Lovastatin induces neuroprotection through tumor necrosis factor receptor 2 signaling pathways

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Abstract

Statins are widely used as medication to lower cholesterol levels in human patients. In addition, it was recently reported that they can also reduce the incidence of stroke and progression of Alzheimer disease when prophylactically administered. To date there is only limited information available on how statins exert this beneficial effect. Therefore, we investigated the neuroprotective effect of lovastatin in primary cortical neurons. We found that lovastatin protects cortical neurons in a concentration-dependent manner against glutamate-mediated excitotoxicity. Interestingly, lovastatin with or without glutamate and/or tumor necrosis factor-alpha (TNF-α) increased TNF receptor 2 (TNF-R2) expression in cortical neurons. It was previously shown that activation of TNF-R2 signaling, which includes phosphorylation of protein kinase B (PKB)/Akt and activation of nuclear factor-kappa B (NF-κB), protects neurons against ischemic or excitotoxic insults.

To investigate if the lovastatin-induced neuroprotection is mediated by TNF-R2 signaling, primary cortical neurons were isolated from TNF-R1−/− or TNF-R2−/− mice. We could show that lovastatin is neuroprotective in TNF-R1−/− neurons, while protection is completely absent in TNF-R2−/− neurons. Furthermore, lovastatin-mediated neuroprotection led to an increase in PKB/Akt and NF-κB phosphorylation, whereas inhibition of PKB/Akt activation entirely abolished lovastatin-induced neuroprotection.

Thus, lovastatin induced neuroprotection against glutamate-excitotoxicity via activation of TNF-R2-signaling pathways.
5.1 Introduction

Hitherto, nine statins are available as pharmaceutical agents: lovastatin, mevastatin, simvastatin, pravastatin, fluvastatin, atorvastatin, rosuvastatin, pitavastatin and cerivastatin. Statins are commonly used as cholesterol lowering drugs (Endo, 1992). They inhibit 3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase, a key-regulating enzyme in cholesterol biosynthesis. Besides this well-known effect on cholesterol synthesis, statins exert several cholesterol-independent effects, including anti-thrombotic (Kunieda et al., 2003) and anti-inflammatory effects (Leung et al., 2003), stimulation of endothelial nitric oxide synthase (eNOS) (Harris et al., 2004; Hernández-Perera et al., 1998) and inhibition of inducible nitric oxide synthase (iNOS) (Vaughan and Delanty, 1999). They are also able to inhibit small G protein activation, such as activation of Rho, Ras and Rac by preventing their isoprenylation and thus their translocation from the inactive GDP-bound forms located in the cytoplasm to the active GTP-bound forms in the plasma membrane (Maltese, 1990; Seabra, 1998). Interestingly, clinical studies showed that statins slow the progression of mild-to-moderate Alzheimer’s disease (AD) in patients treated with these drugs as prophylactic agents (Sparks et al., 2006). These data were paralleled by the finding that statins markedly attenuate amyloid beta deposition in the PSAPP transgenic mouse model of Alzheimer’s amyloidosis (Petanceska et al., 2002).

During Alzheimer’s disease pathology, several pro-inflammatory cytokines are highly expressed in the brain (Perry et al., 2001). TNF-α initiates signaling cascades by binding to its receptors: TNF-R1 and TNF-R2 (Eisel et al., 2006; MacEwan, 2002a,b; Wajant et al., 2003). Although TNF receptors are barely detectable under basal conditions, we reported recently that the expression level of both TNF receptors is drastically increased after retinal ischemia/reperfusion injury (Fontaine et al., 2002). While TNF-R1 expression is modulated by post-translational shedding (Madge et al., 1999), TNF-R2 expression is mainly regulated by transcriptional processes (Hehlgans et al., 2001). We showed that in retinal-induced ischemia the TNF-R1-stimulated pathway promotes neuronal degeneration whereas activation of TNF-R2 signaling leads to neuronal survival (Fontaine et al., 2002). TNF-R2-associated neuroprotective effects are mediated by the activation of PKB/Akt (Fontaine et al., 2002) and phosphorylation of NF-κB (Marchetti et al., 2004).

