CHAPTER 2

The kinetics of neuroprotective tumor necrosis factor
TNF-α molecular signaling

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Submitted
Abstract

We have previously shown that tumor necrosis factor-alpha (TNF-α) protects neurons in vivo in an retinal ischemia model. In vitro studies showed that long term TNF-α (24 h) promotes beneficial effects on neuronal survival against a glutamate insult. To elucidate the neuroprotective signaling pathways of TNF-α we studied the kinetics of this protective TNF-α effect. Using a glutamate-induced excitotoxicity model we showed that TNF-α is able to increase neuronal survival after 6-8 h of treatment. Furthermore, phosphorylation levels of PKB/Akt and the expression of PTEN were profoundly altered upon TNF-α treatment. Since, the mammalian brain is endowed with three PKB/Akt isoforms we investigated which PKB/Akt isoform is important for TNF-α induced neuroprotective effect. From experiments using TNF-α expressing neurons from TNF-transgenic mice, which have constitutively activated PKB/Akt signaling and which were transfected with specific siRNA probes for PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 we concluded that PKBα/Akt1 and PKBγ/Akt3 isoforms are important in TNF-α-mediated neuroprotective effect.
2.1 Introduction

TNF-α is a proinflammatory cytokine that has been associated with the pathology of several autoimmune disorders, including rheumatoid arthritis, spondylitis, Crohn’s disease, psoriasis and asthma (Eisel et al., 2006). TNF-α expression is up-regulated in the brain of human patients with neurodegenerative disorders such as Alzheimer’s disease, Parkinson’s disease (Perry et al., 2001; Nagatsu and Sawada, 2005; Comabella et al., 2006) suggesting a potential pathogenic role of TNF-α in neurodegenerative diseases as well. These findings were paralleled by in vivo models for neurodegeneration, such as the mouse model of retinal ischemia (Fontaine et al., 2002). Recent studies showed that TNF-α contributes to both neuroprotection and neurodegeneration (Cheng et al., 1994; Hermann et al., 2001). Excess TNF-α causes cell death of human cortical neurons and oligodendrocytes (Chao and Hu, 1994) and together with ceramide and NF-κB can induce apoptotic neuronal cell deaths in cultured dopaminergic neurons (Hunot et al., 1997). On the other hand, cortical neurons isolated from NR2B/TNF mice, which have locally restricted TNF-α activity are entirely resistant to neuronal death induced by toxic doses of glutamate. In addition, in isolated cell culture of purified wild-type neurons long TNF-α exposure time exhibit a neuroprotective effect against glutamate-mediated excitotoxicity (Marchetti et al., 2004).

Until now, the molecular mechanisms underlying these beneficial effects of TNF-α are largely unknown, but at least PKB/Akt and NF-KB activation are associated with TNF-α-mediated neuroprotective pathways (Fontaine et al., 2002; Marchetti et al., 2004). TNF-α exerts its biological functions through the action of two main receptors, TNF-R1 and TNF-R2 (Eisel et al., 2006). In recent studies we proposed new functions for TNF-R2 pathway. We showed that TNF-R2 exerts neuroprotection in vivo in a retinal ischemia model (Fontaine et al., 2002) and in vitro in a glutamate-induced excitotoxicity model (Marchetti et al., 2004) in a PKB/Akt-dependent manner.

In the present study we investigated in more detail the kinetics of the TNF-α neuroprotective effect. We concentrated on the kinetics of PKB/Akt activation and on the kinetics of the phosphatase and tensin homologue deleted from chromosome 10 (PTEN), the main negative regulator of PKB/Akt. In particular, we checked whether PTEN activation precedes PKB/Akt activation during long-term TNF-α treatment. Since three isoforms of PKB/Akt have been identified and described in mice and humans (Brazil and Hemmings, 2001) we investigated which PKB/Akt isoform is important in TNF-α-induced neuroprotective pathways.

