

Role of p38 MAPK in the Regulation of Apoptosis Signaling Induced by TNF- α in Differentiated PC12 Cells

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TNF- α elicits various responses including apoptosis, proliferation, and differentiation according to cell type. In neuronal PC12 cells, TNF- α induces moderate apoptosis while lipopolysaccharide or trophic factor deprivation can potentiate apoptosis that is induced by TNF- α . TNF- α initiates various signal transduction pathways leading to the activation of the caspase family, NF- κ B, Jun N-terminal kinase, and p38 MAPK via the death domain that contains the TNF- α receptor. Inhibition of translation using cycloheximide greatly enhanced the apoptotic effect of TNF- α . This implies that the induction of anti-apoptotic genes for survival by TNF- α may be able to protect PC12 cells from apoptosis. Accordingly, Bcl-2, an anti-apoptotic Bcl-2 family member, was highly expressed in response to TNF- α . In this study, we examined the anti-apoptotic role of p38 MAPK that is activated by TNF- α in neuronal PC12 cells. The phosphorylation of p38 MAPK in response to TNF- α slowly increased and lasted several hours in the PC12 cell and DRG neuron. This prolonged and slow phosphorylation of p38 MAPK was distinct from other non-neuronal cells. The specific inhibitor of p38 MAPK, SB202190, significantly enhanced the apoptosis that was induced by TNF- α in PC12 cells. This indicates that the activation of p38 MAPK could protect PC12 cells from apoptosis since there is no known role of p38 MAPK in response to TNF- α in neuron. This discovery could be evidence for the neuroprotective role of the p38 MAPK.

Keywords: Apoptosis, Neuronal PC12 cells, p38 MAPK, SB202190, TNF- α

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Introduction

The tumor necrosis factor- α (TNF- α) is a pleiotropic cytokine, which elicits various cellular responses according to cell type through the TNF- α receptor (TNF-R). The TNF-R family includes the low-affinity nerve growth factor receptor, type I (p55) and type II TNF (p75) receptors. It is considered that p55 TNF-R is a major receptor type, which is responsible for various cellular effects of TNF- α . These include apoptosis, cytokine production, proliferation, and differentiation according to the responding cell type.

In the nervous system, TNF- α has a diverse range of functions. In a developing brain TNF- α is expressed transiently and reduces the survival of serotonin neurons (Gendron *et al.*, 1991; Jarskog *et al.*, 1997). The expression of TNF- α in developing mice showed neurobehavioral alterations (Fiore *et al.*, 1996). The overexpression of the wild-type murine or human TNF- α transgenes by resident astrocytes or neurons is sufficient to trigger a neurological disorder that is characterized by ataxia, seizures, and paresis with histopathological features of chronic CNS inflammation and white matter degeneration (Probert *et al.*, 1997). TNF- α is normally absent in the adult brain; but, it can be induced by CNS injury, and cause subsequent neurological dysfunction (Lafortune *et al.*, 1996; Tchelingierian *et al.*, 1996; Knoblach *et al.*, 1999; Dennis, 2001). It is also reported that the entry of TNF- α through the blood-brain barrier is increased after spinal cord injury (Pan *et al.*, 1999). In summary, TNF- α regulates the CNS development and is involved in pathological conditions in the adult brain.

Type I TNF-R contains the death domain, which interacts with various signaling molecules. These include the TNFR-associated death domain protein (TRADD), TNF-R associated factor (TRAF), and Fas-associated death domain protein (FADD). Through these molecules, TNF- α can activate the Jun N-terminal Kinase (JNK), p38 mitogen-activated protein kinase (MAPK), NF- κ B, and caspases.

The upstream activator of p38 MAPK and JNK is supposed

to be a receptor-interacting protein (RIP) (Yuasa *et al.*, 1998), and that of NF- κ B is considered to be a NF- κ B-inducing kinase (NIK) (Malinin *et al.*, 1997; Koh *et al.*, 2000). TRAF2 acts as an adaptor molecule for both RIP and NIK, which are responsible for the activation of p38 MAPK, JNK, and NF- κ B (Arch *et al.*, 1998; Baud *et al.*, 1999). Interestingly, TNF- α can activate cytosolic phospholipase A₂ (cPLA₂) in a caspase-dependent manner, and inhibition of this activation of cPLA₂ prevents the apoptosis that is induced by TNF- α (Wissing *et al.*, 1997).