In an initial experiment we found that lovastatin had a neuroprotective effect in a glutamate-induced excitotoxicity model using primary cortical neurons. Glutamate-mediated excitotoxicity is a major apoptosis-inducing event that occurs under several pathological conditions including Alzheimer’s disease (Hynd et al., 2004). Interestingly, in primary human endothelial cells (HUVEC) lovastatin treatment was shown to increase the expression of TNF-R2 proteins, without affecting TNF-R1 expression levels (Nübel et al., 2005). Therefore, we investigated whether lovastatin can also induce an increase in TNF-R2 expression levels in primary cortical neurons and whether activation of TNF-R2 signaling might be a mechanism that underlies the neuroprotective effect exerted by lovastatin.
5.2 Materials and methods

Materials. Neurobasal medium and B27 supplement were purchased from Invitrogen (Carlsbad, CA). LY294002 and lovastatin were bought from Calbiochem (San Diego, CA). Mouse TNF-α was purchased from HBT (Uden, The Netherlands). Primary antibodies used were a rabbit polyclonal antibody specific for PKB/Akt (9272, Cell Signaling, Danvers, MA, 1:2 000 dilution), anti-p-Akt (Ser473, 9271, Cell Signaling, 1:2 000 dilution), mouse monoclonal anti-TNF-R1 (8436, Santa Cruz Biotechnology, Santa Cruz, CA, 1:500 dilution), rabbit polyclonal anti-TNF-R2 (3727, Cell Signaling, 1:2000 dilution), mouse monoclonal anti-p-p65 (Ser536, 3036, Cell Signaling, 1:2 000 dilution), rabbit polyclonal anti-p65 (sc-372, Santa Cruz Biotechnology, Santa Cruz, CA, 1:2 000 dilution) and a monoclonal mouse antibody specific for actin (MP Biomedicals, Irvine, CA, 1:100 000 dilution). The neutralizing rat anti mouse TNF-α antibody V1q was provided by Werner Falk and was used at a final dilution of 2 µg/ml. The secondary antibodies used were alkaline phosphatase (AP)-conjugated goat anti-mouse (Applied Biosystems, Bedford, MA, 1:10 000 dilution) and AP-conjugated goat anti-rabbit (Applied Biosystems, 1:10 000 dilution). The chemi-luminiscence detection kit (Nitroblock II and CDP-Star) was purchased from Applied Biosystems. Complete mini protease inhibitor cocktail tablets were purchased from Roche-Diagnostics (Indianapolis, IN). All other materials were from Sigma.

Animal experiments. All experiments were performed using C57BL/6J (Harlan, Horst, The Netherlands), TNF-R1−/− or TNF-R2−/− mice (Erickson et al., 1994; Pfeffer et al., 1993; Rothe et al., 1993b). TNF-R1−/− or TNF-R2−/− mice were kept in a C57BL/6J background for more than ten generations. 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 neuronal culture. Primary cortical neurons were prepared from embryonic brains (E15-16) of C57BL/6J, TNF-R1−/− or TNF-R2−/− mice. The meninges were removed and 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 cortical 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 neuronal cultures were used for experiments.

Neurons were incubated over a period of 24 h with different concentrations of lovastatin and/or 100 ng/ml TNF-α. Lovastatin was dissolved in DMSO to a stock
concentration of 10 mM and stored at −20 °C until used. The final concentration of DMSO in all experiments was less than 0.5% (v/v). At this concentration DMSO had no effect on the survival of neuronal cultures. Incubation with PI3K inhibitor LY294002 was performed at a standard concentration of 25 µM and the NF-κB inhibitor BAY11-7082 was applied at 10 µM. Standard neurotoxic challenges were performed by incubating neuronal cultures for 1 h with 50 or 100 µM of glutamate. After treatment, medium was completely removed and fresh medium was added to the cells.

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

**Nuclear fractionation.** Cortical neurons treated in the absence and presence of lovastatin (10 µM) and/or mouse TNF-α (100 ng/ml) for 24 h were challenged by 100 µM glutamate for 1 h. After the glutamate treatment neurons were washed twice with ice cold phosphate-buffered saline (PBS), lysed in lysis buffer (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.05 % NP40 and complete mini protease inhibitor cocktail tablet (pH 7.9)) and centrifuged at 2000g for 10 min at 4 °C. The pellet was re-suspended in 5 mM HEPES, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol (v/v) (pH 7.9) and 300 mM NaCl. The samples were homogenized and centrifuged at 24000g for 20 min at 4 °C. The supernatant, which includes the nuclear fraction was aliquoted and stored before use at 70 °C.

