[Cell Cycle 6:12, 1479-1486, 15 June 2007]; ©2007 Landes Bioscience

Report

TNFα Induces Apoptosis Through JNK/Bax-Dependent Pathway in Differentiated, but Not Naïve PC12 Cells

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Original manuscript submitted: 03/08/07
Revised manuscript submitted: 04/16/07
Manuscript accepted: 04/18/07

Previously published online as a Cell Cycle E-publication:
http://www.landesbioscience.com/journals/cc/abstract.php?id=4302

KEY WORDS

differentiated PC12 cells, naïve PC12 cells, TNFα, apoptosis, Bax, cytochrome C, caspase-8, JNK, caspase-3

ACKNOWLEDGEMENTS

This research is supported by the National Natural Science Foundation of China (30670507; 30600128; 30470494) and the Natural Science Foundation of Guangdong Province (015012). We thank Dr. A.P. Gilmore (University of Manchester, Oxford Road, UK) for providing the YFP-Bax plasmid, Dr. Y. Gotoh (University of Yokyo, Yayoi, Tokyo, Japan) for providing the pDsRed-Mit plasmid, and we also thank Dr. G. J. Gores (Center for Basic Research in Digestive Diseases, Molecular Medicine Program, Mayo Clinic, Rochester Minnesota) for kindly providing the pE-GFP-Cyt-c plasmid.

ABSTRACT

Differentiated PC12 cells have been used widely as a model for the analysis of neuronal degeneration. Some evidences showed that differentiated PC12 cells were more sensitive than naïve PC12 against apoptosis stimuli. However, the apoptosis mechanism of both types of PC12 cells was not fully known. In this study, the signaling pathways involved in tumor necrosis factor-α (TNFα)-induced apoptosis in living differentiated and naïve PC12 cells were investigated using confocal microscope for the first time. Our results showed that during TNFα-induced apoptosis, Bax translocation to mitochondria and cytochrome C (Cyt c) release from mitochondria were observed in differentiated PC12 cells, but not in naïve PC12 cells. Furthermore, the mRNA levels of *bim*, c-Jun N-terminal protein kinase 1 and 2 (JNK1 and JNK2) increased noticeably in differentiated PC12 cells. The apoptosis induced by TNFα was inhibited by Z-IETD-fmk (specific inhibitor of caspase-8) but not SP600125 (specific inhibitor of JNK) in naïve PC12 cells. While in differentiated PC12 cells, the process of apoptosis could only be inhibited effectively by Z-IETD-fmk and SP600125 cotreatment, and SP600125 inhibited the Bax translocation to mitochondria implying that JNK mediated activation of Bax. The experimental data strongly demonstrated that TNFα induced apoptosis through JNK/Bax-dependent pathway in differentiated, but not naïve PC12 cells.

INTRODUCTION

Apoptosis, or programmed cell death is crucial for the normal development of the nervous system.[1] Apoptotic death in both developing and adult neurons occurs through a highly ordered sequence of signaling cascades. Two pathways of cell apoptosis have been elucidated—the 'extrinsic' or death receptor-initiated pathway, and the 'intrinsic' or mitochondrial pathway.[2,3] Specific genes are involved in the death of different types of neurons, and a given neuron might activate distinct death pathways in response to different stimuli.[4] In fact, cell-specific differences in signaling pathways might underlie the selective loss of particular neuronal populations in various neurodegenerative disorders.[1,5] The extrinsic 'death receptor' pathway either directly activates effector caspases or converges with the intrinsic apoptotic pathway at the mitochondrion. The intrinsic pathway results in release of cytochrome c (Cyt c) from mitochondria, leading to formation of the apoptosome complex and subsequent activation of effector caspases. Cyt c is released in response to apoptogenic stimuli by the translocation of BH3 [Bcl-2 (B-cell leukaemia/lymphoma-2) homology domain 3]—only proteins (Bad, Bid, Bim and Hrk) to the mitochondria. BH3-only proteins directly activate pro-apoptotic multi-domain Bcl-2 family members [such as Bax (B-cell lymphoma 2-associated protein X) or Bak (Bcl-2 agonist killer 1)] to form a mitochondrial membrane permeability pore, and indirectly induce pro-apoptotic functions by suppressing the ability of anti-apoptotic Bcl-2 members [Bcl-2 , Bcl-XL (Bcl-2-like protein 1), Bcl-w (Bcl-2-like protein 2) and Mcl-1 (myeloid cell leukaemia-1)] to inhibit the translocation of the pro-apoptotic molecules Bax, Bak, Bok (Bcl-2-related ovarian killer protein) and Bcl-XS (Bcl-like protein XS) to the mitochondrion.[1,6] Recent studies with BH3-only proteins suggest that the simple inhibition of anti-apoptotic functions is insufficient to induce apoptosis unless a direct activator (Bid, Bim) of Bax or Bak is present.[7-9] Tumor necrosis factor-α (TNFα) elicits a wide range of biological responses, including neuronal apoptosis and neuroprotection, and this functional pleiotropy is essentially determined by the individual molecular orchestration.[10-13] TNFα mediates its biological effects through activation of two distinct receptors, TNF receptor type I (TNFRI, p55) and TNF receptor type II (TNFRII, p75).[14]