Stimulation by a wide spectrum of stimuli (physicochemical stress and treatment with lipopolysaccharide or proinflammatory cytokines such as interleukin-1 and TNF- α) activate p38 MAPK (Lee and Young, 1996). It has been reported that the activation of p38 MAPK protects cells from TNF- α mediated cytotoxicity in L929-cyt16 cells (Roulston *et al.*, 1998). On the other hand, investigators have shown opposite results - TNF- α could induce apoptosis through the activation of p38 MAPK (Ichijo *et al.*, 1997). In neuronal cells, however, the role of p38 MAPK activation by TNF- α in apoptosis has not been investigated. Furthermore, the activation mechanism of p38 MAPK by TNF- α is still unknown. The differentiated rat pheochromocytoma PC12 cell has been commonly used for the study of neuronal cell death because of its neuronal property (Greene and Tischler, 1976). In this study we examined the activation pathway of p38 MAPK in apoptosis that is induced by TNF- α . We suggest that the activation of p38 MAPK can attenuate apoptosis that is induced by TNF- α in differentiated neuronal PC12 cells.

Materials and Methods

Cell culture and reagents PC12 cells were obtained from the American type cell collection (ATCC, Rockville, USA) and maintained in a RPMI1640 medium that was supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) (Life Technologies, Inc., Gaithersburg, USA). For differentiation, the cells were maintained in the presence of 100 ng/ml nerve growth factor (NGF) (Sigma, St. Louis, USA) for 7 days in RPMI1640 that contained 1% HI-FBS in calf-skin collagen-coated 60-mm dish or 6-well plate (Choi *et al.*, 2000).

Female Sprague-Dawley rats (100-150 g) were anesthetized with ether by inhalation, and killed by decapitation. Dorsal root ganglia (DRG) were dissected from all levels of the lumbar and sacral cord, incubated with 0.15% collagenase, then with 0.125% trypsin in Ca²⁺- and Mg²⁺-free *N*'-2-hydroxyethylpiperazine-*N*'-2-ethane sulfonic acid (HEPES) buffer solution at 37°C. The DRG neurons were then mechanically dissociated with fire-polished Pasteur pipettes and plated on poly-L-lysine coated 24-well dishes. The cells were maintained in a Neurobasal™ medium (Life Technologies, Inc.) that was supplemented with B27 (Life Technologies, Inc.) and L-glutamine 2 mM (Life Technologies, Inc.) under a humidified atmosphere of 95% air/5% CO₂ at 37°C.

The primer for *bcl-2*, *bax*, and β -*actin* were from Life

Technologies, Inc. The cPLA₂ specific inhibitor arachidonyltrifluoromethyl ketone (AACOCF₃) was from Biomol (Plymouth Meeting, USA). TNF- α was from R&D Systems (Minneapolis, USA) and SB202190 was from Calbiochem (La Jolla, USA).

Western blot analysis The p38 MAPK and phosphorylated p38 MAPK were detected by a Western blot analysis. After treatment with an appropriate stimuli, the medium was aspirated, and the cell monolayers were solubilized in a SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.1% w/v bromophenol blue]. The solubilized samples were sonicated for 10-15 s to shear DNA, and centrifuged at 10,000 \times *g* for 15 min. Proteins in the supernatant were separated by SDS/PAGE and transferred to nitrocellulose. After blocking, the membranes were incubated with an phospho-p38 MAPK specific polyclonal antibody (New England Biolab, Beverly, USA), phospho-p38 MAPK specific monoclonal antibody (kind gift from Dr. Rony Seger, WIS, Israel), p38 MAPK-specific antibody (Santa Cruz, Santa Cruz, USA), or Bcl-2 specific monoclonal antibody (Santa Cruz), according to the manufacturer's protocol. The membrane was then incubated with a horse redox peroxidase-conjugated secondary antibody, and binding was detected by enhanced chemiluminescence, as described by the manufacturer (ECL; Amersham, Buckinghamshire, UK).