**Protein analysis.** Primary cortical neurons from C57BL/6J, TNF-R1−/− and TNF-R2−/− mice, which were grown in a 6 well plate, were washed twice with ice cold phosphate-buffered saline (PBS) 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% Triton, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate and complete mini protease inhibitor cocktail). Samples were centrifuged at 9000g for 10 min at 4 °C and the supernatants boiled for 5 min in a Laemmli sample buffer. Protein samples (20 µg total) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a PVDF transfer membrane (Millipore Corporation, Billerica, MA) using a semi-dry blotting device (BioRad, Munich, Germany). Proteins were incubated with primary antibodies overnight at 4 °C, washed and subsequently the membranes were incubated with AP-conjugated secondary antibodies. Proteins were detected using the ECL detection system (Applied Biosystems) according to the manufacturer’s in-
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Instructions. Actin was used as an internal control to correct for variations in protein content.

Integrated optical densities (IOD) were measured using the Leica DFC 320 Image Analysis System (Leica, Cambridge, UK) and densitometric analysis was evaluated using the Leica Qwin program. IOD measurements were corrected for the background intensity. Levels of phosphorylated PKB/Akt were calculated as a ratio of Ser473 p-Akt versus total Akt. IOD values of non-treated controls were set as 100%.

Statistical analysis. Results shown are represented as ±SEM. Independent experiments were repeated at least three times. All statistical analyses were performed using 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.

5.3 Results

5.3.1 Lovastatin mediated neuroprotection against glutamate excitotoxicity.

Glutamate-induced excitotoxicity in cortical neurons is well established as a model mimicking the molecular pathways of neurodegeneration in Alzheimer’s disease or of the cerebral ischemic cell death (Marchetti et al., 2004; Hynd et al., 2004). We examined the effect of lovastatin on the viability of neurons after a glutamate challenge. Neurons were pre-treated for 24 h with different concentrations (0.1 \( \mu \)M, 1 \( \mu \)M, 10 \( \mu \)M, 100 \( \mu \)M) of lovastatin. Afterwards they were exposed to 50 \( \mu \)M glutamate for 1 h. At these concentrations glutamate alone induced cell death in approximately 60% of the neurons. Interestingly, lovastatin significantly decreased cell death elicited by glutamate. This neuroprotective effect could be detected even at concentrations as low as 0.1 \( \mu \)M (Fig. 5.1). Large but submaximal effects were observed at a concentration of 10 \( \mu \)M (Fig. 5.1). Therefore, this concentration was used for the following experiments.

5.3.2 Lovastatin alone or in combination with TNF-\( \alpha \) or glutamate induced the up-regulation of TNF-R2 but not of TNF-R1 in cortical neurons.

In HUVEC cells lovastatin induces the up-regulation of TNF-R2 expression, without affecting the TNF-R1 expression levels (Nübel et al., 2005). Activation of TNF-R2 signaling leads to increased cell survival against glutamate-induced excitotoxicity in neurons (Marchetti et al., 2004). Since lovastatin also has a neuroprotective effect in primary cortical neurons, we investigated whether lovastatin is able to alter TNF-R2 protein levels in neurons. Interestingly, lovastatin increased the expression of TNF-R2 protein (Fig. 5.2(b), lane 2). Furthermore, the combination of 10 \( \mu \)M lovastatin with 100 \( \mu \)M glutamate resulted in a two fold increase in the expression of TNF-R2.
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Figure 5.1: Lovastatin mediates neuroprotection in a concentration-dependent manner. Primary cortical neurons were pre-treated with the indicated lovastatin concentrations for 24 h and subsequently treated with 50 µM glutamate for 1 h. Using an MTT assay, neuronal viability was assessed 24 h later. Each bar represents the mean of 12 absorbance values from a single experiment. Experiments were repeated at least 3 times. Statistical analysis was performed using the Student–Neumann test with a 95% confidence interval followed by one-way ANOVA post-hoc LSD and Dunnett test using the SPSS program. Results shown are represented as ±SEM. *p values of < 0.05 versus glutamate-treated neurons were considered to be significant.