Both isoforms of the receptor have been identified in neurons,[15,16] but their expression also displays cell specificity.[17] The molecular mechanisms of TNFα signaling are fairly well characterized in nonneuronal cells. TNFα triggers the activation of the transcription factors NFκB and AP-1, which in turn regulate many of the genes involved in the inflammatory response, protection, or apoptosis.[18-20] Studies of TNFα-induced apoptosis have demonstrated that after binding to its receptor (TNFR1), TNFα activates a death-inducing signaling complex that subsequently activate caspase-8.[5,21,22] The activated caspase-8 can directly activate caspase-3 or indirectly activate caspase-3 through a mitochondrial apoptotic pathway that leads to the release of Cyt c from mitochondria following the loss of mitochondrial transmembrane potential.[23] Signaling of TNFα mediated stimuli in neurons, however, it is not clearly understood and the contribution to neuronal survival or degeneration is still under controversial discussion.[10,24] The c-Jun N-terminal protein kinase (JNK) is a candidate effector for the intra-neuronal pathological response downstream of TNFα. Recent studies suggest that JNK induces apoptosis by directly phosphorylating BimEL and BimL.[25-29] However, the elucidation of the contribution of JNK to neuronal death is complicated by its involvement in physiological and protective processes such as neurite formation, mitosis, or regeneration.[30,31] The inhibition of JNK by the indirect inhibitor SP600125 offers a novel opportunity to clarify the functions of JNK with particular issue of their pro-apoptotic actions.[32]

Rat pheochromocytoma cell line (PC12) is used widely as a model of the sympathetic and sensory nervous system that responds to nerve growth factor (NGF).[33] Although primary cell culture is a powerful tool to study signal transduction, PC12 cells contain a more homogeneous population, develop a faithful neuronal phenotype, and are available in large amounts for biochemical study. Previous reports suggested apoptosis did not occur rapidly in naïve PC12 cells and found that *bcl-xl*, *bcl-xs*, and *caspase-3* mRNAs were up-regulated by NGF. The increase in *caspase-3* and *bcl-xs* mRNAs levels primed the differentiated PC12 cells for apoptosis.[34-36] Recent evidences demonstrated that JNK mediate this enhanced capacity of TNFα-induced apoptosis pathway in differentiated PC12 cells.[37,38] However, there was no clearly evidence to elucidate the difference between differentiated and naïve PC12 cells.

In this study, the signaling pathway involved in TNFα-induced apoptosis was elucidated in living differentiated and naïve PC12 cells. The roles of caspase-8/JNK and mitochondrial function in TNFα-induced apoptosis were examined. The data showed that inhibition of caspase-8 activity abolished the TNFα-induced apoptosis in naïve PC12 cells but not differentiated PC12 cells. In contrary, inhibition of JNK activity abolished the TNFα-induced apoptosis pathway, leading Bax translocation to the mitochondria in differentiated PC12 cells. This was not observed in the naïve PC12 cells.

MATERIALS AND METHODS

Cell culture, transfection and treatment. Naïve PC12 cells were cultured in a humidified (5% CO^2, 37°C) incubator in Dulbecco's modified Eagle's medium (DMEM) culture medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum and 5% horse serum (Hyclone, Logan, Utah, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml). To obtain neuronally differentiated PC12 cultures, subconfluent cells were differentiated for up to 5 days in DMEM with 0.5% fetal bovine serum and NGF (50 nM) (R&D Systems, MN, USA). To ensure maximum bioavailability, NGF and serum were replaced every other day, and cells were plated at a various densities according to each experimental protocol.

Before differentiated, naïve PC12 cells were transfected with different plasmid by using the LipofectamineTM 2000 Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The solution of LipofectamineTM 2000 reagent was replaced with fresh culture medium after 4 hours. After two days, naïve PC12 cells were differentiated in a custom-built dish for five days before examine but naïve PC12 cells were examined 48 hours after transfection.

CHX is a protein synthesis inhibitor that can enhance the effect of TNFα-induced apoptosis. The concentration of TNFα plus CHX used in our experiments was 40 ng/ml and 15 μg/ml, all the chemicals were kept in the medium throughout the experiments. In the experiments using JNK inhibitor SP600125 (20 μM) (Sigma Chemical Co., St. Louis, MO, USA) and caspase-8 inhibitor Z-IETD-fmk (10 μM) (BioVision, Mountain View, CA), all the chemicals were added to the cells 30 minutes before TNFα/CHX and kept in the medium throughout the experiments.

RNA isolation and quantitative real-time RT-PCR (QT-PCR). Total RNA of differentiated and naïve PC12 cells were isolated using the High Pure RNA Isolation Kit (catalog no. 1828665; Roche Applied Science, Mannheim, Germany) and treated with RNase-free DNase. cDNA synthesis and amplification by QT-PCR was carried out using the LC RNA Master SYBR Green I Kit (catalog no. 3064760; Roche Applied Science, Mannheim, Germany). Briefly, for cDNA synthesis and QT-PCR, each capillary contained 0.5 μl (0.911 μg) of total RNA temple, 0.65 μl $Mn(OAc)_2$ stock solution (3.25 mM), 3.75 μl LightCycler RNA Master SYBR Green I, 0.5 μl (0.3 μM) each of the primers in a total volume of 10 μl. The following PCR primers were used.

GAPDH as a house keeper

-GAPDH-1:5'-TGGAGTCTACTGGCGTCTT-3';

-GAPDH-2:5'-GTCTTCTGAGTGGCAGTGAT-3';

-bim-1:5'-TCCCTACAGACAGAATCGC-3'; *-bim*-2: 5'-CCTCCTCGTGTAAGTCTCATT-3'; -JNK1-1: 5'-AATGCTGGTGATAGATGCG-3'; -JNK1-2: 5'-TGACGCCATTCTTAGTTCG-3'; -JNK2-1: 5'-GTCAGAATCCGAACGAG-3'; -JNK2-2: 5'-TCTTCCCAGTCCATCA -3'; *-bax*-1: 5'- CTGCAGAGGATGATTGCTGA -3'; *-bax*-2: 5'-GAGGAAGTCCAGTGTCCAG-3'. In PC12 cells, the expected sizes of the generated fragments are 274 bp for GAPDH, 222 bp for *bim*, 215 bp for JNK1, 252 bp for JNK2, and 207 bp for *bax*, respectively. The cDNA synthesis and QT-PCR reactions were performed using LightCycler (Roche Applied Science, Mannheim, Germany) for reverse transcription of the template RNA at 61°C for 20 min, denaturation of the cDNA/RNA hybrid at 95°C for 1 min, amplifying the target DNA for 45 cycles at 95°C for 5 s, annealing temperature at 55°C for 8 s (GAPDH, JNK1,JNK2,*bax*), at 53°C for 8 s (*bim*), extension at 72°C for 15 s. A negative control (H_2O) was included to check for cross contamination in every run. Amplification specificity was checked using melting curve, according to the manufacturer's instruction. Since each specific product melts at a higher temperature than the primer dimmers, the melting curves thus allow discrimination between the primer dimers and a specific product. For the mathematical model it is necessary to determine the crossing points (CP) for each transcript. CP is defined as the point at which the fluorescence rises appreciably above the background fluorescence. 'Fit Point Method' must be performed in the LightCycler software v.3.5 (Roche Applied Science, Mannheim, Germany) at which CP will be measured at constant fluorescence level.

