NEUROPROTECTION BY TRANSFORMING GROWTH FACTOR-β1 INVOLVES ACTIVATION OF NUCLEAR FACTOR-κB THROUGH PHOSPHATIDYLINOSITOL-3-OH KINASE/AKT AND MITOGEN-ACTIVATED PROTEIN KINASE-EXTRACELLULAR-SIGNAL REGULATED KINASE1,2 SIGNALING PATHWAYS

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Abstract—**Prevention of neuronal apoptosis has been introduced as a new therapeutic strategy for neurodegenerative disorders. We have previously reported anti-apoptotic effects of transforming growth factor-β1 (TGF-β1), a multifunctional cytokine, in models of cerebral ischemia and in cultured neurons and recently focused on the mechanisms underlying the anti-apoptotic effect of TGF-β1. The anti-apoptotic transcriptional factor nuclear factor kappa B (NF-κB) shows high impact in the cell survival function of multiple cytokines and growth factors. The present study explored whether NF-κβ is a target of TGF-β1 and which signaling pathways involved in the activation of NF-κβ are triggered by TGF-β1. We demonstrated that TGF-β1 increased the transcriptional activity of NF-κβ in cultured hippocampal neurons in a time- and concentration-dependent manner. Furthermore, TGF-β1 induced translocation of p65/NF-κβ to the nucleus and enhanced NF-κβ transcriptional activity in the presence of apoptotic stimuli. TGF-β1-mediated NF-κβ activation was blocked by wortmannin and U0126, indicating the involvement of both phosphatidylinositol-3-OH kinase (PI3k)/Akt and mitogen-activated protein kinase (MAPK)/extracellular-signal regulated kinase (Erk)1,2 pathways in the action of TGF-β1. TGF-β1 produced a concomitant increase in the phosphorylations of IκB kinase (IKKα/β) and IκBα with a subsequent degradation of IκBα. Interestingly, the increased phosphorylation of IKKα/β and IκBα was abrogated by wortmannin, but not by U0126, suggesting that PI3k/Akt and MAPK/Erk1,2 pathways triggered by TGF-β1 regulated the activation of NF-κβ through different mechanisms. Of note, wortmannin and U0126, as well as κβ-decoy DNA, abolished the anti-apoptotic effect of TGF-β1, corroborating the notion that both PI3k/Akt and MAPK/Erk1,2 pathways, and NF-κβ activity are necessary for the anti-apoptotic activity of TGF-β1. © 2004 IBRO. Published by Elsevier Ltd. All rights reserved.**

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Abbreviations: BSA, bovine serum albumin; E, embryonic day; Erk, extracellular-signal regulated kinase; IκB, inhibitor of NF-κB; IKK, IκB kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PI3k, phosphatidylinositol-3-OH kinase; RLU, relative light unit; STS, staurosporine; TBS, Tris-buffered saline; TGF-β1, transforming growth factor-β1.

Transforming growth factor-β1 (TGF-β1) is a cytokine capable of modulating multiple cellular processes (Mattson et al., 1997; Flanders et al., 1998; Krieglstein et al., 2002). We and others have demonstrated the neuroprotective activity of TGF-β1 after cerebral injuries (Henrich-Noack et al., 1996; Pang et al., 2001; Ruocco et al., 1999; Tyor et al., 2002; Zhu et al., 2002) and in cultured neurons after various stimuli (Prehn et al., 1996; Ren and Flanders, 1996). The neuroprotective mechanism of this cytokine has been coupled to the transcriptional induction of plasminogen activator inhibitor-1 (Buisson et al., 1998; Docagne et al., 2002), the regulation of the Ca^{++} homeostasis, the induction of Bcl-2 and Bcl-xl (Prehn et al., 1994), and the inhibition of Caspase 3 activation (Zhu et al., 2001). More recently, we have demonstrated that TGF-β1 inactivates the proapoptotic protein Bad via activation of mitogen-activated protein kinase (MAPK)/extracellular-signal regulated kinase (Erk)1,2 pathway (Zhu et al., 2002). Accumulating evidence indicates that TGF-β1 activates multiple pathways including MAPK and phosphatidylinositol-3-OH kinase (PI3k)/Akt signaling pathways (Xiao et al., 2002; Yu et al., 2002) besides the classic receptor-activated Smad signaling.

The transcription factor nuclear factor-κB (NF-κB) regulates various genes involved in immunoresponses, cell proliferation and apoptosis. The activity of NF-κB is tightly controlled by a family of inhibitory proteins termed inhibitor of NF-κB (IκBs) including IκBα, IκBβ and IκBε as the most prominent members (Whiteside and Israel, 1997). The central paradigm of NF-κB activation has been linked to the dissociation of IκB from the IκB–NF-κB complex and the subsequent translocation of liberated NF-κB from the cytoplasm to the nucleus. This process requires the advanced phosphorylation and the subsequent degradation of IκB (Woronicz et al., 1997). The phosphorylation of IκB is mostly regulated by a protein kinase complex IκB kinase (IKKs) that consists of two catalytic subunits (IKKα and IKKβ) and the regulatory subunit IKKγ (Yamaoka et al., 1998). IKKα and IKKβ target serine residues of IκBα (Ser32/Ser36) and IκBβ (Ser19/Ser23). Recent attempts to identify the upstream kinase of IKK have revealed an involvement of MEK kinase 1 (MEKK1) (Nemoto et al., 1998), PI3k/Akt (Kane et al., 2002) and NF-κB-inducing kinase (Woronicz et al., 1997).

NF-κB triggers a number of anti-apoptotic genes which interrupt the apoptotic cascade at multiple levels (Mattson

et al., 2000; Karin and Lin, 2002; Wu, 2003), and a pivotal role of NF-κB in the regulation of cell survival and death has therefore been suggested. Although findings reported are controversial, strong evidence supports the notion that NF-κB functions as an anti-apoptotic transcription factor in various cell populations including neurons (Kaltschmidt et al., 1999; Glazner et al., 2000; Mattson et al., 2000).

The role of TGF-β1 in activation of NF-κB has been studied predominantly in non-neuronal populations and results appear diverse depending on the cell type and the experimental conditions (Chang, 2000; Arsura et al., 2003). Little is known about the effect of TGF-β1 on neuronal cells in this regard. In the present study, we attempted to elucidate whether TGF-β1 triggered signaling pathways leading to activation of NF-κB in cultured hippocampal neurons, and furthermore, whether NF-κB activation contributes to the anti-apoptotic effect of TGF-β1.

