bid cleavage and the mitochondrial translocation together with Bax [23]. Bax translocation should ultimately promote mitochondrial fragmentation and cell death. However, our results show that Fas-signaling activation did not trigger cytochrome C release, caspase-3 activation, or apoptosis. These results suggest that Fas activation selectively triggers mitochondrial fragmentation without propagation of the signal toward the execution of apoptosis. Considering that mitochondrial fragmentation serves for the control of cellular energy homeostasis [4, 36], we propose that Fas signaling may be involved in the control of neuronal energy homeostasis.

It is known that one of the substrates for caspase-8, Bap-31, is localized on the endoplasmic reticulum (ER), and death signaling, including Fas-induced cell death, cleaves Bap31 via caspase-8 activation. The P20, which is resultant product of Bap31 cleavage, promotes calcium release from the ER [37]. Calcium release from the ER can stimulate mitochondrial fragmentation via activation of dynamin-related protein 1 (Drp1) or inactivation of fusion-promoting protein Opa1 [38], raising Bap31 as a candidate for mediating caspase-8-induced mitochondrial fragmentation in neurons. However, it is known that overexpression of p20 promotes mitochondrial fragmentation and subsequent cytochrome c release via activation of Bcl-2 family molecules [37]. Therefore, it remains unclear whether limited Bap-31 activation is involved in Fas-dependent mitochondrial fission in neurons, or whether other molecule(s) are involved in Fas-dependent mitochondrial fragmentation. In this respect, it is clear that further studies are required to identify the missing molecular links between caspase-8 activation and mitochondrial fragmentation.

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