Creation of the coefficient file and quantification. The total RNA of differentiated PC12 cells was diluted serially from 7000 ng to 280 ng using five points with RNase-free H_2O. Then added different concentrations of total RNA temple, 0.65 μl $Mn(OAc)_2$ stock solution (3.25 mM), 3.75 μl LightCycler RNA Master SYBR Green I, 0.5 μl (0.3 μM) each of the primers in a total volume of 10 μl. The cDNA synthesis and QT-PCR reactions were performed as described above. The experimental standard curves were analyzed with Real Quant Software (Roche Applied Science, Mannheim, Germany) to create five coefficient files (GAPDH, *bim*, JNK1, JNK2, and *bax*).

The results were analyzed using efficiency correction with the LightCycler Software v.3.5 and the Real Quant Software.

Time-lapse confocal fluorescence microscopy. GFP, YFP and DsRed emissions were monitored confocally using a laser scanning microscope (LSM 510/ConfoCor 2) combination system (Zeiss, Jena, Germany) equipped with a Plan-Neofluar 40x/1.3 NA Oil DIC objective. Excitation wavelength and detection filter settings for each of the fluorescent indicators were as follows. GFP was excited at 488 nm with an argon ion laser and its fluorescence emission was recorded through a 500-550 nm band-pass filter. YFP was excited at 488 nm with an argon ion laser and its fluorescence emission was recorded through a 500–530 nm band-pass filter. DsRed was excited at 543 nm with a helium-neon laser and its emitted light was recorded through a 600–650 nm band-pass filter.

For time-lapse imaging, culture dishes were mounted onto the microscope stage equipped with a temperature-controlled chamber (Zeiss, Jena, Germany).

YFP-Bax translocation assay. Differentiated and naïve PC12 cells were transiently cotransfected with YFP-Bax (a generous gift from Dr. A.P. Gilmore of University of Manchester, Oxford Road, UK) and DsRed-Mit (a generous gift from Dr. Y. Gotoh of University of Tokyo, Tokyo, Japan). Before detection, cells were treated by TNFα/CHX. Live cell imaging was performed by the Zeiss LSM 510