EXPERIMENTAL PROCEDURES

Neonatal rat and embryonic mouse hippocampal cultures

Animal care followed official governmental guidelines. All experimental procedures involving animals were approved by the government ethics committee, and all efforts were made to minimize suffering and number of the rats. Hippocampal cultures were prepared from neonatal Fischer 344 rats (P1–P2; Zhu et al., 2001) or from κB-luciferase reporter mice on embryonic day 14 (E14; Culmsee et al., 2002). Briefly, the isolated hippocampi were dissected and incubated at 37 °C for 20 min in Leibovitz's L15 medium supplemented with 1 mg/ml papain and 0.2 mg/ml bovine serum albumin (BSA). Thereafter, the cell suspension was layered onto growth medium containing 1% trypsin inhibitor plus 10% BSA and then centrifuged at $200 \times g$ for 10 min. The pellet was resuspended and seeded at a density of 2×10^4 cells/cm^2 into poly-L-lysine-coated Petri dishes and cultured in a humidified atmosphere (5% CO_2 and 95% air at 37 °C) in neurobasal medium supplemented with 0.5 mM glutamine, B27 supplement as well as 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Karlsruhe, Germany). Experiments were performed 7–8 days after the preparation. At that time there were about 60% neurons and 40% astrocytes in rat hippocampal cultures, whereas more than 95% cells were neurons in mouse hippocampal cultures.

Cell treatment

TGF-β1 (R&D Systems, Wiesbaden, Germany) was administered to the cells on days 7–8 of the culture. To induce apoptosis, staurosporine (STS) was added to the cells at a final concentration of 100 nM in the absence of TGF-β1 or 24 h after the onset of TGF-β1 treatment. To block NF-κB activation, double-strand oligonucleotide with a specific NF-κB-binding consensus sequence (decoy, 5′-AGTTGAGGGACTTTCCCAGGC-3′; MWG-Biotech AG, Munich, Germany) was added to the culture medium at the final concentration of 5 μM 2 h before and remained in the medium after the onset of TGF-β1 treatment. As a control, the single-base mutated double-strand oligonucleotide (mismatch, 5′-AGTT-GAGCGACTTTCCCAGGC-3′) was given to the medium at the same concentration. To block the activation of Erk1,2 or Akt, U0126 (Cell Signaling Technology, Frankfurt, Germany) or wortmannin (Calbiochem, Bad Soden, Germany) at a final concentration of 20 μM or 60 nM, respectively, was applied to the cultures 2 h prior to the TGF-β1 treatment. Control cultures received vehicle only.

Western blotting

Cells were harvested in lysis buffer containing 10% glycerol, 3% SDS, 0.5 M Tris, 1 mM phenylmethylsulfonyl fluoride, 1 μM calpain inhibitor I and 7 μg/ml trypsin inhibitor. Protein was measured using a BCA kit (Perbio Science, Bonn, Germany). Samples containing an equal amount of total protein were loaded on 12.5% or 15% SDS-polyacrylamide gels. Following electrophoresis, the proteins were transferred onto a nitrocellulose membrane. The protein transfer was controlled by staining the membrane with ponceau S. Unspecific binding was blocked by a buffer containing 0.1% Tween-20, 2% BSA and 5% non-fat dry milk in Tris-buffered saline (TBS). The blots were then incubated with primary antibodies diluted in blocking buffer overnight at 4 °C. The following antibodies were used in the present study: rabbit anti-phospho-p44/42 MAPK (Thr202/Tyr204, P-Erk1/2; 1:200), rabbit anti-phospho-Akt (Ser473, P-Akt, 1:1000), rabbit anti-phospho-IκBα (Ser32, P-IκBα; 1:1000) and rabbit anti-phospho-IKKα (Ser181)/IKKβ (Ser180) (P-IKKα/β; 1:1000) were purchased from Cell Signaling Technology. Rabbit anti-IκBα and anti-IKKα/β were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). α-Tubulin was detected by using a mouse anti-α-tubulin (1:5000; Sigma, Deisenhofen, Germany) to control the amount of protein loaded on each lane of the gel. After washing the membranes with 0.1% Tween-20 in TBS, the blots were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:2500; Amersham Biosciences, Freiburg, Germany) at room temperature for 1 h. Peroxidase activity was detected by the ECL detection system (Amersham Biosciences, Braunschweig, Germany).

Immunocytochemistry

Cells were fixed with methanol for 20 min at −20 °C. After washing with ice-cold phosphate-buffered saline (PBS), cells were incubated with a blocking buffer containing 2% BSA and 10% normal goat serum for 1 h at 37 °C. Rabbit anti-p65/NF-κB antibody (1:100; Roche Molecular Biochemicals, Mannheim, Germany) was applied to cells and incubated at 4 °C overnight. For negative control, cells were incubated with the blocking buffer omitting the primary antibody. After washing the cells with PBS, biotin-conjugated anti-rabbit IgG was added to the cells followed by incubation with fluorescein avidin D. The cells were finally mounted with a mounting medium (Dako, Hamburg, Germany) and analyzed by confocal laser scanning microscopy (Zeiss, Jena, Germany). The number of p65-nuclear positive cells was counted in four different areas with the size 326×326 μm. Five Petri dishes were used in each group.

Luciferase activity assay

Luciferase activity was measured in protein extracts from cultured hippocampal neurons prepared from E14 κB-luciferase reporter mice using a luciferase detection kit according to the manufacturer's protocol (Promega, Mannheim, Germany). Briefly, Cells were harvested in an ice-cold luciferase reporter lysis buffer. The homogenates were centrifuged at $15,000 \times g$ for 5 min at 4 °C. The protein content in the supernatants was determined using a BCA kit. Equal amount of protein from the extracts was mixed with 100 μl of luciferase assay buffer containing the luciferase substrate and ATP. Luminescence in this mixture was immediately detected over 60 s and expressed as relative luciferase units (RLU).

Nuclear staining

Cells were fixed with methanol and incubated with Hoechst 33258 (10 μg/ml; Sigma, Deisenhafen, Germany) at 37 °C for 10 min followed by washing with methanol and PBS. Thereafter, the nuclear morphology was analyzed under a fluorescence micro-

scope. Cells showing condensed chromatin or fragmented nuclei were counted as apoptotic cells.