2.2 Materials and methods

Materials. Neurobasal medium, B27 supplement, L-glutamine and Penicillin/Streptomycin were purchased from Invitrogen (Carlsbad, CA). LY294002 was from Calbiochem (San Diego, CA) and Complete mini protease inhibitor cocktail tablets from Roche (Indianapolis, IN). TNF-α was purchased from HBT (Uden, The Netherlands).
Primary antibodies used were a rabbit polyclonal antibody specific for total PKB/Akt (9272, Cell Signaling, Danvers, MA, 1:2000 dilution), anti p-Akt (Ser473, 9271, Cell Signaling, Danvers, MA, 1:2000 dilution), anti PKBo/Akt1 (5919, Abcam, 1:2000 dilution), anti PKBβ/Akt2 (5920, Abcam, 1:2000 dilution), anti PKBγ/Akt3 (5922, Abcam, 1:2000 dilution) and a monoclonal mouse antibody specific for actin as internal standard (MP Biomedicals, Irvine, CA, 1:100000 dilution). The secondary antibodies used were alkaline phosphatase (AP)-conjugated goat anti-mouse (Applied Biosystems, Bedford, MA, 1:10000 dilution) and AP-conjugated goat anti-rabbit (Applied Biosystems, Bedford, MA, 1:10000 dilution). The chemi-luminiscence detection kit (Nitroblock II and CDP-Star) was purchased from Applied Biosystems (Bedford, MA). All other materials were from Sigma.

Animal experiments. All experiments were performed using C57BL/6J (Harlan, Horst, The Netherlands), TNF-R1−/−, TNF-R2−/− and TNF-α overexpressing mice (Rothe et al., 1993; Pfeffer et al., 1993; Erickson et al., 1994). The procedures concerning animal care were in accordance with the regulation of the Ethical Committee for the use of experimental animals of the University of Groningen, The Netherlands. Mice were individually housed in standard macrolon cages and maintained on a 12 h light/dark cycle. They received food and water ad libitum.

Cell cultures. Primary cortical neurons were isolated from embryonic brains (E15-16) of C57BL/6J, TNF-R1−/− TNF-R2−/− and TNF-α overexpressing mice. The meninges was removed and the PCNs were separated by mechanical dissociation. Cortical neurons were plated in a density of 12 × 10⁴ cells/well (96 well plates) and 2 × 10⁶ cells/well (6 well plates) on 2 µg/ml poly-D-lysine coated plates. Neurobasal medium with B27-supplement, 0.5 mM L-glutamine, 50 units/ml Penicillin/Streptomycin and 2.5 µg/ml amphotericin B was used as a culture medium. After 48 h cells were treated with 10 µM cytosine arabinoside for another 48 h to inhibit non-neuronal cell growth. Subsequently, the medium was completely changed and after 6 days of in vitro culture, cortical neurons were used for experiments. Acquisition of primary cultures was under the regulation of the Ethical Committee for the use of experimental animals of the University of Groningen, The Netherlands (DEC 4048).

Cortical neurons were incubated with 100 ng/ml TNF-α for different time intervals. Excitotoxicity was induced by 50 µM glutamate for 1 h. After treatment, neuronal cells were either lysed (and subsequently subjected to western blot analysis) or allowed to recover for another 24 hours, as shown in the schematic diagram 2.1(a).

Determination of cell viability. 24 h after glutamate treatment viability of the neurons was determined by the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-lium bromide) assay as described previously (Mosmann, 1983). 1.25 mg/ml MTT solution was added to each well of a 96 well plate. After 2-4 h of incubation, cells were lysed by adding 120 µl of isopropyl-HCl solution (37% (v/v)
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HCl/isopropyl alcohol: 1/166) for 15 min. The absorbance of each well was determined with an automated ELISA reader (Bio-Rad, Munich, Germany) at 595 nm with a background correction at 620 nm.

**siRNA experiments.** siRNA probes targeted to PKBα/Akt1 (L-040709-00), PKBβ/Akt2 (L-040782-00) and PKBγ/Akt3 (L-040891-00) were purchased from Dharmacon (Dharmacon, Inc. Lafayette, CO, USA). A scrambled siRNA probe was used as a control in all siRNA transfection experiments. Primary cortical neurons were transfected with siRNAs using the Amaxa nucleofector technology (Amaxa, Cologne, Germany). Cells were cultured for 4 days after nucleofection, lysed and subsequently analysed for protein content.