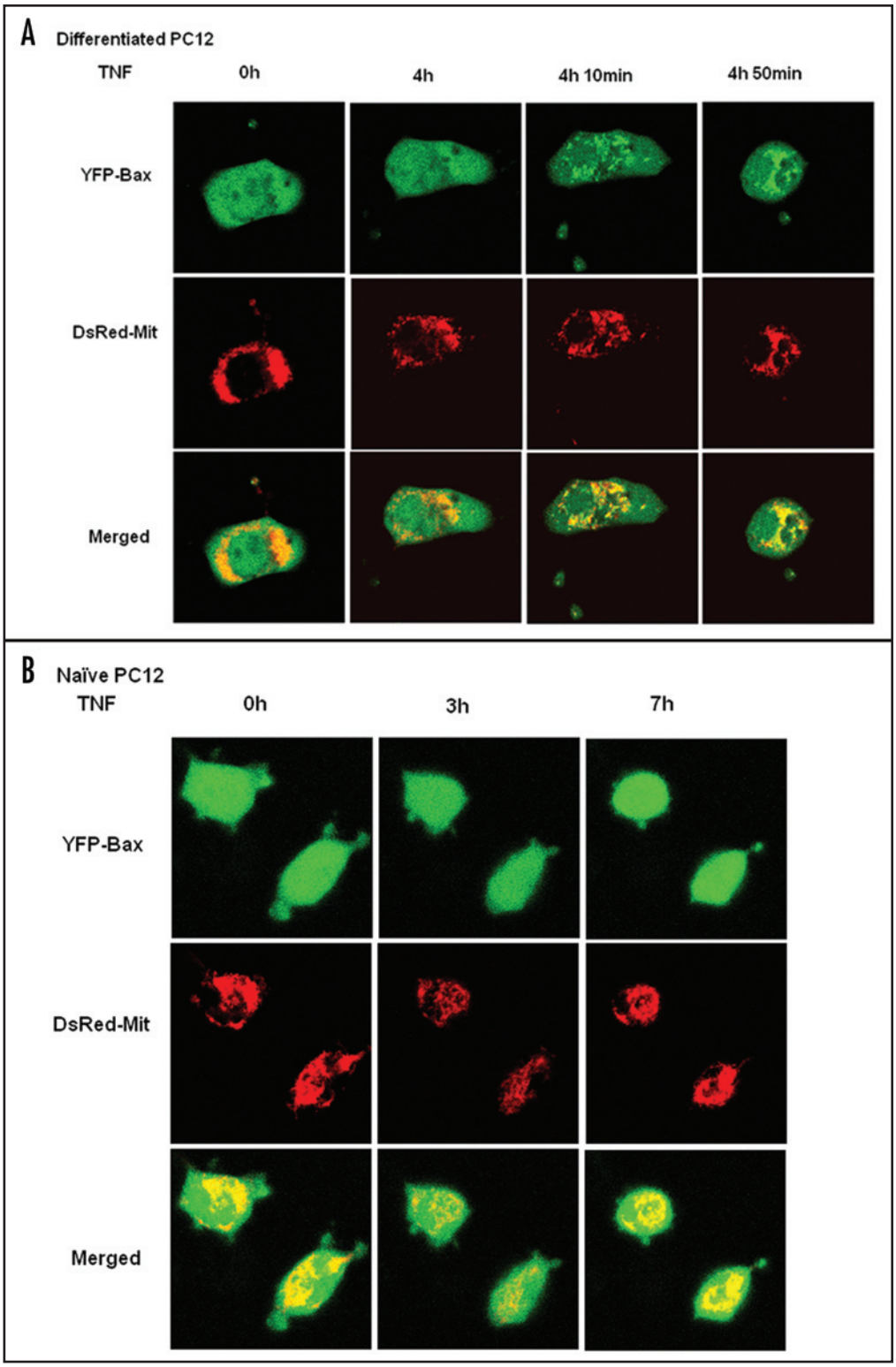

Figure 1. Translocation of YFP-Bax in differentiated and naïve PC12 cells, coexpressing YFP-Bax and pDsRed-Mit, treated with TNFα/CHX. Translocation of YFP-Bax was performed by laser fluorescence confocal microscopy. (A) Differentiated PC12 cells treated with TNFα/CHX for 4 h, YFP-Bax translocated to mitochondria noticeably within 10 min and the cell apoptosis after 50 min. (B) Naïve PC12 cells treated with TNFα/CHX for 7h, YFP-Bax didn't translocate to the mitochondria until the cell apoptosis. Data are representative of three independent experiments.

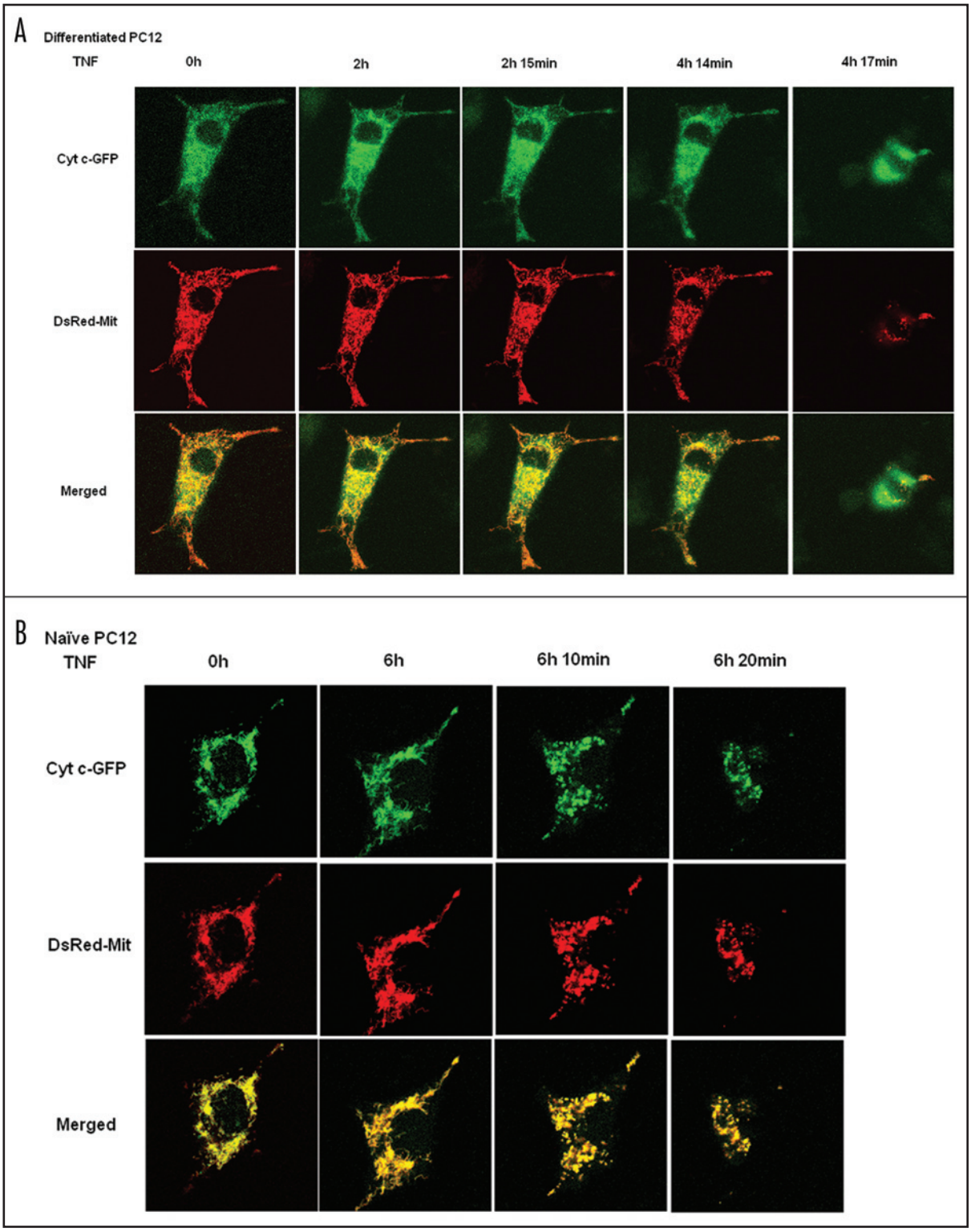

Figure 2. Dynamics of Cyt c release in differentiated and naïve PC12 cells, coexpressing E-GFP-Cyt-c and DsRed-Mit, treated with TNFα/CHX. (A) Examination by laser fluorescence confocal microscopy revealed that differentiated PC12 cells began to release Cyt c from mitochondria after treatment with TNFα/CHX for 2 h. (B) Naïve PC12 cells treated with TNFα/CHX, the Cyt c didn't release from mitochondria until the cell apoptosis. Data are representative of three independent experiments.

confocal microscope at 37°C. The images of YFP-Bax and DsRed-Mit were obtained separately and then merged. The Bax translocation was determined based on the overlap of YFP-Bax and DsRed-Mit fluorescence images. Then, the cotransfected differentiated PC12 cells and naïve PC12 cells treated with Z-IETD-fmk or/and SP600125 before TNFα/CHX treatment respectively, the Bax translocation was determined based on the image and the mitochondrial YFP-Bax fluorescence intensity.