Statistics

Data were presented as means±S.D. Statistical analysis of apoptotic damage was evaluated by analysis of variance test followed by Scheffé-test. Student's *t*-test was used to analyze the changes of RLU between each matched control and corresponding TGF-β1-treated group. $P<0.05$ was considered significant.

RESULTS

TGF-β1 mediates a time- and concentration-dependent activation of NF-κB in cultured mouse hippocampal neurons

To evaluate the effect of TGF-β1 on activation of NF-κB, the transcriptional activity of NF-κB was evaluated by luciferase activity assay in cultured hippocampal neurons prepared from E14 κB-luciferase reporter mice. The time course was established using 10 ng/ml of TGF-β1 (Fig. 1A), the most neuroprotective concentration established previously (Zhu et al., 2001). A significant increase in the luciferase activity was detected as early as 3 h after TGF-β1 treatment ($P<0.05$), which was similar to that caused by the positive control TNFα at the same time point ($P<0.05$). Luciferase activity was further enhanced 3.5-fold ($P<0.01$) and 3.8-fold ($P<0.01$) at 6 h and 24 h, respectively, after adding TGF-β1 (Fig. 1A). Western blot analysis of luciferase protein levels using the same probes confirmed this time course (Fig. 1B). According to this time course, a concentration-dependency study was carried out at 6 h after the onset of the treatment using the TGF-β1 concentration at the range 0.1–10 ng/ml. Approximate 2.1-fold ($P<0.05$), 3.1-fold ($P<0.01$) and 3.9-fold ($P<0.01$) increases in luciferase activity were detected in the extracts of cultured hippocampal neurons treated with 0.1, 1.0 and 10 ng/ml of TGF-β1, respectively.

Since we have previously shown that TGF-β1 protected cultured hippocampal neurons from STS-induced apoptosis, it was of interest whether TGF-β1 could activate NF-κB in the presence of apoptotic inducer STS. Luciferase activity was thus measured in the extracts of hippocampal neurons treated with STS alone or a combination of TGF-β1 with STS. We found that luciferase activity was not influenced at 3 h and 6 h, but moderately decreased at 24 h after challenging with STS ($P<0.05$). TGF-β1 (10 ng/ml) administered to the cultures 24 h prior to the STS treatment not only prevented the inhibitory effect of STS but also further markedly increased the transcriptional activity of NF-κB at all tested time points ($P<0.01$; Fig. 2A). In support, immunocytochemistry with the antibody against the p65 subunit of NF-κB revealed a significant enhanced nuclear location in TGF-β1-treated cells as compared with controls or with STS-treated cells ($P<0.05$; Fig. 2B–c). As a positive control, TNFα also significantly increased the nuclear translocation of p65/NF-κB. These results indicate that TGF-β1 is capable of activating NF-κB under normal culture conditions as well as in the presence of STS.
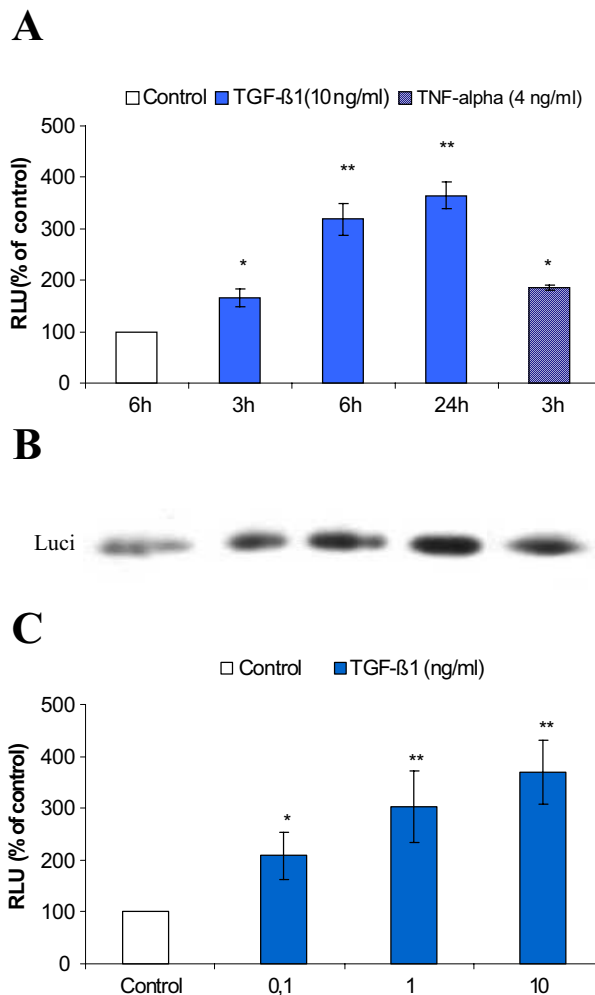
Fig. 1. Time- and concentration-dependent activation of NF-κB by TGF-β1 in cultured hippocampal neurons. A and B: Time course of NF-κB activation. TGF-β1 at a final concentration of 10 ng/ml was added to the media of hippocampal cultures prepared from E14 mouse κB-luciferase-reporter mice. Control cultures received vehicle only. The proteins were extracted at the indicated time points for both luciferase activity assay (A) and Western blotting analysis (B) of luciferase (Luci) protein levels. C: Concentration-dependent activation of NF-κB by TGF-β1. Cultured mouse hippocampal cells were treated with different concentrations of TGF-β1 (0.1, 1.0 and 10 ng/ml) or vehicle, and the cells were harvested 6 h later for luciferase activity assay. * $P<0.05$ and ** $P<0.01$ compared with the control.

