TNF-α mediates neuroprotection against glutamate-induced excitotoxicity via NF-κB-dependent up-regulation of SK2 channels

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Abstract

Previous studies have shown that TNF-α induces neuroprotection against excitotoxic damage in primary cortical neurons via sustained NF-κB activation. The transcription factor NF-κB can regulate the expression of small conductance calcium-activated potassium (SK) channels. These channels reduce neuronal excitability and as such may yield neuroprotection against neuronal overstimulation. In the present study we investigated whether TNF-α-mediated neuroprotective signaling is inducing changes in the expression of SK channels. Interestingly, the expression of SK2 channel was up-regulated by TNF-α treatment in a time-dependent manner whereas the expression of SK1 and SK3 channels was not altered. Furthermore, activation of SK channels by NS309 or CyPPA induced neuroprotection against a glutamate challenge and inhibition of SK channels with apamin reverted the neuroprotective effect elicited by TNF-α. The increase in SK2 channel expression after TNF-α treatment was shown to be NF-κB-dependent since the NF-κB inhibitor BAY11-7082 blocked the TNF-α-induced increase in SK2 channel expression. We conclude that treatment of primary cortical neurons with TNF-α leads to increased SK2 channel expression which renders neurons more resistant to excitotoxic cell death.
4.1 Introduction

TNF-α is a proinflammatory cytokine involved in a wide range of cellular responses including inflammation, cellular differentiation and apoptosis. TNF-α perse can be synthesized and released in the brain by several types of cells, including astrocytes, microglia and neurons. TNF-α was shown to be upregulated in a number of neurodegenerative disorders including Alzheimer’s disease, stroke and multiple sclerosis. It exerts its biological functions through the stimulation of two receptors, TNF-R1 and TNF-R2. Both TNF-R1 and TNF-R2 induce activation of the NF-κB pathway (Marchetti et al., 2004; Eisel et al., 2006). However, the activation kinetics of NF-κB stimulation reveals characteristic differences: while activation of the TNF-R1 pathway leads to a transient NF-κB stimulation (first 20-40 min during 24 h TNF-α treatment), TNF-R2 signaling promotes persistent and long lasting NF-κB activation (persisting even after 24 h of TNF-α treatment) (Marchetti et al., 2004).

Several studies showed that TNF-α is able to promote neuroprotection against glutamate-induced excitotoxicity in cortical neurons (Cheng et al., 1994; Marchetti et al., 2004; Barger et al., 1995). Until now, the molecular mechanisms underlying the neuroprotective effects of TNF-α against glutamate-induced excitotoxicity are largely unknown but at least PKB/Akt activation and subsequent sustained NF-κB activation were shown to play a major role (Marchetti et al., 2004).

In a recent study, small conductance Ca\(^{2+}\) activated potassium (SK) channels were identified as one of the downstream targets of NF-κB mediated promoter regulation (Kye et al., 2007). SK channels are important regulators of neuronal excitability. These channels are voltage insensitive, but open in response to low (< 1 µM) intracellular Ca\(^{2+}\) levels. In many central nervous system neurons, SK channel activity dampens excitability by contributing to the afterhyperpolarization, affecting interspike intervals during a burst of action potentials as well as the length of the burst (reviewed by Stocker (2004)). In the mammalian brain, SK channels are the product of three paralogous genes, namely SK1, SK2 and SK3 genes. SK1 and SK2 channel subunits show extensive colocalization. They are mainly found in the entorhinal cortex, the subiculum, in pyramidal cortical neurons, the CA1 - CA3 region from the hippocampus and in the thalamus. SK3 channel subunits have a complementary distribution, and are mainly located in the brain stem and in monoaminergic neurons (Sailer et al. (2002); Sailer et al. (2004)). In certain neuronal cell types, SK channels are present at dendritic spines where they co-localise with NMDA receptors. There, SK channel activity suppresses the amplitude of evoked synaptic potentials by inhibiting NMDA receptor-dependent activation (Faber et al., 2005; Lin et al., 2008; Ngo-Anh et al., 2005). Thus, enhancing SK channel expression during an excitotoxic insult could be protective due to hyperpolarization and diminished excitability. In an initial study Lee and colleagues (Lee et al., 2003) showed indeed that overexpression of SK channels can prevent kainic acid and glutamate-induced excitotoxicity.