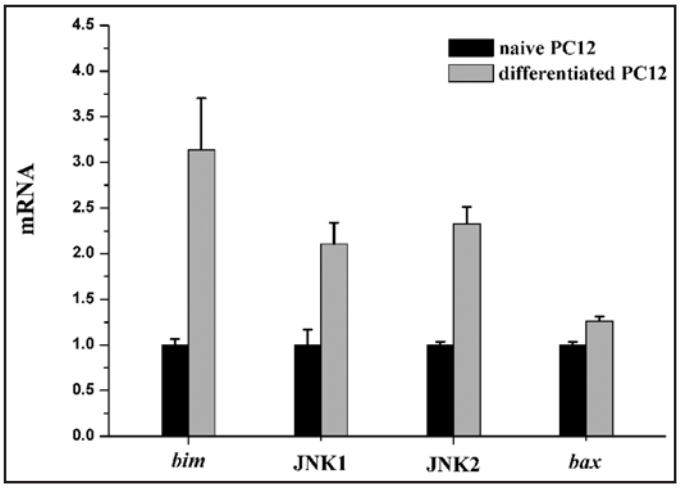

Figure 3. Expression of *bim*, JNK1, JNK2, and *bax* mRNA in naïve and differentiated PC12 cells measured by QT-PCR. The amount of each product was normalized to the reference gene GAPDH and data were normalized to that of mRNA in naïve PC12 cells. Data shown are mean ± SEM of three independent experiments.

Detection of Cyt c release. Differentiated and naïve PC12 cells were transiently cotransfected with DsRed-Mit and E-GFP-Cyt-c (a generous gift from Dr. G.J. Gores of Center for Basic Research in Digestive Diseases, Molecular Medicine Program, Mayo Clinic, Rochester, Minnesota). Before detection, cells were treated by TNFα/CHX then imaged by the Zeiss LSM 510 confocal microscope at 37°C. The images of GFP-Cyt-c and DsRed-Mit were obtained separately and then merged. The Cyt-c released from mitochondria was determined based on the overlap of GFP-Cyt-c and DsRed-Mit fluorescence images.

Western blot analysis. Differentiated and naïve PC12 cells were treated with TNFα/CHX alone or in the presence of Z-IETD-fmk or/and SP600125 for 12 h before lysed in a buffer containing 50 mM Tris•HCl pH8.0, 150mM NaCl, 1%TritonX-100, 100 μg/ml PMSF.

Lysates were separated by SDS-PAGE, and transferred to BioTrace NT Nitrocellulose Membranes (Pall). The membrane was first blocked with 5% skim milk for 1 h, followed by incubation with primary antibodies overnight at 4°C. Primary antibodies used were mouse polyclonal β-actin antibody (Santa Cruz Biotechnology), rabbit polyclonal caspase-3 antibody (Cell Signaling Technology). β-actin antibody was used at 1:500 dilution, caspase-3 antibody was used at 1:1000 dilution. Primary antibody incubation was performed in 5% bovine serum albumin in TBS-T (Tris base saline, pH 7.4, 0.1% Tween 20). A designated secondary antibody diluted at the recommended ratio, in TBS-T and 5% bovine serum albumin for 60 min at room temperature. Washing with TBS-T was performed between all steps. The signals were detected by use of ODYSSEY Infrared Imaging System (U.S.A).

RESULTS

TNFα promote Bax translocation in differentiated but not naïve PC12 cells. Bax translocation to mitochondria during TNFα-induced apoptosis in single living differentiated and naïve PC12 cells were investigated by using confocal microscope and subcellular fractionation methods, respectively. The cells were coexpressed with YFP-Bax and DsRed-Mit. The YFP-Bax was distributed uniformly throughout the cytosol in the normal differentiated PC12 cells (Fig. 1A, 0 h, green fluorescence). After TNFα/CHX treatment for 4 h, YFP-Bax translocated to mitochondria markedly in differentiated PC12 cells (Fig. 1A). However, in naïve PC12 cells, YFP-Bax did not translocate to mitochondria until cell apoptosis (Fig. 1B). The results suggested that TNFα-induced apoptosis pathway in differentiated PC12 cells was distinctly different from that in naïve PC12 cells. In naïve PC12 cells, TNFα induce apoptosis through the extrinsic 'death receptor' pathway which directly activates effector caspases but not converges with the intrinsic apoptotic pathway at the mitochondrion.

Dynamics of Cyt c release. To further determine the apoptosis pathway, we examined the release of Cyt c from mitochondria into the cytosol in single living differentiated and naïve PC12 cells coexpressed with DsRed-Mit and E-GFP-Cyt-c during TNFα-induced apoptosis. Using confocal microscope and subcellular fractionation methods, the pattern of Cyt c-GFP fluorescence was found that it is indistinguishable from that of DsRed-Mit initially. These confocal images demonstrated that the Cyt c-GFP fusion protein was translocated to mitochondria (Fig. 2A and B, 0 h). With the TNFα/CHX treated time prolonged, the pattern of Cyt c-GFP fluorescence in differentiated PC12 cells began to change from punctate to diffuse, indicating release of Cyt c from the mitochondria (Fig. 2A, 2 h). In this process, Cyt c-GFP fluorescence was not fully diffuse, and some punctate Cyt c-GFP remained in mitochondria. However, release of Cyt c did not occur in naïve PC12 cells until cell apoptosis (Fig. 2B).