Quantification of apoptosis For DNA staining, the cells were fixed with PBS that contained 4% paraformaldehyde for 20 min at room temperature. Ten μ g/ml Hoechst 33285 (Calbiochem) in PBS was added to the cell monolayer. After 10 min, the cells were washed with PBS and examined under UV illumination on a fluorescence microscope. The cells with condensed and fragmented nuclei were counted in randomly selected regions; the counts were made with no knowledge of the treatment history.

The assay of apoptosis was also performed using a Cell Death Detection ELISA Kit (Boehringer Mannheim, Germany).

Results

TNF- α induces moderate apoptosis in differentiated PC12 cells TNF- α induced apoptosis in differentiated PC12 cells in a dose dependent manner (Fig. 1a), but the intensity of apoptosis was moderate compared to the apoptosis that was induced by Fas-ligand (Fas-L) in other cell types (Roulston *et al.*, 1998). Both Fas and p55 TNF-R contain the death domain and activate the caspase family in a protease cascade manner, which needs no expression of new proteins. Therefore, we postulated that TNF- α could also activate survival pathways, which lead to the attenuation of the death pathway that is mediated by the death domain and that the survival pathways may involve a new gene expression. If this postulation is true, then the blockade of the protein expression would enhance the cell death that is induced by TNF- α in PC12 cells. As expected, the results showed that the inhibition of the protein expression by cycloheximide greatly increased the apoptosis that was induced by TNF- α (Fig. 1b, TNF + CHX), compared

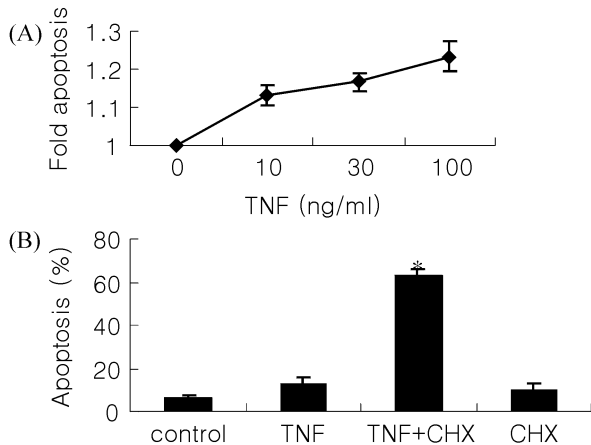


Fig. 1. TNF- α induces moderate apoptosis in PC12 cells. (A) Differentiated PC12 cells were incubated with TNF- α for 12 h. The intensity of apoptosis was determined by measuring mono- and oligo-nucleosomes in the cell lysates, using a Cell Death Detection ELISA Kit. Data are presented as means \pm S.E. of fold apoptosis with respect to untreated cells. (B) PC12 cells were incubated with the indicated agent (TNF 30 ng/ml, cycloheximide (CHX) 5 μ g/ml) for 12 h and assayed for the apoptosis by nuclear staining, described in Experimental Procedures. Data are means \pm S.E. * P <0.01 versus control.

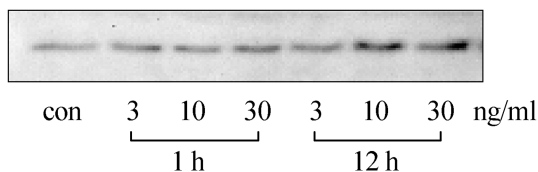


Fig. 2. The induction of bcl-2 in response to TNF- α . Dose- and time-dependent expression of bcl-2 that was induced by TNF- α was determined by a Western blot analysis. Thirty micrograms of protein were loaded in each lane.

to the apoptosis level that was induced by TNF alone (Fig. 1b, TNF). Cycloheximide itself induced no significant apoptosis up to 12 h (Fig. 1b, CHX).