Activation of NF-κB contributes to the anti-apoptotic effect of TGF-β1

After demonstrating the capacity of TGF-β1 on activation of NF-κB, we were interested in the role of NF-κB activation in the neuroprotection by TGF-β1. To address this issue, the effect of TGF-β1 on neuronal apoptosis induced by STS was evaluated in both cultured rat (Fig. 3A) and mouse (Fig. 3B) hippocampal cells in the presence and absence of κB-decoy DNA which specifically blocks the activation of NF-κB. As a control, a mismatch DNA was applied to the cultures at the same concentration. In both cultures, neither κB-decoy DNA nor mismatch DNA alone showed any toxicity at the tested concentration (5 μM),
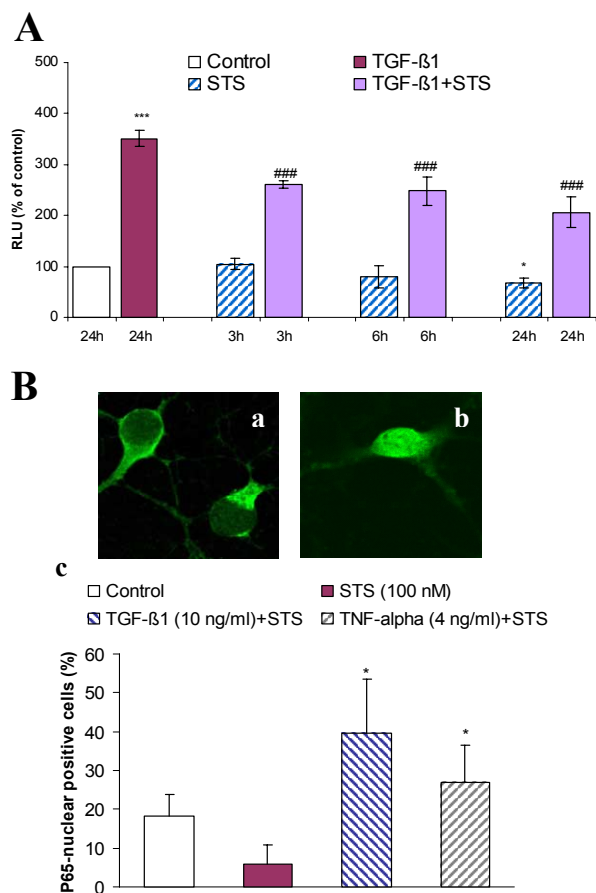
A

B

Fig. 2. TGF-β1 preserves NF-κB activity in neurons challenged with STS. A: TGF-β1-induced transcriptional activity of NF-κB in the presence of STS. Cultured mouse hippocampal cells received vehicle or STS (100 nM) alone, or 10 ng/ml TGF-β1 24 h prior to challenging with STS. The cells were harvested at different time points after adding STS for luciferase activity assay. $*$ $P<0.05$ and $***$ $P<0.001$ compared with control. $\#\#\#$ $P<0.001$ compared with the corresponding group treated with STS alone. RLU: relative luciferase unit. B: Induction of nuclear translocation of p65/NF-κB by TGF-β1 in cultured rat hippocampal cells. Cells were similarly treated as described above. As a positive control, TNF-α (final concentration: 4 ng/ml) was added to the cultures 3 h before the onset of STS treatment. Immunocytochemistry of p65/NF-κB was carried out 6 h after challenging with STS. In control, most of cells exhibited location of p65/NF-κB immunoreactivity in cytoplasm (B–a). Cells with positive p65/NF-κB immunoreactivity in the nucleus were counted as p65/NF-κB-nuclear positive cells (B–b). The number of p65/NF-κB-positive cells was presented as the percentage of total number of cells (B–c). Cells in four different areas (326×326 μm each) per dish and five dishes per group were analyzed. $*$ $P<0.05$ compared with STS alone.

whereas STS (100 nM) caused about 55% of cells undergoing apoptotic death as revealed by Hoechst 33258 nuclear staining ($P<0.001$). TGF-β1 (10 ng/ml) significantly protected cultured rat and mouse hippocampal neurons from STS-induced apoptotic damage ($P<0.001$ and $P<0.05$, respectively). Notably, this protective effect was abolished in both cultures by κB-decoy DNA, but not by the mismatch DNA, suggesting that activation of NF-κB contributed to the anti-apoptotic effect of TGF-β1. To confirm that the blockage of the anti-apoptotic effect of TGF-β1 by κB-decoy DNA was based on the inhibition of NF-κB acti-

vation, luciferase activity was detected in sister cultures (Fig. 3C). Again, TGF-β1 (10 ng/ml) alone significantly increased luciferase activity 24 h after the treatment ($P<0.05$), and this increase was completely blocked by κB-decoy DNA, but not by mismatch DNA, given to the cultures 2 h before the TGF-β1 treatment, indicating that κB-decoy DNA specifically and sufficiently blocked TGF-β1-mediated NF-κB activation under those experimental conditions. These results indicate a crucial role of NF-κB activation in the neuroprotective mechanism of TGF-β1.

TGF-β1 increases phosphorylation and subsequent degradation of IκBα

We next attempted to reveal how TGF-β1 mediated the activation of NF-κB in cultured neurons. Phosphorylation and the subsequent degradation of IκB are suggested to be key steps in the process of NF-κB activation. Therefore, we detected P-IκBα and IκBα by Western blotting in parallel in neonatal rat hippocampal cultures at different time points after the onset of TGF-β1 treatment. As shown in Fig. 4A, an increase in P-IκBα level was detected as early as 3 h, and remained at the higher level from 6 h to 24 h after adding TGF-β1. In contrast, expression of IκBα fell to levels below the baseline after incubation with TGF-β1 (Fig. 4A). The concentration-dependency study showed that an increase in P-IκBα and a decrease in IκBα were already seen with 0.1 as well as with 1.0 and 10 ng/ml of TGF-β1 (Fig. 4B), which was consistent with the results showing enhanced NF-κB activity in the luciferase activity assay (Fig. 1C). The effects of TGF-β1 on phosphorylation and degradation of IκBα were also studied in the presence of STS. As a broad spectrum kinase inhibitor, STS clearly suppressed the phosphorylation of IκBα, whereas IκBα expression was enhanced by STS in comparison with the controls, suggesting a reduced degradation of IκBα. Interestingly, the inhibition of IκBα phosphorylation mediated by STS was prevented by TGF-β1, and as a consequence, the degradation of IκBα was increased in the presence of TGF-β1 (Fig. 4C). These data suggest that TGF-β1-mediated activation of NF-κB involves the phosphorylation and the degradation of IκBα.