In the present study we have tested the hypothesis whether increased SK2 channel expression is part of the mechanism of TNF-α mediated neuroprotection against
glutamate-induced excitotoxicity in cortical neurons.

4.2 Materials and methods

Materials. Neurobasal medium and B27 supplement were purchased from Invitrogen (Carlsbad, CA, USA). Complete mini protease inhibitor cocktail tablets were from Roche (Indianapolis, IN) and mouse TNF-α was purchased from HBT (Uden, The Netherlands). Cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine (CyPPA) was bought from Specs (Delft, Netherlands). Primary antibodies used to detect SK channels were rabbit anti-SK1, rabbit anti-SK2 (both kindly provided by H-G. Knaus (Sailer et al., 2004)), and anti-SK3 (APC-025; Alomone Laboratories, Jerusalem, Israel). For the detection of actin a monoclonal mouse antibody from MP Biomedicals (691001; Irvine, CA, USA) was used. Alkaline phosphatase (AP)-conjugated goat anti-mouse (Applied Biosystems, Bedford, MA, USA) and AP-conjugated goat anti-rabbit (Applied Biosystems, Bedford, MA, USA) were used as secondary antibodies. The chemi-luminiscence detection kit (Nitroblock II and CDP-Star) was purchased from Applied Biosystems (Bedford, MA, USA). Immunohistochemical studies were performed using SK2 (LifeSpan Biosciences, Seattle, WA, USA), NR2B antibodies (BD Transduction Laboratories, Franklin Lakes, NJ, USA) and Alexa antibodies (Invitrogen, Carlsbad, CA, USA). All other materials were from Sigma.

Animal experiments. All experiments were performed using C57BL/6J mice (Harlan, Horst, The Netherlands). All procedures were in accordance with the regulation of the Ethical Committee for the use of experimental animals of the University of Groningen, The Netherlands (Licence number DEC 4048). Mice were housed in standard macrolon cages and maintained on a 12 h light/dark cycle. They received food and water ad libitum.

Primary cortical neuron culture. Primary cortical neurons were prepared from embryonic brains (E15-16) of C57BL/6J mice. The meninges were removed and the cortical neurons were separated by mechanical dissociation. Cells were plated in a density of $12 \times 10^4$ cells/well (96 well plates) and $2 \times 10^6$ cells/well (6 well plates) on poly-D-lysine pre-coated plates. Neurobasal medium with B27-supplement, 0.5 mM glutamine, 1% penicillin/ streptomycin and 2.5 µg/ml amphotericin B was used as a culture medium. After 48 h neurons were treated with 10 µM cytosine arabinoside for another 48 h to inhibit non-neuronal cell growth. Subsequently, the medium was completely exchanged and after 6 days of in vitro culture, the neurons were used for experiments.

Neurons were incubated for the indicated periods of time with 100 ng/ml mouse TNF-α. Some cortical neurons were incubated with NF-κB inhibitor BAY11-7082
(10 µM). Where indicated, neurons were pre-incubated with NS309 (6,7-dichloro-1H-indole-2,3-dione 3-oxime) 50 µM or CyPPA (with indicated concentrations) for 30 min. Apamin was used where indicated in a concentration of 20 µM. Pilot experiments using different concentrations of apamin showed that, at a concentration of 10 µM, apamin already partially blocked the TNF-mediated neuroprotective effect but the effect was even stronger when a concentration of 20 µM was used. 50 µM apamin had a similar effect as 20 µM apamin. The final concentration of DMSO in all experiments was less than 0.5% (v/v). In this concentration DMSO had no effect on glutamate-induced cell death. Excitotoxicity was induced by 1 h treatment with glutamate (50 µM). After treatment, the medium was completely removed and fresh media was added to the cells.