Accordingly, the expression of anti-apoptotic gene, bcl-2, also increased 12 h after the treatment of TNF- α in a dose-dependent manner (Fig. 2).

p38 MAPK is activated in response to TNF- α The p38 MAPK and JNK are phosphorylated and activated in response to TNF- α in various cell types, including PC12 cells (Fig. 3, Modur *et al.*, 1996; Roulston *et al.*, 1998). It was reported that the activation of JNK and p38 MAPK, in response to TNF- α , is very rapid. Also, the early activation of JNK and p38 MAPK usually appeared within 10 min and slowly decreased in the L929-cyt16 cells (Modur *et al.*, 1996; Roulston *et al.*, 1998). However, in the differentiated PC12 cells, the phosphorylation of p38 MAPK slowly increased up to 6 h after the treatment of TNF- α (Fig. 3). The phosphorylation pattern of p38 MAPK in our system was somewhat different

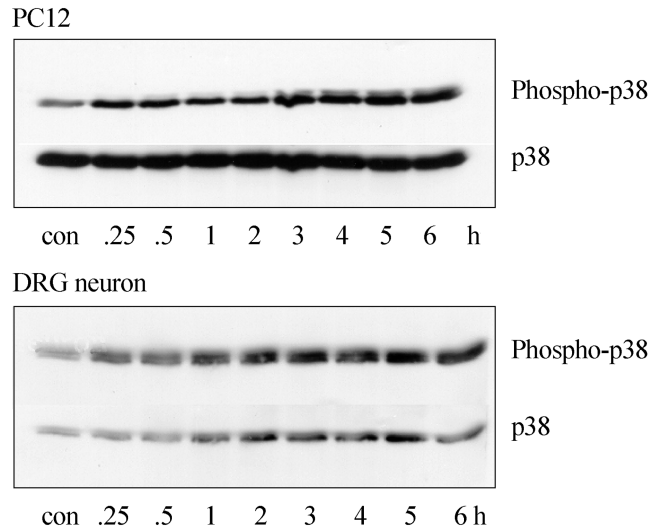


Fig. 3. Time course phosphorylation of p38 MAPK in PC12 cell and DRG neuron in response to TNF- α . Cells were treated with TNF- α (30 ng/ml) for various durations. Western blotting was performed as indicated in Materials and Methods. Identical results were obtained in the other two experiments.

than that of others. To examine whether this slow increase in the phosphorylation of p38 MAPK in response to TNF- α was confined to PC12 cells, we also performed the same experiment in a primary dorsal root ganglia neuron. The phosphorylation pattern of the DRG neuron was exactly the same as in the PC12 cells (Fig. 3).

Activation of p38 MAPK partially prevents TNF- α induced apoptosis In order to investigate the role of p38 MAPK in the TNF- α -induced apoptosis event in the neuronal systems, we used the p38 MAPK inhibitor SB202190 along with the TNF- α treatment to the differentiated PC12 cells (Lee *et al.*, 1994; Cuenda *et al.*, 1995). According to cell types and stimuli that induces apoptosis, the activation of p38 MAPK was reported to either contribute to apoptosis or protect the cells from the cell death (Roulston *et al.*, 1998; Jun *et al.*, 1999). In our experiment, SB202190 increased the TNF- α -induced apoptosis in differentiated PC12 cells, compared to the increment of the apoptosis level between TNF alone (Fig. 4, TNF) and the SB202190 co-treatment group (Fig. 4, TNF + SB). SB202190 itself did not affect the viability of the cells (Fig. 4, SB). This indicated that p38 MAPK may act as an anti-apoptotic protein in response to TNF- α in differentiated PC12 cells. Until now, nothing has been known about the role of p38 MAPK in response to TNF- α in neuronal cells. Therefore, this study is the first to report data regarding the function of the TNF- α activated p38 MAPK in neuronal cells.