TGF-β1-mediated activation of NF-κB is blocked by Mek1,2/Erk1,2 and PI3k inhibitors

There are multiple signaling pathways leading to the phosphorylation of IκB and the activation of NF-κB. In the present study, we investigated whether MAPK/Erk1,2 and PI3k/Akt pathways were involved in the activation of NF-κB by TGF-β1. The transcriptional activity of NF-κB was evaluated by luciferase activity assay in cultured mouse hippocampal neurons in the presence or absence of specific inhibitors of Mek1,2/Erk1,2 and PI3k after the administration of TGF-β1. As shown in Fig. 5A, an increase in luciferase activity was significantly reproduced at 3 h ($P<0.05$) and more pronouncedly seen at 6 h ($P<0.001$) after adding TGF-β1 (10 ng/ml). Interestingly, both wortmannin and U0126 reduced not only the basal level of luciferase activity, but also abrogated the enhancement of luciferase activity in response to TGF-β1. It was noted that the inhibitory
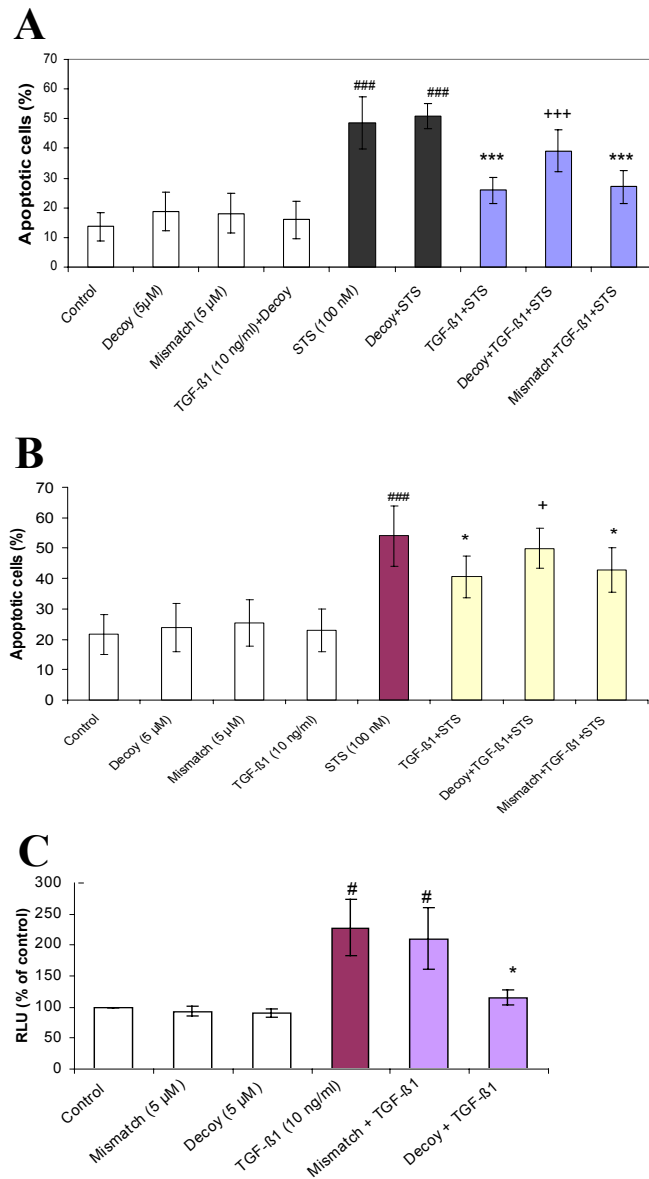
Fig. 3. Pivotal role of NF-κB activity in the anti-apoptotic effect of TGF-β1 in cultured mouse hippocampal neurons. A and B: κB-Decoy DNA diminished the anti-apoptotic effect of TGF-β1 in cultured rat (A) and mouse (B) hippocampal cells. κB-Decoy DNA or mismatch DNA at the final concentration 5 μM were given to the cultures 2 h before and remained in the media up to the onset of TGF-β1 treatment. Cells were challenged with STS (100 nM) 24 h after adding TGF-β1. Controls received vehicle only. Apoptotic damage was evaluated by the nuclear staining with Hoechst 33258 24 h after the exposure of STS. Cells exhibiting condensed or fragmented nuclei were counted as apoptotic cells. ### $P<0.001$ compared with the control; * $P<0.05$ and *** $P<0.001$ compared with STS alone; + $P<0.05$ and +++ $P<0.001$ compared with TGF-β1 plus STS. C: κB-Decoy DNA blocked TGF-β1-mediated NF-κB activation in cultured mouse hippocampal cells. κB-Decoy DNA, mismatch DNA and TGF-β1 were added to the cultures as described above. Cells were harvested 24 h after administration of TGF-β1 for the measurement of luciferase activity. # $P<0.05$ compared with the control; * $P<0.05$ compared with TGF-β1 alone.

effect of wortmannin appeared predominantly stronger than that caused by U0126 at both tested time points after adding TGF-β1. In addition, the levels of P-Erk1,2 and P-Akt were detected in sister cultures exposed to U0126 or wortmannin in order to clarify whether the TGF-β1-mediated increase in NF-κB transcriptional activity depended on the activation of MAPK/Erk1,2 or PI3k/Akt signaling pathways. TGF-β1 (10 ng/ml) increased the levels of P-Erk1,2 and P-Akt at both 3 h and 6 h after the treatment without altering the expression of total Erk1,2 or Akt. TGF-

β1-mediated activation of Erk1,2 was completely abolished by U0126 (Fig. 5B). The increased level of P-Akt by TGF-β1 also clearly declined in the presence of wortmannin in comparison with the baseline and the levels detected after TGF-β1 treatment (Fig. 5C). This evidence supports the notion that TGF-β1-mediated activation of NF-κB in cultured hippocampal neurons involves both MAPK/Erk1,2 and PI3k/Akt signaling pathways.

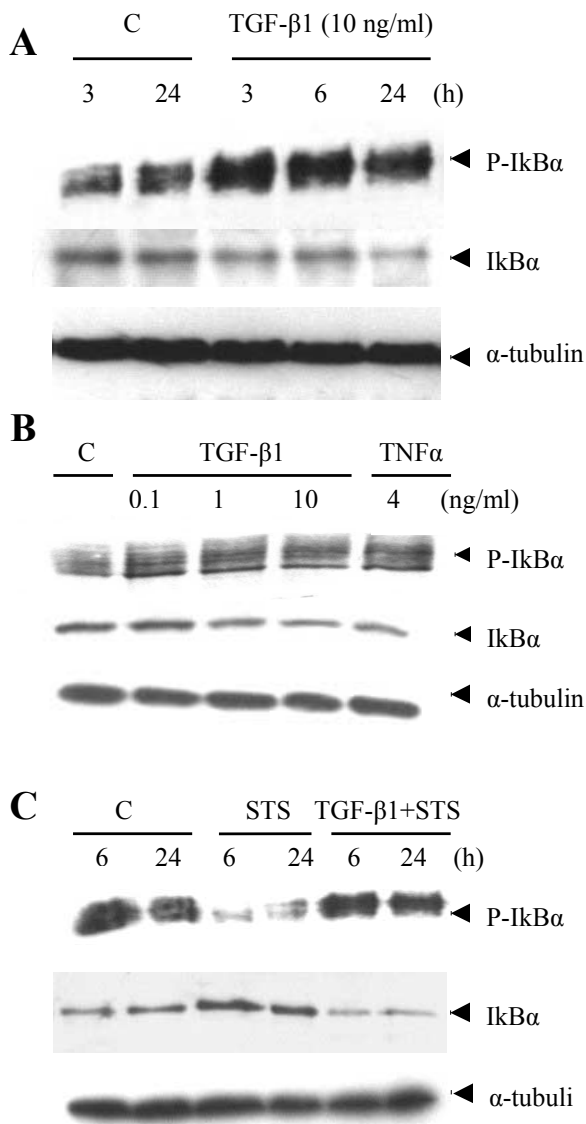Based on these findings, we next addressed whether the increased phosphorylation of Erk1,2 and Akt by