Taken together, we further ascertained that TNFα induced apoptosis through Bax-dependent (or mitochondrial) pathway in differentiated PC12 cells, but not naïve PC12 cells.

Expression of *bim*, JNK1, JNK2, and *bax* mRNA. PC12 cell line is a widely used model of the sympathetic and sensory nervous system that responds to NGF. Some researches have revealed that the NGF mechanism includes regulation of gene expression.[34,35] Therefore, we investigated the levels of *bim*, JNK1, JNK2 and *bax* mRNAs that may be linked to the sensitivity against apoptosis stimuli.

When naïve PC12 cells were induced to be differentiated PC12 cells with the NGF treatment, the levels of *bim*, JNK1, JNK2 mRNAs increased significantly. However, the *bax* mRNA only presented a little change as shown in Figure 3. The levels of these genes mRNA were expressed as the target/reference ratio of the sample. In all of our experiments, GAPDH mRNA was reference.

The data demonstrated that the difference of TNFα-induced apoptosis pathway in differentiated and naïve PC12 cells gained from the selective expression of JNK and *bim*.

Effects of JNK and Caspase-8 inhibitor on regulating TNFα-induced apoptosis in differentiated and naïve PC12 cells. To further determine whether TNFα-induced apoptosis activate the extrinsic 'death receptor' pathway through caspase-8 which directly activates effector caspases in naïve PC12 cells and activate both the extrinsic 'death receptor' pathway and the intrinsic apoptotic pathway at the mitochondrion in differentiated PC12 cells, we examine the effects of SP600125 (specific inhibitor of JNK) and Z-IETD-fmk (specific inhibitor of caspase-8) on regulating TNFα-induced apoptosis in differentiated and naïve PC12 cells using confocal microscopy.

Differentiated and naïve PC12 cells were coexpressed with YFP-Bax and DsRed-Mit. Then, the cells treated with Z-IETD-fmk or/and SP600125 before TNFα/CHX treatment, respectively. The Bax translocation was determined based on the images as shown in (Fig. 4A).

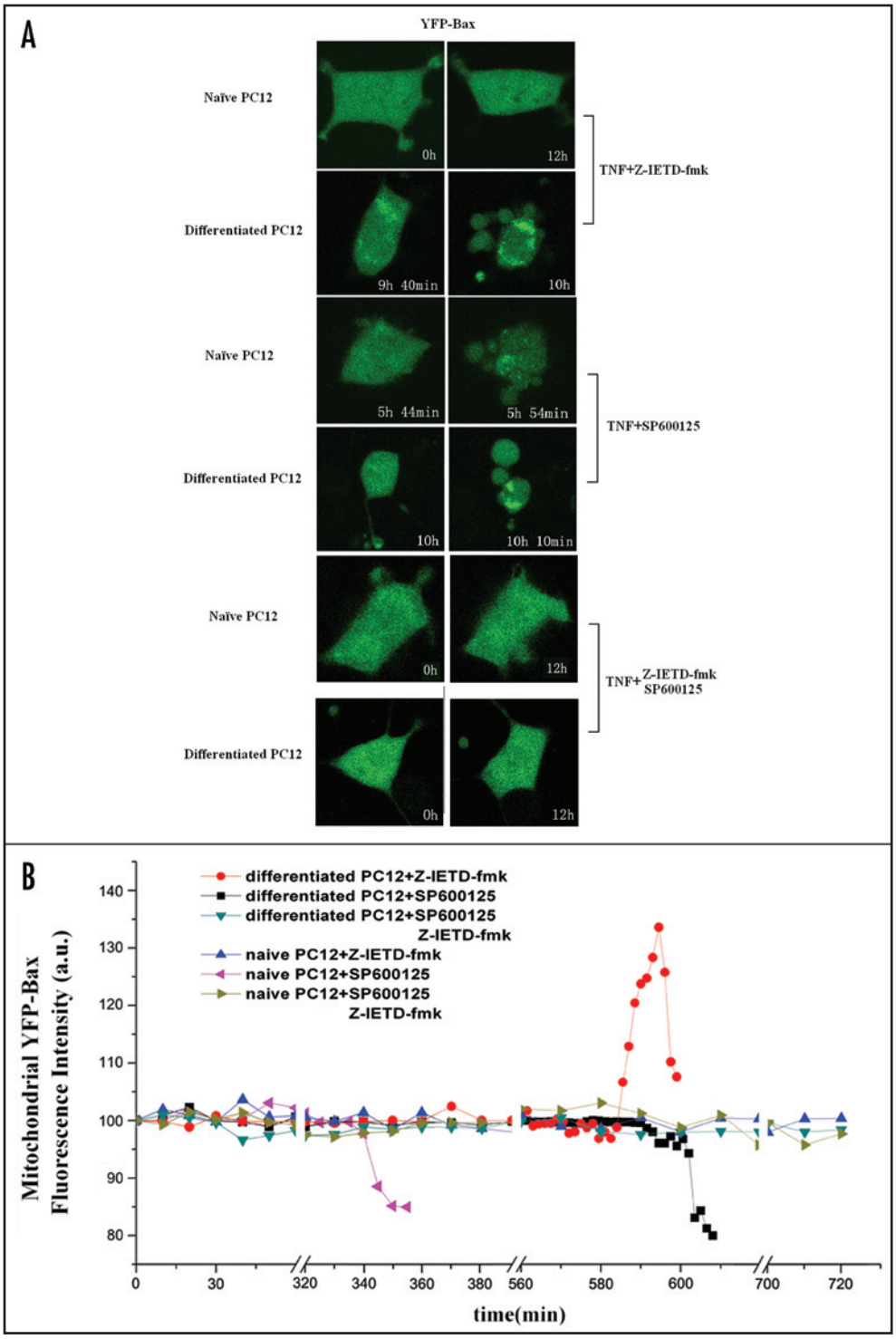

Figure 4. Translocation of YFP-Bax treated with Z-IETD-fmk or/and SP600125. (A) Differentiated and naïve PC12 cells coexpressing YFP-Bax and DsRed-Mit were treated with Z-IETD-fmk or/and SP600125 before TNFα/CHX treatment. Translocation of YFP-Bax was performed by laser fluorescence confocal microscopy. (B) The mitochondrial YFP-Bax fluorescence intensity during treatment that comes from the overlay image supplies the result shown in (A). Data are representative of three independent experiments.