**Confocal microscopy.** Cortical neurons were grown on PDL-coated coverslips for 6 days. Neurons were either incubated or not with 100 ng/ml TNF-α for 6 h. Subsequently, they were fixated with ice-cold methanol for 10 min at 20 °C. Neurons were permeabilized with 0.05% TritonX-100 in phosphate-buffered saline (PBS) and blocked with 10% (v/v) normal donkey serum and 2% (v/v) bovine serum albumin (BSA) in PBS (pH 7.4) for 30 min at room temperature (RT). Slices were incubated overnight at 4 °C with antibodies raised against SK2 (LifeSpan Biosciences, Seattle, WA, USA, 1:500 dilution) and NR2B (BD Transduction Laboratories, Franklin Lakes, NJ, USA, 1:500 dilution). The next day, cells were incubated with secondary antibodies (Alexa 488, 555 and 633, Invitrogen, Carlsbad, CA, USA, 1:500 dilution). For mounting we used PreLong Gold (Invitrogen, Carlsbad, CA, USA). The confocal images were analysed with a Leica TCS SP2 confocal microscope using an Ar/ArKr laser (488 nm) or a HeNe laser (543 and 633 nm). To avoid overlap in emission spectra we used sequential scanning between frames.

**Determination of cell viability.** 24 h after glutamate treatment neuronal viability was determined by the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium 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% 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.

**Protein analysis.** Primary cortical neurons from C57BL/6J mice were washed twice with ice cold PBS after indicated periods of time of TNF-α incubation 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) TritonX, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate and complete mini protease inhibitor cocktail tablet (Roche, Indianapolis, IN, USA)). The samples were centrifuged at 9000 g for 10 min at 4 ºC.
and the supernatants boiled for 5 min in Laemmli’s sample buffer. Twenty µg of total protein was separated by 10% SDS-polyacrylamide gel electrophoresis. After transfer to a PVDF transfer membrane (Millipore Corporation, Billerica, MA, USA) proteins were linked with primary antibodies overnight at 4°C. Subsequently, the blots were incubated with AP-conjugated secondary antibodies (1:10000). Proteins were detected with an ECL detection system according to the manufacturer’s instructions (Applied Biosystems, Bedford, MA, USA). Actin (1:100000 antibody dilution) was used as an internal control to correct for variations in protein content.

Integrated optical densities (IOD) were measured with the Leica DFC 320 Image Analysis System (Leica, Cambridge, UK) and densitometric analysis was performed using the Leica Qwin program. Quantification of IOD was done only with images in which saturation of the signal had not occurred. IOD measurements were corrected for background intensity.

Statistical analysis. Each experiment was performed with at least two different batches of neurons and MTT and Western blot were repeated at least twice for each batch. Results are represented as mean ±SEM. Statistical analysis was performed by the Student-Neumann test with a 95% confidence interval followed by one-way ANOVA post-hoc LSD and Dunnett test using the SPSS program. p values of < 0.05 were considered to be significant.

4.3 Results

4.3.1 TNF-α protects neurons against glutamate-induced excitotoxicity.

To assess the time-dependent effect of TNF-α pretreatment on cortical neuronal cultures challenged with glutamate, we exposed cortical neurons to mouse TNF-α (100 ng/ml) for 2, 4, 6, 8 or 24 h. After TNF-α incubation, cortical neurons were challenged with glutamate (50 µM, for 1 h). Neuronal viability was determined 24 h following glutamate exposure by the colorimetric MTT assay. Preincubation of neurons with TNF-α for up to 6 h had no significant protective effect against glutamate-induced excitotoxicity (Fig. 4.1). The number of surviving neurons gradually increased when the TNF-α pre-treatment lasted between 6-24 h. Treatment with TNF-α (100 ng/ml) alone for 24 h did not change neuronal viability (Fig. 4.1). TNF-α treatment after the glutamate challenge did not protect cortical neurons against glutamate-induced toxicity (data not shown).