Fig. 4. TGF-β1 increases phosphorylation and degradation of IκBα in cultured neonatal rat hippocampal cells in the absence and presence of STS. A: Time course of phosphorylation and degradation of IκBα. Cells were harvested at the indicated time after adding TGF-β1 (10 ng/ml) for Western blotting of P-IκBα and IκBα. Controls (C) received the vehicle only. B: Concentration-dependent effect of TGF-β1 on IκB phosphorylation and degradation. TGF-β1 was administered to cells at concentrations of 0.1, 1.0 and 10 ng/ml. TNFα (final concentration: 4 ng/ml) was used as a positive control. The cells were harvested 6 h after the treatment with TGF-β1 or TNFα. C: TGF-β1 prevented STS-mediated decrease in phosphorylation and degradation of IκBα. Cells were challenged with STS (100 nM) 24 h after TGF-β1 treatment. Proteins were extracted from the cells 6 h and 24 h after the onset of STS treatment for Western blotting. α-Tubulin was detected to control the amount of protein loaded in each lane of the gel.

TGF-β1 was associated with the enhanced phosphorylation of IKKα/β and IκBα. As shown in Fig. 6, TGF-β1 (10 ng/ml) increased the levels of P-Erk1,2 and P-Akt, which was accompanied by an enhanced expression of P-IKKβ and P-IκBα. P-IKKα was detected at a very low level in cultured rat hippocampal cells and was slightly
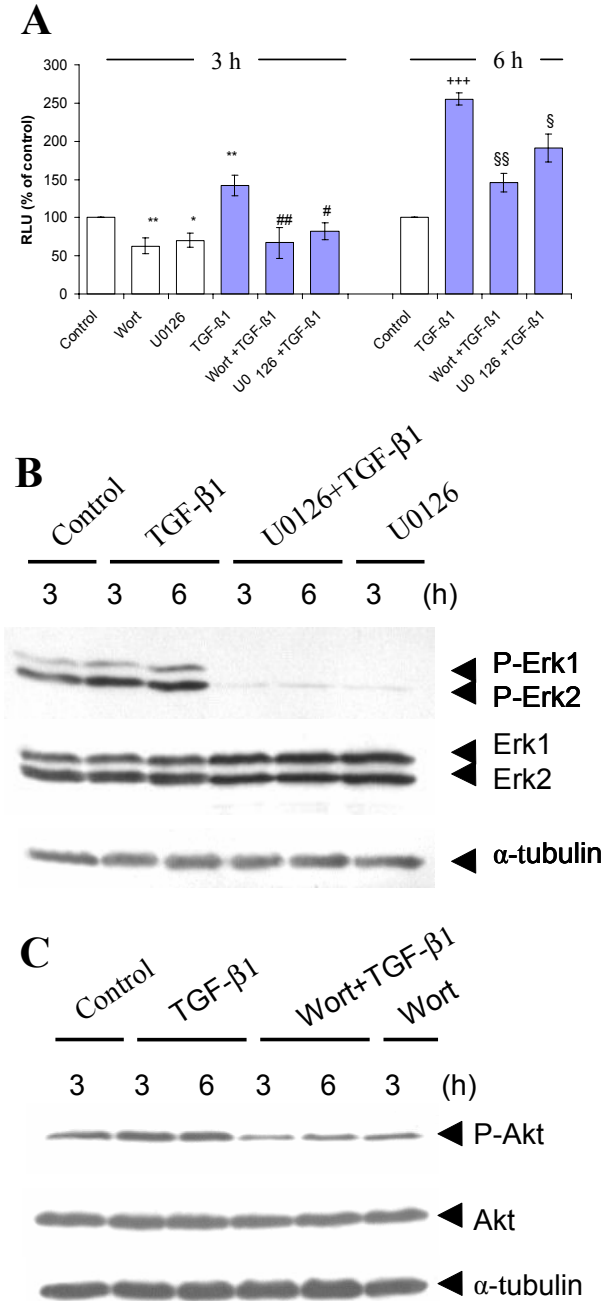