**Protein analysis.** Cortical neurons were washed twice with ice cold phosphate-buffered saline and subsequently lysed by the addition of 0.15 ml lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate and Complete mini protease inhibitor cocktail tablet (Roche). The samples were centrifuged at 9 000g for 10 min at 4 °C and the supernatant boiled for 5 min in Laemmli’s sample buffer. Twenty µg of total protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After transfer to a PVDF transfer membrane (Millipore Corporation, Billerica, MA) proteins were linked with primary antibodies overnight at 4 °C. Phosphorylation of PKB/Akt was evaluated as the ratio of p-Akt Ser473/total Akt. Actin was used as an internal control to correct for variations in protein content. The blots were incubated with alkaline phosphatase-conjugated goat anti-mouse (Applied Biosystems, Bedford, MA, 1:10 000 dilution) or alkaline phosphatase-conjugated goat anti-rabbit (Applied Biosystems, Bedford, MA, 1:10 000 dilution). Proteins were detected with an enhanced chemoluminescence detection system according to the manufacturer’s instructions. Integrated optical densities (IOD) were measured by the Leica DFC 320 Image Analysis System (Leica, Cambridge, UK) and densitometric analysis was evaluated by the Leica Qwin program. Quantification of IOD was performed only in images in which saturation of signal had not occurred. IOD measurements were corrected for the background intensity.

**Statistical analysis.** Results shown represent mean ±S.E.M. Statistical analysis was performed by Statistical Package for the Social Science (SPSS) program. One-Sample T Test procedure tests were used to check whether the mean of a single variable differs from the control samples, taken as the specific constant. These tests were followed by one-way ANOVA LSD post-hoc program. p values of 0.05 were considered to be significant.
2.3 Results

2.3.1 Neuroprotective time window initiated by TNF-α in cortical neurons.

Exposure of cortical neurons to cytotoxic glutamate concentrations triggers cell death in a dose and time dependent manner (Rogers et al., 2004). In an initial study, 24 h of TNF-α treatment of primary cortical neurons was shown to induce neuroprotection against the glutamate-mediated excitotoxicity (Marchetti et al., 2004). Therefore, here we investigated the TNF-α-mediated effects after shorter exposure time in an *in vitro* excitotoxic model.

Cortical neurons were treated as shown in the schematic diagram (2.1(a)). After 6 days in culture, neurons were pre-incubated with TNF-α for 1, 2, 4, 6, 8 or 24 h and they were glutamate-challenged after each time point. The first 4 h of TNF-α pre-incubation did not protect neurons against glutamate excitotoxicity (Glu: 36.4±3.2% compared with TNF-α and Glu 35.7±1.8% of non-treated neurons; n = 6), while 8 h of TNF-α incubation rescued neurons from the cell death (Glu: 36.4±3.2% compared with TNF-α and Glu: 55.7±8.7% of non-treated neurons; n = 6). Neuronal resistance to apoptosis was observed, to an even greater extent after 24 h TNF-α treatment (Glu: 36.4±3.2%, TNF-α and Glu: 65.2±6.3% of non-treated neurons; n = 6). Exposure of cortical neurons to TNF-α after the glutamate challenge was not able to inhibit the cellular death (data not shown). TNF-α alone for 24 h had a slight toxic effect on primary neurons (TNF-α: 89.5±6.4% of non-treated neurons; n = 6).

2.3.2 TNF-R2 pathway increases PKB/Akt phosphorylation.

To gain further insight into TNF-α-mediated neuroprotective mechanisms, we investigated the effects of TNF-α on PKB/Akt phosphorylation status since PKB/Akt is a downstream molecule of TNF-TNFR axis. Increased PKB/Akt phosphorylation is linked with cell survival, proliferation and metabolic functions (Lawlor and Alessi, 2001). The activation of enzyme activity of PKB/Akt is due to phosphorylation of two sites: Thr\textsuperscript{308} and Ser\textsuperscript{473} (Lawlor and Alessi, 2001; Franke et al., 1997). Here we evaluated the activation of PKB/Akt as a ratio of phosphorylated-Akt Ser\textsuperscript{473} (p-PKB/Akt)/total Akt.