4.3.2 TNF-α increases SK2 channel expression.

Activation of SK channels in cortical neurons has been shown to suppress hyperexcitability (Pedarzani et al., 2001), which could be a mechanism to enhance neuronal survival in conditions of overstimulation. Therefore, we determined whether TNF-α treatment leads to a change in expression of the three SK channels SK1, 2 or 3. Similar to a previous study in mouse brain (Sailer et al., 2004), we found that anti-SK1_{12−29}...
FIGURE 4.1: TNF-α-mediated neuroprotection against glutamate-induced excitotoxicity.

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-Newmann test with a 95% confidence interval using the SPSS program. p values of < 0.05 were considered to be significant.

antibody detects three separate bands with molecular weights of 65, 58 and 43 kDa. The anti-SK2(array index=538−555) antibody detects a band with an apparent Mr value of 64 kDa and the anti-SK3(array index=504−522) antibody recognises a protein running at the height of 75 kDa. Glutamate treatment of neurons did not affect SK channel expression (data not shown) and treatment of cortical neurons with TNF-α (100 ng/ml) for 2, 4, 6, 8 and 24 h had no effect on the expression of SK1 and SK3 channels (Fig. 4.2). However, SK2 channel expression increased significantly when neurons were treated with TNF-α for 6, 8, and 24 h. Shorter incubation of TNF-α showed no effect on SK2 channel expression when compared to untreated neurons.

These data were extended by confocal studies showing increased SK2 channel immunoreactivity in TNF-α (100 ng/ml) treated cortical neurons in comparison to untreated neurons (Fig. 4.3(a)). In addition, SK2 channel expression was also augmented in dendrites where they appeared to be co-localised with NR2B subunits (Fig. 4.3(b)).

4.3.3 SK channel activation leads to neuronal survival.

To investigate whether increased SK2 channel activity can be neuroprotective, cortical neurons were treated for 30 min. with different concentrations of NS309, an activator of SK and intermediate conductance Ca²⁺-activated K⁺ (IK) channels (Strøbaek et al., 2004), followed by addition of glutamate (50 µM) to the medium for
TNF-α-induced neuroprotection is mediated by SK2 channels

Figure 4.2: Exposure to TNF-α increases SK2 channel expression in cortical neurons. Neurons were treated with murine TNF-α (100 ng/ml) for indicated periods of time. (a) Blots and bar graphs show the quantification of the 65 kDa SK1 band. Accordingly, integrated optical densities for the other two SK1 bands at 58 and 43 kDa showed no changes in response to the indicated TNF-α incubation times. (b) Quantification and representative immunoblot for SK2 with apparent Mr of 64 kDa after exposure to TNF-α for indicated periods of time. (c) Quantification and representative immunoblot for SK3. Results shown represent mean ±SEM from three independent experiments. Statistical analysis was performed by the Student-Neumann test with a 95% confidence interval followed by one-way ANOVA post-hoc LSD and Dunnett test using the SPSS program. p values of < 0.05 were considered to be significant.

1 h. NS309 strongly reduced glutamate-induced neuronal death in a concentration-dependent manner (Fig. 4.4(a)). Since NS309 does not distinguish between the SK subtypes and also activates the related IK channels, we repeated this experiment with CyPPA, a compound which is selective for stimulating SK2 and SK3 (Hougaard et al., 2007). Treatment with CyPPA also led to increased neuronal survival upon glutamate
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Figure 4.3: TNF-α treatment increases SK2 channel immunoreactivity. (a) Untreated cortical neurons and neurons treated with TNF-α for 6h were fixed, permeabilized and stained with anti-SK2 antibody coupled to Alexa 555. Boxed area represents the localization of panel (b) TNF-α treated neurons were stained with anti-SK2 antibody coupled to Alexa 555 and anti-NR2B antibody coupled to Alexa 488 as indicated in the respective panels. Images were collected from stained neurons by confocal microscopy and merged in the respective color image.

treatment in a concentration-dependent manner although to a somewhat lower extent than NS309. Higher CyPPA concentrations than 50 µM, however, induced neuronal death (Fig. 4.4(b)).