In order to determine the significance of the relationship between the early and late phases of the phosphorylation of p38 MAPK in attenuating the cell death, which is induced by TNF- α , we blocked the late phase activation of p38 MAPK by

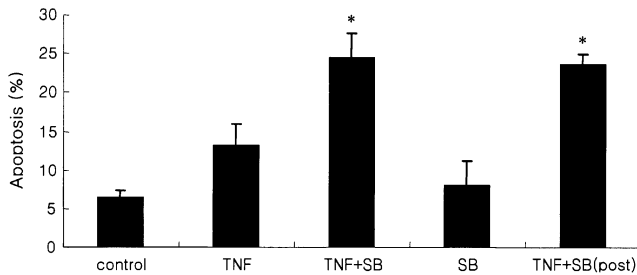


Fig. 4. Inhibitor of p38 MAPK, SB202190 enhanced the apoptosis induced by TNF- α in PC12 cells. Cells were treated with TNF- α (30 ng/ml) and SB202190 (10 μ M) for 12 h. The apoptotic cell was assayed by nuclear staining. In group TNF + SB(post), SB202190 was treated 3 h after the TNF- α stimulus. Data are means \pm S.E. * P <0.01 versus control.

adding the inhibitor, SB202190, after a 3 hour-incubation in TNF- α . As shown in Fig. 4, we found no significant difference in the apoptosis level between the two groups, TNF + SB and TNF + SB (post). From this result, it is apparent that the late phase of the p38 MAPK activation by TNF- α played an important role in preventing apoptosis in differentiated PC12 cells.

Activation of cPLA₂ suppresses the phosphorylation of p38 MAPK Reportedly, TNF- α can activate the cPLA₂ that promotes cell death in breast carcinoma and fibrosarcoma cells (Wissing *et al.*, 1997). But, it is not known how the activation of cPLA₂ can promote cell death in response to TNF- α . Since our results (Fig. 4) showed that the activation of p38 MAPK could protect PC12 cells from apoptosis, we hypothesized that the activation of cPLA₂ could promote apoptosis by down-regulating the activity of p38 MAPK. In order to investigate the effect of the activation of cPLA₂ on the activity of p38 MAPK in PC12 cells, we inactivated cPLA₂ with its specific inhibitor arachidonyltrifluoromethyl ketone (AACOCF₃). The down-regulation of cPLA₂ with AACOCF₃ greatly enhanced the phosphorylation of p38 MAPK that is induced by TNF- α (Fig. 5). Based on these results, it is possible to conclude that the activation of cPLA₂ suppresses the activation of p38 MAPK by a mechanism that is still unknown, thus delaying the activation of p38 MAPK; therefore, apoptosis may be promoted in response to TNF- α .

Discussion

The PC12 cell is widely used in various types of neurochemical studies, and the neuronal cell death model, because of its neuronal property (Green, 1982). The PC12 cell can be reversibly differentiated into the neuronal cell by the prolonged treatment of NGF. The differentiated PC12 cell expresses many neuronal markers and enzymes that are specifically expressed in neurons (Shafer and Atchison, 1991). We examined apoptosis that is induced by TNF- α in this cell line. TNF- α is a pro-inflammatory cytokine that mediates the

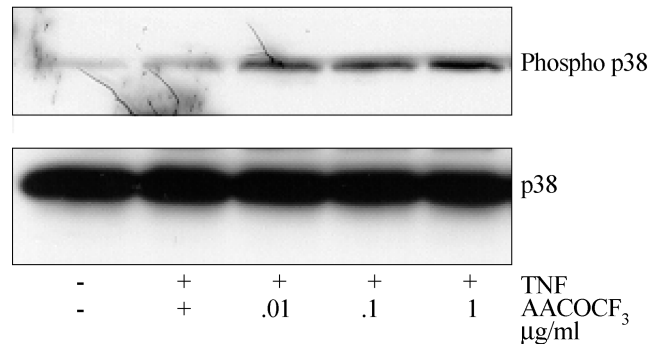


Fig. 5. The inhibitor of cPLA₂, AACOCF₃ enhanced the phosphorylation of p38 MAPK in response to TNF- α . Cells were treated with AACOCF₃ at indicated concentrations for 1 h. The Western blotting for phospho-p38 MAPK was performed as indicated in Materials and Methods.

inflammatory response. In CNS, TNF- α is induced in the glial cells (Lafortune *et al.*, 1996; Tchelingirian *et al.*, 1996; Knobloch *et al.*, 1999), and is also transported across the blood-brain barrier after injury (Pan *et al.*, 1999). An exact knowledge of TNF- α is important, because it is the major cytokine that elicits the inflammatory process after CNS injury (Feuerstein *et al.*, 1998; Knobloch *et al.*, 1999).