The involvement of TNF-R2 in lovastatin-mediated neuroprotection was confirmed in neurons isolated from TNF-R1−/− or TNF-R2−/− mouse embryo brains (Fig. 5.3(b) and (c)). Although these data cannot exclude an indirect effect due to the absence of one of the receptors, the finding that the selective up-regulation of TNF-R2 induces neuroprotection and that the selectivity of TNF-R2 signaling in neuroprotection was shown previously (Marchetti et al., 2004) by the treatment of wild-type neurons with TNF-R2 specific agonistic antibodies, makes this possibility highly unlikely. These neurons were treated with lovastatin (10 µM) or with lovastatin and TNF-α for 24 h. After the drug incubation, neurons were stimulated with glutamate (100 µM) for 1 h. In TNF-R1−/− neurons, lovastatin was able to rescue neurons from glutamate-mediated excitotoxicity, with or without TNF-α treatment (Fig. 5.3(b)). The neuroprotective
effect of treatment with lovastatin alone may be explained by activation of TNF-R2 signaling by endogenous TNF-α levels. However, in TNF-R2−/− neurons lovastatin with or without TNF-α treatment did not protect neurons against glutamate-induced excitotoxicity (Fig. 5.3(c)).

5.3.3 The neuroprotective effect of lovastatin is PKB/Akt and TNF-α dependent.

Previous studies of cellular survival pathways indicated that the activation of PKB/Akt by PI3K is one of the key steps involved in the induction of transcription factors involved in anti-apoptotic gene transcription (Marchetti et al., 2004; Gustin et al., 2004; Song et al., 2005). We have recently shown that TNF-α-induced neuroprotection against glutamate excitotoxicity in primary cortical neurons (Marchetti et al., 2004) is dependent on TNF-R2 activation and that this effect is associated with PI3K-dependent PKB/Akt phosphorylation (Fontaine et al., 2002) and NF-κB activation (Marchetti et al., 2004).

To check if the neuroprotective effect of lovastatin is also mediated by the PI3K pathway, cortical neurons were pre-treated with a PI3K inhibitor (LY294002) and then challenged with glutamate (Fig. 5.3). The neuroprotective effect elicited by pre-incubation with lovastatin with or without TNF-α was completely reverted by LY294002 treatment (Figs. 5.3(a) and (b)).

To investigate whether TNF-α was involved in the neuroprotective effect induced by lovastatin treatment we used TNF-α neutralising antibodies. Lovastatin-mediated neuroprotection was partially reverted when TNF-α neutralizing antibodies were incubated together with lovastatin for 24 h (Fig. 5.3(a), lane 5).

PKB/Akt becomes activated by phosphorylation at two different sites Thr308 and Ser473. Activation of PKB/Akt was evaluated as a ratio of phosphorylated-Akt-Ser473 (phospho-Akt)/ total PKB/Akt. We chose the serine site to determine the PKB/Akt activation, as this site is primarily linked to the PI3K pathway (Franke et al., 1997; Lawlor and Alessi, 2001). Analysis of PKB/Akt activation in wild-type neurons pre-treated with lovastatin revealed an increase in PKB/Akt phosphorylation (Fig. 5.4(a), lane 10). However, lovastatin-treated neurons subjected to a glutamate challenge showed a 2-fold enhanced PKB/Akt phosphorylation compared to non-treated neurons (Fig. 5.4(a), lane 2). Co-incubation of TNF-α with lovastatin led to a similar activation of PKB/Akt (Fig. 5.4(a), lane 4). Glutamate alone did not significantly alter PKB/Akt phosphorylation (Fig. 5.4(a), lane 11). However combining TNF-α with glutamate treatment did lead to an increase in PKB/Akt phosphorylation (Fig. 5.4(a), lane 6). The ability of LY294002 to reverse lovastatin-mediated neuroprotection (Figs. 5.3(a) and (b)) and to suppress PKB/Akt phosphorylation after lovastatin treatment (Fig. 5.4(a)) suggested that PKB/Akt activation is involved in the neuroprotective mechanism of lovastatin.