Fig. 5. Wortmannin and U0126 block TGF-β1-mediated activation of NF-κB in cultured mouse hippocampal neurons. To block the activation of Akt or MAPK/Erk1,2, wortmannin (Wort) or U0126 at a final concentration of 60 nM or 20 μM, respectively, was added to the cells 2 h prior to treatment with TGF-β1 (10 ng/ml, T). Control cultures received vehicle only. Cells were harvested 3 h and 6 h after adding TGF-β1 for luciferase activity assay or for Western blotting of P-Akt, Akt, P-Erk1,2, Erk1,2 and α-tubulin. A: TGF-β1-mediated activation of NF-κB was blocked by Wort and U0126. * $P<0.05$ and ** $P<0.01$ compared with the control (3h); # $P<0.05$ and ## $P<0.01$ compared with TGF-β1 alone (3h); +++ $P<0.001$ compared with the control (6h); § $P<0.05$ and §§ $P<0.01$, compared with TGF-β1 alone (6h). B and C: U0126 (B) and Wort (C) blocked the TGF-β1-mediated activation of Akt and Erk1,2. Cells in sister cultures were treated as described above and harvested for Western blotting at indicated time points.
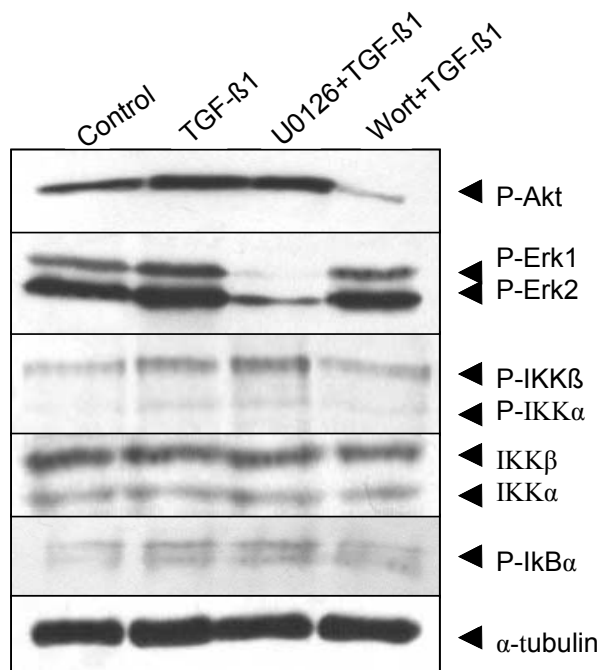
Fig. 6. Wortmannin (Wort), but not U0126, inhibits TGF-β1-mediated increase in phosphorylation of IKKs and IκBα in cultured rat hippocampal neurons. Wort or U0126 at the final concentration of 60 nM or 20 μM, respectively, was added to the cells alone or 2 h prior to the TGF-β1 treatment (10 ng/ml, T). Control cultures received vehicle only. Cells were harvested 3 h after adding TGF-β1 for Western blotting analysis of P-Akt, P-Erk1,2, P-IKKα/β, IKKα/β, P-IκBα and α-tubulin.

increased after incubation with TGF-β1. Of note, TGF-β1-mediated activation of Erk1,2 and Akt was again abolished by U0126 and wortmannin, respectively. However, the increased P-IKKβ and P-IκBα levels mediated by TGF-β1 were only blocked by wortmannin but not by U0126 (Fig. 6), suggesting that only Akt acted as an upstream kinase phosphorylating IKKβ in our experimental conditions, whereas the Mek1,2/Erk1,2 signaling pathway participated in TGF-β1-mediated activation of NF-κB through a distinct and yet undefined mechanism.

Blockage of Erk1,2 and Akt activation diminishes the anti-apoptotic effect of TGF-β1

We have demonstrated the involvement of both Mek1,2/Erk1,2 and PI3k/Akt signaling pathways in the activation of NF-κB by TGF-β1. These findings raised the question whether these two signaling pathways are necessary for the neuroprotective effect of TGF-β1. We evaluated the anti-apoptotic effect of TGF-β1 in the presence of wortmannin or U0126. Wortmannin or U0126 alone at the final concentration of 60 nM or 20 μM, respectively, did not show any toxicity. TGF-β1 (10 ng/ml) consistently protected cultured rat hippocampal cells from STS-induced apoptosis ($P<0.01$), and this protective effect was completely blocked by wortmannin and U0126 (Fig. 7).
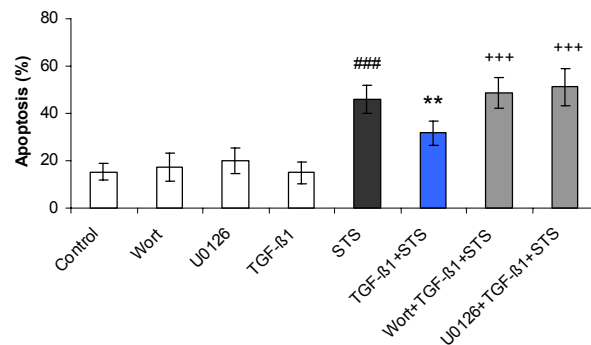

Fig. 7. Wortmannin (Wort) and U0126 abolish the anti-apoptotic effect of TGF-β1 in cultured rat hippocampal cells. Wort or U0126 at a final concentration of 60 nM or 20 μM, respectively, was added to the cells alone or 2 h prior to TGF-β1 treatment (10 ng/ml). Cells were then challenged with STS (100 nM) 24 h later. Controls received vehicle only. Apoptotic damage was evaluated by Hoechst 33258 staining 24 h after adding STS. ### $P<0.001$, compared with the control; ** $P<0.01$ compared with STS alone; +++ $P<0.001$ compared with TGF-β1 plus wortmannin or with TGF-β1 plus U0126.

DISCUSSION

We have previously reported new intracellular signaling mediating the anti-apoptotic effects of TGF-β1 in neurons which involved the activation of MAPK/Erk1,2 and subsequent inactivation of Bad through enhanced phosphorylation (Zhu et al., 2002). The present study further elucidates an additional novel mechanism underlying the neuroprotective effect of TGF-β1 against apoptotic insults. We hypothesized that NF-κB is a target of TGF-β1 and mediates the anti-apoptotic effect of TGF-β1. This proposal is based on the following facts: (i) the anti-apoptotic function of NF-κB in neurons has been well documented (Taglialatela et al., 1997; Glazner et al., 2000; Mattson et al., 2000; Bhakar et al., 2002), although a janus-faced character of this transcription factor was reported under certain conditions; and (ii) NF-κB activity is regulated by TGF-β1 in multiple types of non-neuronal cells (Azuma et al., 1999; Saile et al., 2001; Arsura et al., 1996, 2003). These studies indicate a controversial, yet central role of TGF-β1 in the regulation of NF-κB activation. However, the role of TGF-β1 in NF-κB activation has not been identified in neuronal cells. In the present work, we provided evidence that TGF-β1 increased the transcriptional activity of NF-κB in a time- and concentration-dependent manner in cultured mouse hippocampal neurons (Fig. 1). Since more than 95% of cells in this hippocampal culture are neurons, we suggest that this is a direct action of TGF-β1 on hippocampal neurons. Furthermore, TGF-β1 efficiently prevented the suppression of the nuclear translocation of p65/NF-κB by STS and activated NF-κB in the presence of STS (Fig. 2). Importantly, we demonstrated an essential role of NF-κB activation in the anti-apoptotic effect of TGF-β1. This is based on the findings: i) the anti-apoptotic activity of TGF-β1 was blocked by the specific NF-κB activity inhibitor κB-decoy DNA (Fig. 3A and 3B), and ii) this abrogation of the anti-apoptotic activity of TGF-β1 by κB-decoy DNA was due to the significant suppression of the transcrip-