First, Z-IETD-fmk inhibited TNFα-induced apoptosis in naïve but not differentiated PC12 cells. The differentiated PC12 cells still underwent apoptosis through YFP-Bax translocation. Second, SP600125 was found no effect on TNFα-induced apoptosis either in naïve or in differentiated PC12 cells. However, it could inhibit YFP-Bax translocation to mitochondria during apoptosis in differentiated PC12 cells. Third, Z-IETD-fmk and SP60012 used together could inhibit apoptosis in differentiated PC12 cells. Detailed time courses of the YFP-Bax translocation in above situations were shown in Figure 4B. We used the mitochondrial YFP-Bax fluorescence intensity which was confirmed in the overlay image to supply the image results (Fig. 4A). Figure 4B showed that just when differentiated PC12 cells treated with Z-IETD-fmk could make the YFP-Bax translocation to mitochondria, which was indicated by the quick increase in fluorescence intensity of the mitochondrial YFP-Bax.

Activation of Caspase-3. Because caspase-3 is one of the key executioners of apoptosis, the caspase-3 activation was analyzed by Western blot in naïve and differentiated PC12 cells following the induction of apoptosis. From Figure 5A and B, we found that both forms of procaspase-3 (35 and 32KD) were cleaved in naïve and differentiated PC12 cells after the TNFα-induced apoptosis. Z-IETD-fmk inhibited the cleavage of caspase-3 in naïve but not differentiated PC12 cells. SP600125 did not inhibit the cleavage of caspase-3 in both of naïve and differentiated PC12 cells. However, Z-IETD-fmk and SP60012 used together could inhibit the cleavage of caspase-3 not only in naïve PC12 cells but also in differentiated PC12 cells. These results of Western blot consist with that of previous studies and further testify that TNFα-induced apoptosis occurs through JNK/Bax-dependent pathway in differentiated, but not naïve PC12 cells.

DISCUSSION

To our best knowledge, this is the first time that the signaling pathways of TNFα-induced apoptosis in differentiated and naïve PC12 cells are investigated by using the real-time single-cell analysis. TNFα, a potent prodegenerative cytokine in the brain, is released following various acute and chronic, mainly inflammatory, insults.[37] It has been widely considered as a potent inducer of apoptosis contributing to neurodegenerative disorders such as Alzheimer's and Parkinson's disease.[1] Two main pathways mediated TNFα-induced apoptosis—the 'extrinsic' or death receptor-initiated pathway, and the 'intrinsic' or mitochondrial

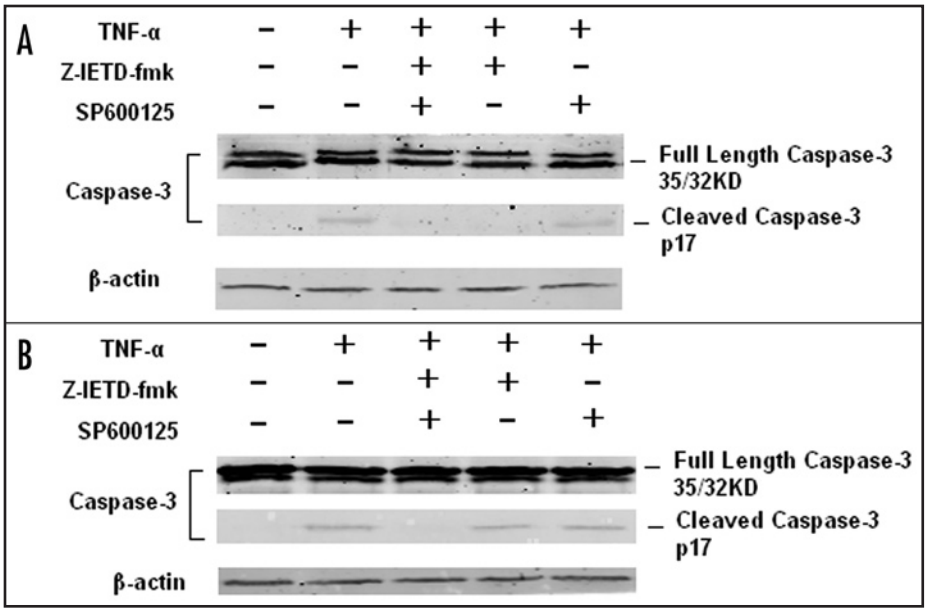

Figure 5. Caspase-3 activation in naïve and differentiated PC12 cells induced by TNFα/CHX alone or in the presence of Z-IETD-fmk or/and SP600125. (A) Naïve and (B) Differentiated PC12 cells were treated with TNFα/CHX in the absence or presence of Z-IETD-fmk or/and SP600125, then the cell lysates were prepared and western blotting performed to analyze activation of caspase-3 using rabbit specific anti-caspase-3 monoclonal antibody. The positions of procaspase-3 and active subunits are indicated. Data are representative of three independent experiments.