In TNF-R1−/− neurons lovastatin treatment alone did not significantly alter the basal levels of PKB/Akt phosphorylation (Fig. 5.4(b), lane 2). When lovastatin-
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Figure 5.2: Lovastatin increases TNF-R2 protein levels, without affecting TNF-R1. Primary cortical neurons were pre-treated with 10 μM lovastatin alone or with lovastatin and 100 ng/ml mouse TNF-α. Afterwards the cortical neurons were challenged with 100 μM glutamate for 1 h. Where indicated, neurons were pre-incubated with for 24 hours with 10 μM of the NF-κB inhibitor BAY11-7082. Immediately after the glutamate challenge, all neurons were lysed. Representative immunoblots are shown at the lower part of the quantified IOD of the (a) TNF-R1 and (b) TNF-R2 protein expression resulted from different experiments (n = 3, *p < 0.05 versus non-treated neurons).
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Figure 5.3: Lovastatin mediates neuroprotection in C57BL/6J, TNF-R1<sup>-/-</sup>, but not in TNF-R2<sup>-/-</sup> neurons. Primary cortical neurons from (a) C57BL/6J (wild-type), (b) TNF-R1<sup>-/-</sup> and (c) TNF-R2<sup>-/-</sup> mice were pre-incubated with 10 µM lovastatin and/or 100 ng/ml mouse TNF-α for 24 h. After the drug incubation, neurons were challenged with glutamate. Where indicated, neurons were also pre-incubated with 25 µM of the PI3K inhibitor LY294002. Neuronal viability was checked 24 h later after the glutamate challenge using an MTT assay. *p values of < 0.05 versus glutamate-treated neurons were considered to be significant.

treated TNF-R1<sup>-/-</sup> neurons were challenged with glutamate PKB/Akt phosphorylation was strongly increased (Fig. 5.4(b), lane 3). Lovastatin in combination with TNF-α led to a slightly stronger PKB/Akt phosphorylation in glutamate-challenged TNF-R1<sup>-/-</sup> neurons (Fig. 5.4(b), lane 4). Augmented PKB/Akt phosphorylation was also detected in TNF-R1<sup>-/-</sup> neurons treated with TNF-α and glutamate (Fig. 5.4(b), lane 5). Furthermore, LY294002 was able to revert the neuroprotection mediated by
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Figure 5.4: Lovastatin induces PKB/Akt activation in glutamate-challenged C57BL/6J and TNF-R1\(^{-/-}\) neurons. Primary cortical neurons were incubated with 10 \(\mu\)M lovastatin and/or 100 ng/ml mouse TNF-\(\alpha\) for 24 h and subsequently challenged with 100 \(\mu\)M glutamate for 1 h. Where indicated, neurons were also pre-incubated with 25 \(\mu\)M of the PI3K inhibitor LY294002. Immediately after the glutamate challenge all neurons were lysed and the protein lysates analyzed in a western blot. Phosphorylation of PKB/Akt was monitored using an anti-phospho PKB/Akt antibody (phosphorylated-AktSer473). Total cellular PKB/Akt served as loading control. Relative optical densiometric values of phosphorylated PKB/Akt from (a) C57BL/6J, (b) TNF-R1\(^{-/-}\) and (c) TNF-R2\(^{-/-}\) neurons are shown in the upper panels. Data is presented as percentage of each respective ratio between optical density value of phospho-Akt band intensity and optical density value of the matched Akt band intensity. *\(p\) values of < 0.05 versus non-treated neurons were considered to be significant.

Lovastatin in TNF-R1\(^{-/-}\) neurons (Fig. 5.3(b)).

In TNF-R2\(^{-/-}\) neurons PKB/Akt was not activated upon lovastatin treatment
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alone or in combination either with glutamate or TNF-α (Fig. 5.4(c)). Combining TNF-α with glutamate treatment decreased the basal PKB/Akt phosphorylation level (Fig. 5.4(c), lane 5).

5.3.4 Lovastatin increased TNF-R2-induced NF-κB activation.

Several studies have shown that both TNF-R1 and TNF-R2 signaling activate the canonical NF-κB pathway (Wajant et al., 2003; Marchetti et al., 2004). 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 (even persisting after 24 h of TNF-α treatment) (Marchetti et al., 2004). Furthermore, TNF-R2-induced NF-κB activation is dependent on PI3K-PKB/Akt stimulation, whereas TNF-R1-mediated NF-κB activation is not (Marchetti et al., 2004).