To confirm the involvement of SK channels in TNF-α-induced neuroprotection, we analyzed the viability of cortical neurons that were treated with 100 ng/ml mouse TNF-α for 24 h and challenged with glutamate (50 µM, for 1 h) in the presence or absence of an inhibitor of SK channel activity (Fig. 4.4(c)). As inhibitor we used apamin, an 18-amino-acid bee-venom toxin with a high selectivity for SK channels. Application of apamin (20 µM) alone had no significant effect on cell survival. The detrimental effect of glutamate on cell survival was also not changed by apamin treatment. Glutamate-induced neuronal death was partially reverted by preincubation of TNF-α, whereas suppression of SK channel activity by apamin (20 µM) prevented the TNF-α induced neuroprotective effect (Fig. 4.4(c)).
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4.3.4 Inhibition of NF-κB attenuates TNF-α induced upregulation of SK2 channel expression.

The activation kinetics of the transcription factor NF-κB in response to TNF-α treatment in neurons was shown by several groups (Marchetti et al., 2004; Wang et al., 2005) (reviewed by Kaltschmidt et al. (1999)). It has been reported that long-term (24 h) exposure of neurons to TNF-α promotes persistent and long lasting NF-κB
activation, which is associated with increased protection against glutamate-induced neurotoxicity (Marchetti et al., 2004). Since SK2 channel expression is regulated by NF-κB (Kye et al., 2007), we determined whether the observed increase in SK2 channel expression after TNF-α incubation was NF-κB dependent. SK2 protein expression was evaluated by Western blot analyses in TNF-α treated neurons in the presence or absence of BAY11-7082, an inhibitor of NF-κB activity. SK2 levels in cortical neurons were not increased by application of TNF-α (100 ng/ml) when BAY11-7082 (10 µM) was co-applied (Fig. 4.5). BAY11-7082 treatment alone did not affect SK2 channel expression. These findings suggest that inhibition of NF-κB prevented the TNF-α-induced increase in SK2 channel expression (Fig. 4.5).

4.4 Discussion

In the present study we have demonstrated that an NF-κB-dependent increase in SK2 channel expression in cortical neurons contributes to the neuroprotective effect of TNF-α against glutamate-induced excitotoxicity. TNF-α has been implicated as contributing to both neuroprotection and neurodegeneration, depending on the tissue and experimental paradigm and conditions (Chao and Hu, 1994; Cheng et al., 1994; Hermann et al., 2001; Marchetti et al., 2004; Zou and Crews, 2005). In our in vitro
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model of glutamate-induced cell death of primary cortical neurons, TNF-α was shown to have neuroprotective properties. Although the involvement of TNF-R2, PKB/Akt and NF-κB was already suggested (Dolga et al., 2008; Fontaine et al., 2002; Marchetti et al., 2004), only limited data are available on potential neuroprotective downstream targets of NF-κB.

In our study, the time-dependent neuroprotective effect of TNF-α against a glutamate challenge was paralleled by a selective increase in SK2 channels, whereas the expression of SK1 and SK3 channels remained unaffected. The specific SK channel blocker apamin blocked the neuroprotective effect of TNF-α against glutamate-induced excitotoxicity. SK channels have different affinities for apamin with SK2 proteins being the most sensitive, SK3 intermediate and SK1 being the least sensitive to apamin (Finlayson et al., 2001; Strøbaek et al., 2004). Given the finding that in cortical neurons SK1 and SK2 channels are more abundant than SK3 channels (Sailer et al. (2002); Sailer et al. (2004) and SK2 channels are more sensitive to apamin than SK1 channels (D’hoedt et al., 2004), reduction of the TNF-α mediated neuroprotection by apamin could be mainly attributed to the inhibition of SK2 channels.