In this study, we identified the activation of p38 MAPK in response to TNF- α , and its role in differentiated PC12 cells. Surprisingly, the phosphorylation kinetics of p38 MAPK in response to TNF- α in the differentiated PC12 cells (Fig. 3) are different from other cell types (Roulston *et al.*, 1998). Yuasa *et al.* (1998) demonstrated that the activation of p38 by TNF- α is mediated by RIP, which can be associated with TRAF2 through the death domain. It also activates MKK6, the direct activator of p38 MAPK. The slowly increased phosphorylation of p38 MAPK in our experiment (Fig. 3) did not coincide with this model, because these phosphorylation cascades that were initiated from the receptor activation were a more rapid process. These results imply that the activation of p38 MAPK in the differentiated PC12 cell may use different pathways, such as a signaling pathway through a second messenger, which accumulates by enzymatic activity.

Unlike Fas-L, the TNF- α -mediated apoptosis was greatly enhanced by the inhibition of the transcription or translation in PC12 cells (Fig. 2), which was also shown by another investigator (Roulston *et al.*, 1998). Therefore, a new gene expression was needed to protect the PC12 cells from apoptosis in response to TNF- α . In hippocampal neurons, TNF- α protects the cells from hypoxia-induced cell death, and induces the anti-apoptotic protein Bcl-2 and Bcl-x expressions (Tamatani *et al.*, 1999). In differentiated PC12 cells, Bcl-2 was also induced by TNF- α (Fig. 2). TNF- α , therefore, activates both the death and survival pathways.

Interestingly, the activation of p38 MAPK shows two different actions with opposite manners, according to stimuli and cell types. To be more specific, the activation of p38

MAPK by the overexpression of Ask1 was shown to induce the apoptosis of the cell, while the activation by TNF- α was shown to protect the L929-cyt16 cell from apoptosis (Ichijo *et al.*, 1997; Roulston *et al.*, 1998). However, in neuronal cells, the role of p38 MAPK activation in apoptosis that was induced by TNF- α has not yet been reported. SB202190 is widely used as a p38 MAPK inhibitor (Lee *et al.*, 1994). Using this inhibitor, we examined the effect of the activation of p38 MAPK in response to TNF- α in differentiated PC12 cells. Although SB202190 itself did not induce apoptosis in the differentiated PC12 cell, the apoptosis that was induced by TNF- α increased about 3-fold in the presence of SB202190. Collectively, these results show the delayed activation of p38 MAPK. It can be seen that the late activation of p38 MAPK after caspase activation may attenuate the apoptosis that is induced by TNF- α . These results highlight the potential use of p38 MAPK as a protection against apoptosis, even after the apoptosis inducer has already been introduced into the system.

We have shown that the phosphorylation of p38 MAPK is regulated by cPLA₂, which produces arachidonic acid (AA) by cleaving phospholipid. AA exerts many biological actions. These include the modulation of the activities of protein kinases and ion channels, inhibition of neurotransmitter uptake, and enhancement of synaptic transmission. AA also serves as a precursor of a variety of eicosanoids, which are formed by the oxidative metabolism of AA. The AA cascade is activated under several pathological conditions in the brain, such as ischemia and seizures. It also may be involved in irreversible tissue damage (Katsuki and Okuda, 1995). Our results present one possible mechanism for enhancing the neuronal cell death by AA through the regulation of p38 MAPK. However, how AA attenuates the phosphorylation of p38 MAPK is unknown.

In summary, our study demonstrated that the weak apoptotic activity of TNF- α in differentiated PC12 cells is due to the protective role of p38 MAPK that is activated by TNF- α . This activation of p38 was regulated by cPLA₂, which was also activated by TNF- α . Therefore, the inactivation of cPLA₂ could be a possible therapeutic target in many neurodegenerative diseases that are mediated by TNF- α .

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