It was previously shown that the phosphorylated p65 subunit from nuclear fraction can be used as an indication for NF-κB activation (Wang et al., 2005). In our experiments, lovastatin altered basal NF-κB phosphorylation (Fig. 5.5, lane 2). In combination with glutamate it induced an even stronger phosphorylation of NF-κB (Fig. 5.5, lane 3). Co-incubation of lovastatin with TNF-α resulted in highest levels of p65 phosphorylation when neurons were also challenged with glutamate. (Fig. 5.5, lane 4).

As expected, the suppression of NF-κB phosphorylation by a specific inhibitor, BAY11-7082, reverted the lovastatin-mediated neuroprotective effect (data not shown). Interestingly, inhibition of NF-κB activation by BAY11-7082 prevented the up-regulation of TNF-R2 protein normally observed after lovastatin and glutamate treatment (Fig. 5.2(b)).

5.4 Discussion

In this study, we show that pre-treatment with lovastatin can protect cultured cortical neurons against glutamate-induced excitotoxicity. Glutamate-mediated excitotoxicity is a major apoptosis-inducing event that occurs under several pathological conditions including Alzheimer’s disease and cerebral ischemia (Marchetti et al., 2004; Hynd et al., 2004; Harkany et al., 2000; Mattson, 2004). Hitherto, only few studies reported neuroprotective mechanisms of statins against a glutamate challenge (Zacco et al., 2003; Lim et al., 2006). These studies described the effect of acute and chronic simvastatin treatment on glutamate-induced neuronal damage. Acute treatment with simvastatin inhibited the production and cytotoxicity of the final product of lipid peroxidation, 4-hydroxynonenal (HNE). Chronic treatment with simvastatin results in neuroprotection against glutamate-induced excitotoxicity by depletion of the cellular cholesterol pool (Zacco et al., 2003). Here we show, that lovastatin resistance against glutamate-induced excitotoxicity is elicited by activation of TNF-R2 signaling, which
Figure 5.5: Lovastatin treatment induces NF-κB activation. Cortical neurons were treated with 10 μM lovastatin and/or with 100 ng/ml mouse TNF-α for 24 h and, where indicated, subsequently challenged with 100 μM glutamate for 1 h. Protein lysates from the nuclear fractions were analyzed in a western blot. Phosphorylation of NF-κB was monitored by detection using an anti-phospho p65 antibody (phosphorylated p65 Ser536). *p values of < 0.05 versus non-treated neurons were considered to be significant.

includes signaling via PI3K-PKB/Akt and activation of NF-κB. However, from our study we can not exclude that these lovastatin-induced neuroprotective mechanisms are dependent on cholesterol depletion or inhibition of HNE.

The PKB/Akt pathway is known to be essential for several physiological processes, including insulin signaling, cell differentiation and cell survival (Lawlor and Alessi, 2001). Simvastatin-induced PKB/Akt translocation promotes activation of PKB/Akt by phosphorylation at Ser473, in endothelial cells (Kureishi et al., 2000), but not in smooth muscle cells (Skaletz-Rorowski et al., 2003). Our data showed that in cortical neurons, lovastatin treatment alone was able to activate PKB/Akt at Ser473. This activation was further increased when neurons were pre-incubated with a combination of lovastatin and TNF-α and afterwards stimulated with glutamate. In TNF-R1−/− neurons lovastatin also increased PKB/Akt activation when the neurons were stimulated with glutamate, whereas in TNF-R2−/− neurons lovastatin did not alter the basal PKB/Akt phosphorylation. The ability of LY294002, an inhibitor of PKB/Akt, to suppress the PKB/Akt phosphorylation and lovastatin-induced neuroprotection, confirmed the importance of PKB/Akt activation in lovastatin-mediated neuroprotection. Furthermore, LY294002 was able to inhibit the up-regulation of TNF-R2 expression in lovastatin and glutamate-challenged neurons. Together these data suggest that lovastatin-mediated neuroprotection is dependent on the activation of PKB/Akt by TNF-R2 signaling pathways.
In HUVEC cells, lovastatin is able to specifically up-regulate the expression of TNF-R2, but not of TNF-R1 (Nübel et al., 2005). Here, we showed that lovastatin together with glutamate was also able to selectively increase TNF-R2 protein levels without affecting the expression of TNF-R1 in cortical neurons. Sequence analyses of the 5’ non-coding region of the two TNF-α-receptors indicated that the TNF-R1 gene is a non-inducible gene (Rothe et al., 1993a). However, under several circumstances, such as a retinal ischemia its expression is strongly, but transiently and locally, up-regulated (Fontaine et al., 2002). While TNF-R1 expression is mainly regulated by post-translational processes, TNF-R2 is believed to be controlled by transcriptional factors such as NF-κB (Rasmussen et al., 2001; Santee and Owen-Schaub, 1996).