The neuroprotective function of SK channels becomes evident by the finding that the SK channel activators, NS309 and CyPPA, elicited neuroprotection against glutamate-induced excitotoxicity in primary cortical neurons. NS309 facilitates Ca\(^{2+}\)-dependent activation of IK and SK channels, being most potent on IK and 1020 times less active on the SK subtypes (Strøbaek et al., 2004). CyPPA is a selective positive modulator of SK3 and SK2 channels, which does not affect SK1 or IK channels (Hougaard et al., 2007). Although we can not exclude the involvement of SK3 channel activity in the neuroprotective effect elicited by CyPPA, the distribution profile of SK2 and SK3 in cortical neurons (Sailer et al., 2002) suggests a major role of SK2 in the CyPPA-induced neuroprotective effect. The neuroprotective effect of CyPPA was similar to the neuroprotective effect of TNF-α whereas the effect of NS309 was much larger. This result may be explained by the possible neuroprotective effect of IK channel activation in NS309-treated neurons.

Based on our results we can only speculate on how increased SK2 channel expression can contribute to the neuroprotective effect elicited by TNF-α. However an SK2-mediated decrease in neuronal excitability is likely to be part of the mechanism. Indeed, the neuroprotective potential of SK2 has also been observed in an earlier study showing that overexpression of SK2 channels in cultured hippocampal neurons increases cellular survival against a kainic acid challenge by blunting the kainic acid-induced increase in excitability (Lee et al., 2003). We and others showed a colocalization of SK2 channels with NMDA receptor subunits in dendritic spines (Faber et al., 2005; Lin et al., 2008; Ngo-Anh et al., 2005). Glutamate treatment of neurons triggers ionotropic glutamate (e.g. NMDA, AMPA) receptor dependent activation which leads to excessive Ca\(^{2+}\) influx into the cell. SK2 channels can be activated by this Ca\(^{2+}\) influx. It was shown that the repolarizing effect of SK2 channel activity can oppose the depolarizing effect of AMPA receptor activity, favoring
Mg$^{2+}$ reblocking of NMDA receptors and thus reducing the Ca$^{2+}$ transient (Faber et al., 2005; Lin et al., 2008; Ngo-Anh et al., 2005). In this way SK2 channels may decrease glutamate-induced excitotoxicity. We hypothesize that increased SK2 channels expression/activity may lead to decreased susceptibility to glutamate-induced excitotoxicity via its coupling with NMDA receptors.

Our data correspond well with the results from Houzen and colleagues (Houzen et al., 1997) who reported that the neuroprotective effect of TNF-α against NMDA-induced toxicity can be attributed to an increase in outward potassium current (A-current) density in rat cortical neurons. The A-current is shown to be involved in the regulation of the neuronal firing rates in such a way that an increase in A-current density reduces cell excitability (Houzen et al., 1997). In addition, 4-aminopyridine (4-AP), a blocker of the potassium current, which can also inhibit the activation of all SK channel subtypes, reverted the observed TNF-α-mediated neuroprotection (Houzen et al., 1997). The increase in A-current density was inhibited by cycloheximide, a blocker of gene translation. Therefore, it was concluded that the effect of TNF-α might be mediated by de novo synthesis of channel protein itself and/or modulating proteins associated with the channel activities.

The observed increase in SK2 expression after TNF-α treatment is most likely mediated by NF-κB because BAY11-7082, a specific inhibitor of NF-κB, blocked the TNF-α mediated increase in SK2 channel levels. NF-κB might interact with two NF-κB binding sites, which were recently reported as regulatory parts of the murine SK2 promoter (Kye et al., 2007).

Altogether, the TNF-α/NF-κB pathway may recruit several downstream targets in order to produce neuroprotective effects (Mattson, 2005) and the present study identified SK2 as a central and novel candidate.

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References

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