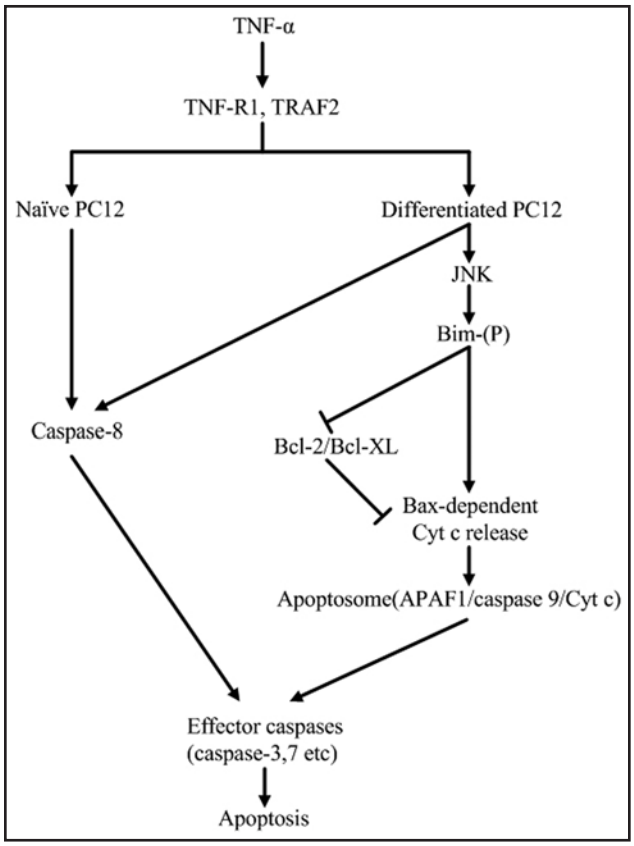

Figure 6. A model of TNFα-induced apoptosis pathways in differentiated and naïve PC12 cells. In naïve PC12 cells, TNFα induced apoptosis through extrinsic apoptotic pathway which independent of mitochondria; In differentiated PC12 cells, TNFα induced apoptosis through both extrinsic apoptotic and intrinsic or mitochondrial pathway.

pathway. The activation of the specific pathway is related to the different cell types and apoptotic stimulus.[2-5] When treated in the culture with NGF, naïve PC12 cells stop dividing and develop sympathetic neuronlike characteristics such as neurite outgrowth. Although many reports suggested the apoptotic behaviors of differentiated PC12 and naïve PC12 cells were different, there was no clearly evidence to elucidate the difference.[37,38] Our results demonstrated that TNFα induced apoptosis through JNK/Bax-dependent signal pathway in differentiated but not in naïve PC12 cells.

Bax translocation from cytosol to mitochondria is a critical step in many drug-mediated apoptosis.[39,40] Bax contributes to apoptosis by interacting with Bcl-2/Bcl-XL, and then controls mitochondrial protein export. In many cells, Bax locates predominantly in the cytosol, but translocates to mitochondria during apoptosis process.[41-43] In our experiments, the results showed that Bax translocated to mitochondria after TNFα/CHX treatment in differentiated PC12 cells but not naïve PC12 cells (Fig. 1). This observation is the foundation of our hypothesis that, TNFα induced apoptosis involves Bax-dependent (or mitochondrial) pathway in differentiated PC12 cells, but not in naïve PC12 cells.

To further determine the apoptosis pathway, we investigated the release of Cyt c from mitochondria in differentiated and naïve PC12 cells. Cyt c released from mitochondria to cytosol during TNFα-induced apoptosis was observed in differentiated PC12 cells but not naïve PC12 cells (Fig. 2). These data supported that TNFα induced apoptosis in naïve PC12 cells through extrinsic apoptotic pathway which is independent of mitochondria. And that TNFα induced apoptosis in differentiated PC12 cells through both extrinsic apoptotic and intrinsic or mitochondrial pathway.

JNK is activated when cells are exposed to multiple forms of stress, and this signaling pathway has been implicated as a mediator of TNFα-induced apoptosis.[44,45] Bax is essential for the release of Cyt c in JNK-stimulated apoptosis.[26,46] One target of JNK is Bim, a BH3-only protein that is transcriptionally up-regulated in neurons undergoing JNK-dependent apoptosis.[25,47,48] Therefore, the levels of *bim*, JNK1, JNK2 and *bax* mRNAs were investigated to ascertain whether JNK pathway made the difference in the two type cells. The data demonstrated that the difference gained from the selective expression of JNK1, JNK2 and *bim* (Fig. 3).

Further experiments provided evidence that SP600125 as an inhibitor of JNK could inhibit the Bax translocation and reduce the TNFα-induced apoptosis in differentiated PC12 cells, but it had no effect on apoptosis in naïve PC12 cells. Caspase-8 inhibitor Z-IETD-fmk could inhibit the apoptosis in naïve PC12 cells but differentiated PC12 cells treated with Z-IETD-fmk still undergo apoptosis through Bax translocation. SP600125 and Z-IETD-fmk used together could inhibit apoptosis both in differentiated and naïve PC12 cells (Fig. 4). The same results were also obtained from Western blot analysis of caspase-3 activation (Fig. 5). Furthermore, it is found that the apoptosis in naïve PC12 cells was never observed after TNFα/CHX treatment for 5 h even after SP600125

treatment. However, the apoptosis in differentiated PC12 cells was often observed after TNFα/CHX treatment for 5 h and SP600125 or Z-IETD-fmk treatment can delay the apoptotic time until 10 h after TNFα/CHX treatment (Fig. 4B). The difference of apoptotic rate also showed that TNFα induced apoptosis through two apoptosis pathways in differentiated cells.

In conclusion, TNFα induced apoptosis through JNK/Bax-dependent pathway in differentiated PC12 cells, but the JNK/Bax-dependent pathway was not involved in the TNFα-induced apoptosis in naïve PC12 cells (Fig. 6). Although our study has focused on the differences in the JNK/Bax-dependent pathway, other pathways such as JNK/jBid/Smac-dependent pathway, JNK/Bad-dependent pathway and p38/MAPK-dependent pathway might be also responsible for the differences in the apoptotic behaviors of the two types of cells.[45,49] The differences of apoptosis pathway between differentiated and naïve PC12 cells are likely to be important mechanism of differentiated PC12 cells more sensitive against apoptosis stimuli.

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