Primary cortical neurons were treated with TNF-α for a time period ranging from 2 to 24 h. In wild-type cortical neurons, in the first 4 h of TNF-α treatment, p-PKB/Akt levels were slightly reduced (2.2(a)). After 6 h of TNF-α treatment we observed a significant increase in PKB/Akt phosphorylation (2.2(a)).

To investigate the contribution of each TNF-Rs in TNF-α-mediated neuroprotective signaling we checked PKB/Akt phosphorylation in TNF-R1\textsuperscript{-/-} and TNF-R2\textsuperscript{-/-} neurons. Upon 24 h of TNF-α treatment the phosphorylation of PKB/Akt was down-regulated in TNF-R2\textsuperscript{-/-} neurons (as seen in Fig. 2.2(b)). However, in TNF-R1\textsuperscript{-/-} neurons PKB/Akt phosphorylation was increasing with TNF-α treatment time (Fig. 2.2(b)).
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Figure 2.1: Long term TNF-α treatment increases neuronal survival. (a). Cortical neurons were treated as shown in the schematic diagram. (b). Cortical neurons were treated with TNF-α (100 ng/ml) for the indicated periods of time and subsequently challenged with glutamate (50 µM, 1 h). Neuronal survival was assessed 24 h following the exposure to glutamate by an MTT assay. Values represent mean ±SEM of determinations made in three separate cultures. Statistical analysis was performed by the Student-Neumann test with a 95% confidence interval using the SPSS program. p values of < 0.05 were considered to be significant.

2.3.3 PTEN is a negative regulator of neuroprotective signalling.

Several studies showed that PI3K-PKB/Akt signaling is inhibited by PTEN (Lawlor and Alessi, 2001; Fayard et al., 2005). Our next step, therefore, was to investigate whether increased levels of PTEN are associated with the neuronal sensitivity towards glutamate neurotoxicity. We observed an increase in PTEN levels in the first 2-4 h of TNF-α-treated neurons Fig. 2.3(a), while after 24 h of TNF-α treatment PTEN expression was diminished Fig. 2.3(a).

Since PKB/Akt phosphorylation is modulated differently by TNF-Rs signaling, we investigated whether PTEN expression is changed as well during TNF-α treatment
Figure 2.2: Long term TNF-α treatment increases PKB/Akt phosphorylation. PKB/Akt phosphorylation in wild-type neurons (a) and TNF-R1/− or TNF-R2/− neurons (b) treated with 100 ng/ml mouse TNF-α for indicated periods of time. Representative immunoblots are shown below the quantified data of PKB/Akt phosphorylation at the Ser473 site (αp473-Akt) analyzed from three separate experiments. The bars indicate the mean ± S.E.M. of IOD of protein bands corresponding to α p473-Akt as percentage of total PKB/Akt (depicted as total Akt) (Univariate Analysis of Variance and LSD test, p < 0.05 versus non-treated neurons).

in TNF-Rc/− neurons. Western blot analysis showed that in TNF-R2/− neurons, PTEN levels were significantly up-regulated after 6-8 and 24 h of TNF-α pre-incubation Fig. 2.3(b), while in TNF-R1/− neurons, PTEN expression was down-regulated starting from 2 h of TNF-α incubation (Fig. 2.3(b)) up to an even stronger down-regulation after 24 h of TNF-α treatment (Fig. 2.3(b)).

2.3.4 Neuroprotection in neurons is dependent on PKBα/Akt1 and PKBγ/Akt3 expression.

In an initial study, we found that TNF-α-induced PKB/Akt phosphorylation promoted neuroprotection against glutamate-induced neurotoxicity (Marchetti et al., 2004; Dolga et al., 2008). Until now, three isoforms of PKB/Akt have been identified and described in mice and humans (Brazil and Hemmings, 2001). The next step, therefore, was to investigate which PKB/Akt isoform is important for TNF-α-mediated neuroprotective signaling. Using NR2B/TNF neurons that are endowed with constitutively activated PKB/Akt (Marchetti et al., 2004) we silenced each PKB/Akt isoform and then checked the phosphorylation of PKB/Akt. First we ascen-
2.4. Discussion

In this study we show the kinetics of the neuroprotective TNF-α effect against glutamate-induced toxicity in primary cortical neurons. We investigated in particular the dual effect of PTEN and PKB/Akt in this neuroprotective TNF-α signaling.