NF-κB is also a key regulator in apoptotic and anti-apoptotic signal transduction cascades. It is considered to play an important role in neuroprotection (Mattson and Camandola, 2001). As an example, the cellular inhibitors of apoptosis proteins (cIAPs), well-known NF-κB-target gene(s) upregulated by TNF-α, have been recently identified as facilitators in regulating apoptosis sensitivity within neurons (Yang et al., 2005). Its pleiotropic effects are mostly attributed to a large variety of upstream stimuli and its effects are dependent on the specific cell type (Gustin et al., 2004). Although in astrocytes or in tumor cells activation of NF-κB leads to apoptotic events, NF-κB can promote either cell death or cell survival in cortical neurons (Marchetti et al., 2004; Mattson and Camandola, 2001; Grilli et al., 1996). Several studies have shown that a long lasting TNF-α treatment resulted in neuroprotection against glutamate excitotoxicity or oxidative injury (Cheng et al., 1994; Barger et al., 1995; Bruce et al., 1996) in a NF-κB-dependent manner (Marchetti et al., 2004). This persistent NF-κB activation was induced by TNF-R2 signaling (Marchetti et al., 2004). Several studies carried out in vivo and in different cell lines revealed the ability of statins to modulate NF-κB activity (Gnad et al., 2001; Nübel et al., 2004; Sironi et al., 2006). They can suppress NF-κB activity by the inhibition of small GTPases of the Rho family (Maltese, 1990; Seabra, 1998). In contrast, Lim and colleagues demonstrated that acute treatment with simvastatin inhibits the lipid peroxidation product HNE, which results in NF-κB activation (Lim et al., 2006). In our study, we observed that lovastatin treatment increased NF-κB phosphorylation by the activation of PKB/Akt. Furthermore, BAY11-7082, an NF-κB inhibitor was able to inhibit the up-regulation of TNF-R2 expression in glutamate-challenged neurons pre-incubated with lovastatin. Thus, lovastatin-induced NF-κB activation was involved in the up-regulation of TNF-R2 protein. Treatment of glutamate challenged cortical neurons with lovastatin and a TNF-α neutralizing antibody (V1q) (Echternacher et al., 1990) resulted in a substantially reduced neuroprotective effect as compared to lovastatin treatment alone. This effect suggests that endogenous TNF-α and here most likely the membrane bound form of TNF-α in an auto- or juxtatropic way may be involved in stimulating TNF-R2 upon lovastatin treatment and that this effect plays an important role in lovastatin mediated neuroprotection. A similar effect of endogenous and protective TNF-α expression has been observed and described in an earlier study.
The present study describes a novel neuroprotective mechanism of statins. Lovastatin was shown to exert a neuroprotective effect via the activation of the TNF-R2 signaling pathway. TNF-R2 activation increased PI3K-PKB/Akt and NF-κB phosphorylation and activation. Interestingly, NF-κB activation in turn led to increased TNF-R2 expression suggesting a positive feedback loop between lovastatin-induced NF-κB activation and TNF-R2-mediated neuroprotective signaling. Taken together these findings add to the known effects of lovastatin another signaling pathway potentially explaining some of the neuroprotective effects that statins have shown in several clinical studies on Alzheimer’s disease, stroke and even in Multiple Sclerosis (Sparks et al., 2006; Sacks et al., 1996; Vollmer and Singh, 2004).

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