In a previous study we showed that long exposure (24 h) of cortical neurons to TNF-α induced increase cellular survival. Therefore, in this study we investigated the time course of TNF-α-treatment necessary to initiate neuroprotection. Using MTT as-

Figure 2.3: Long term TNF-α treatment decreases PTEN expression. PTEN expression in wild-type neurons (a) and TNF-R1⁻/⁻ or TNF-R2⁻/⁻ neurons (b) treated with 100 ng/ml mouse TNF-α for indicated periods of time. Representative immunoblots are shown below the quantified data of PTEN expression analyzed from three separate experiments. The bars indicate the mean ±S.E.M. of IOD of protein bands corresponding to α PTEN (Univariate Analysis of Variance and LSD test, p < 0.05 versus non-treated neurons).

tained that these NR2B/TNF neurons have indeed higher levels of PKB/Akt phosphorylation than wild-type neurons (Fig.2.4(a)). siRNA probes targeted for PKBo/Akt1, PKBβ/Akt2 and PKBγ/Akt3 significantly reduced each specific isoform after 4 days in vitro culture (Fig.2.4(b)). Here we evaluated the reduction of PKB/Akt isoforms after siRNA experiments as a ratio of PKB/Akt(1/2 or 3)/total Akt. All three PKB/Akt isoforms were silenced approximately 40% as compared with scramble control RNA (Fig.2.4(c)). Interestingly, silencing PKBo/Akt1 and PKBγ/Akt3 drastically reduced PKB/Akt phosphorylation, while silencing PKBβ/Akt2 produced only a slight decrease in PKB/Akt phosphorylation (Fig. 2.4(d)).

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Figure 2.4: Silencing of PKB/Akt1 and PKB/Akt3 decrease PKB/Akt phosphorylation. (a). Primary cortical neurons from NR2B/TNF mice have increased PKB/Akt phosphorylation levels compared to wild-type neurons. (b). NR2B/TNF neurons were transfected with specific PKBα/Akt1 siRNA, PKBβ/Akt2 siRNA or PKBγ/Akt3 siRNA or with scrambled siRNA. After 4 days neurons were lysed and analyzed for PKB/Akt phosphorylation at the Ser473 site (α p473-Akt). Cortical neurons electroporated without siRNA served as additional controls to scramble siRNA. Representative immunoblots (b) and the quantified data (c) of PKBα/Akt1/total PKB/Akt, PKBβ/Akt2/total PKB/Akt and PKBγ/Akt3/total PKB/Akt are shown. Actin was used as an internal control to correct for variations in protein content. (d). The bars indicate the mean ± S.E.M. of IOD of protein bands corresponding to α p473-Akt as percentage of total PKB/Akt (depicted as total Akt) (Univariate Analysis of Variance and LSD test, p < 0.05 versus non-treated neurons). (LSD test, n = 3, p < 0.05 versus control neurons).
say for determining the cellular viability, we observed that short-time exposure (2-4 h) to TNF-α did not rescue neurons from glutamate-mediated excitotoxicity. However, longer TNF-α treatment than 6-8 h promoted neuroprotection in a time dependent manner. In our experiments 6-8 h TNF-α treatment represented a switch point from neuronal death to neuronal survival. To further dissect the molecular mechanisms that allowed neurons to change their condition from a vulnerable to a resistant state when they are glutamate challenged, we investigated PKB/Akt phosphorylation during TNF-α treatment. Our hypothesis that TNF-α treatment may alter PKB/Akt phosphorylation is derived from studies showing that 24 h TNF-α treatment induced strong increase in PKB/Akt phosphorylation (Marchetti et al., 2004). In neurons, PKB/Akt activation is associated with increased neuronal protection and survival against various noxious conditions (Lawlor and Alessi, 2001). PKB/Akt activity can be regulated by several phosphatases, including PTEN, PHLPP or PP2A. PTEN is considered so far the main negative regulator of PKB/Akt phosphorylation (Fayard et al., 2005). Furthermore, an increase in PTEN expression in cortical neurons was shown to induce neuronal death (Gary and Mattson, 2002). In our experiments, short-term TNF-α treatment (2-4 h) induced an increase in PTEN expression and a decrease in PKB/Akt phosphorylation. However, long-term TNF-α treatment (6-8 to 24 h) resulted in a decrease in PTEN expression and an augmented PKB/Akt phosphorylation in a time dependent manner. The fact that high levels of PTEN coincided with low levels of PKB/Akt phosphorylation was in accordance with the inverse relationship between PTEN and PKB/Akt activation. TNF-α is a ligand for both TNF-R1 and TNF-R2. A previous study from our group reported that TNF-R2 is essential for TNF-α-induced neuroprotective pathways. Using TNF-R-/- neurons in this study we investigated the relationship between TNF-receptors and PKB/Akt phosphorylation or PTEN expression. It appeared that in TNF-R1-/- neurons, PKB/Akt was phosphorylated after long term TNF-α treatment with visible effects after 6-8 h, while in TNF-R2-/- PKB/Akt phosphorylation was down-regulated in a TNF-α-treatment time dependent manner (Figs. 2.2 and 2.3). Overall, in wild-type neurons the increase in PTEN expression or the decrease in PKB/Akt phosphorylation observed in the first hours of TNF-α treatment could be attributed to TNF-R1 signaling, while the later decrease in PTEN levels and increase in PKB/Akt phosphorylation are due to TNF-R2 signaling (Figs. 2.2 and 2.3).

Several studies showed the influence of TNF-α-treatment on synaptic transmission (Beattie et al., 2002; Stellwagen et al., 2005; Stellwagen and Malenka, 2006). Interestingly, TNF-α acting on neuronal TNF-R1 receptors, but not on TNF-R2 receptors increases in 20 to 40 min surface AMPARs through a PI3 kinase-dependent pathway (Stellwagen et al., 2005). This fast increase in excitatory synaptic transmission (AMPARs) is concomitant with endocytosis of GABA receptors that weakens the inhibitory synaptic strength. This unique feature of TNF-α action could explain why in our experiments the first hours of TNF-α-treatment were not able to induce neuroprotection against glutamate excitotoxicity.
In neurodegenerative disorders TNF-α expression levels were found elevated in specific brain regions. In a previous study we investigated the consequences of TNF-α increase in specific brain regions by creating a transgenic mouse line NR2B/TNF transgenic mice with a neuron-specific promoter to guide TNF-α expression. In NR2B/TNF transgenic mice TNF-α was expressed under the control of the murine NMDAR subunit NR2B promoter. Interestingly, NR2B/TNF neurons were totally resistant to excitotoxic effects induced by glutamate treatment. This effect was paralleled somehow to a lower extent by exposure of wild-type neurons to soluble TNF-α.

Most important, TNF-α treatment in wild-type neurons induced PKB/Akt phosphorylation while NR2B/TNF neurons have a constitutively high level of activated PKB/Akt compared with wild-type neurons (Marchetti et al., 2004). Since it is unknown which PKB/Akt isoform is engaged in TNF-α-mediated neuroprotective pathways, we used NR2B/TNF neurons to silence each PKB/Akt isoform and check the phosphorylation status of PKB/Akt. Several reports showed that TNF-R2 neuroprotective pathways are dependent on the activation of PKB/Akt signaling, which is associated with increased neuronal survival (Fontaine et al., 2002; Marchetti et al., 2004). In this study we found that silencing PKB/Akt isoforms in NR2B/TNF neurons only PKBα/Akt1 and PKBγ/Akt3 are important in PKB/Akt phosphorylation. These findings demonstrate the important role of PKBα/Akt1 and PKBγ/Akt3 in neuroprotection and associate these isoforms with TNF-R2 pathways.

Taken together, our studies suggest potential for therapeutic manipulation of TNF-α signaling in the treatment of neurodegenerative disorders.

References