tional activity of NF-κB (Fig. 3C). Interestingly, Kaltschmidt and Kaltschmidt (2001) showed recently that TGF-β2, another member of the TGF-β superfamily, repressed NF-κB activity in cultured cerebellar granule cells. The different effects of different TGF-β family members on NF-κB activation in different populations of neurons are not surprising, since it has been well established that the function of TGF-β crucially depends on the cell type, the state of cell maturation as well as the experimental conditions (Krieglstein et al., 2000, 2002). TGF-β1 functions as a survival factor in cultured hippocampal neurons (Zhu et al., 2001, 2002), motoneurons and dopaminergic neurons (Krieglstein et al., 1995) as well as sensory neurons (Chalazonitis et al., 1992). However, TGF-β acted as a promoter of apoptosis in cerebellar granule cells cultured in the medium with low (5 mM) but not with high K^+ (20 mM) concentrations (de Luca et al., 1996), suggesting that the effect of TGF-β on the cerebellar granule cells depended on the state of the cell membrane potential. It is yet unclear whether this pro-apoptotic effect of TGF-β is coupled to the inhibition of NF-κB activation in cerebellar granule cells. Our study demonstrated that TGF-β1-mediated NF-κB activation plays a pivotal role in promoting survival of cultured hippocampal neurons challenged with STS, hence pointing out NF-κB activation as a novel mechanism mediating the anti-apoptotic effect of TGF-β1.

An IκB-dependent pathway for the activation of NF-κB has been well characterized in various types of cells in response to different stimuli (Schmitz et al., 2001; Sun and Andersson, 2002). In this regard, NF-κB activation can be principally influenced at multiple levels: IκB phosphorylation, binding of $E3^{IκB}$, a specified ubiquitin ligase complex, to phospho-IκB, the polyubiquitination reaction and proteasome-mediated degradation of IκB. However, the only regulated step in this cascade is IκB phosphorylation, whereas the activity of the enzymes involved in IκB polyubiquitination and degradation is constitutive. Here we showed that TGF-β1-mediated activation of NF-κB involved an increase in the phosphorylation and the degradation of IκBα, a best characterized member of inhibitory NF-κB family proteins. As shown in Fig. 4A, the phosphorylation of IκBα at Ser32, an essential phosphorylation site on IκBα for the subsequent degradation, was markedly enhanced as early as 3 h, and remained at the increased level up to 24 h after TGF-β1 treatment. The increase in IκBα phosphorylation was associated with an increased degradation of IκBα. These findings were consistently produced in the study as presented in Fig. 4B. Since we observed preservation of NF-κB activity by TGF-β1 also in the presence of the apoptotic stimulus, we further measured TGF-β1-mediated changes in IκBα levels in STS-treated cells. In agreement with the previous report, STS suppressed the phosphorylation of IκBα and accordingly inhibited its degradation. These effects mediated by STS were completely reversed by TGF-β1 (Fig. 4C). Taken together, the activation of NF-κB by TGF-β1 in cultured hippocampal neurons involves the modification of IκBα phosphorylation and degradation.

Our next focus was placed on upstream kinases and signaling pathways leading to the phosphorylation of IκBα. Recent studies revealed a direct association of IKKα/β and the phosphorylation of IκB. In mammalian cells, activation of IKKs depends on the phosphorylation of Ser177 and/or Ser181 in IKKβ and Ser176 and/or Ser180 in IKKα (Delhase et al., 1999). Once activated, both kinases phosphorylate serine residues of IκBα (Ser32/36) and IκBβ (Ser19/23), thereby inducing ubiquitination and degradation of IκBα/β and the concomitant activation of NF-κB. However, it is unclear whether IKKs mediate the increase in IκBα phosphorylation caused by TGF-β1 in cultured hippocampal neurons. We elucidated the effect of TGF-β1 on the expression of P-Ser180-IKKα, P-Ser181-IKKβ and P-Ser32-IκBα in parallel. As predicted, TGF-β1 clearly increased the level of P-Ser181-IKKβ, which was accompanied by a concomitantly enhanced P-Ser32-IκBα expression. Interestingly, the level of P-Ser180-IKKα was almost not detectable in control cultures and only slightly increased by TGF-β1. The different expression patterns of P-Ser180-IKKα and P-Ser181-IKKβ suggest an essential role of the latter in the process of NF-κB activation in hippocampal neurons. Of note, the protein levels of both IKKα and IKKβ were not changed by TGF-β1, indicating that TGF-β1 modulated IKKβ at the posttranscriptional level, i.e. increased phosphorylation of IKKβ.

We demonstrated previously the capacity of TGF-β1 to activate MAPK/Erk1,2/Rsk1 pathway in cultured hippocampal neurons and in mouse brain tissues (Zhu et al., 2002). Here, we attempted to test whether TGF-β1-mediated NF-κB activation is associated with its ability to activate MAPK/Erk1,2 signaling, since there is evidence showing an involvement of MAPK signaling in activation of NF-κB (Ghoda et al., 1997; Zhao and Lee, 1999; Baumann et al., 2000). In addition, we extended our attention to the PI3k/Akt pathway, a major cascade mediating activation of NF-κB and survival signaling in neurons. Akt-mediated induction of NF-κB transcriptional activity has been shown to be necessary and sufficient for the anti-apoptotic effect of PDGF (Romashkova and Makarov, 1999) and TNF (Ozes et al., 1999). Furthermore, a direct association among Akt, IKKβ and NF-κB activation has been demonstrated by Madrid et al. (2000). In this study, we found that the TGF-β1-mediated increase in transcriptional activity of NF-κB was completely abolished by wortmannin, whereas U0126 only partially suppressed the effect of TGF-β1. This indicates that both PI3k/Akt and MAPK/ERk1,2 pathways are involved in the underlying mechanism of NF-κB activation by TGF-β1 with a dominant role for PI3k/Akt. Interestingly, further studies revealed that the increased phosphorylation of IKKα/β and IκBα caused by TGF-β1 was abrogated by wortmannin, but not by U0126. Therefore, it seems that the cascade of TGF-β1-mediated NF-κB activation involves phosphorylation of IKKs through the PI3k/Akt pathway, while the regulation of NF-κB activity by MAPK/Erk1,2 pathway remains unclear. Different mechanisms have been shown in MAPK-mediated NF-κB activation (Ghoda et al., 1997; Baumann et al., 2000). A recent study revealed that activation of mitogen- and stress-acti-
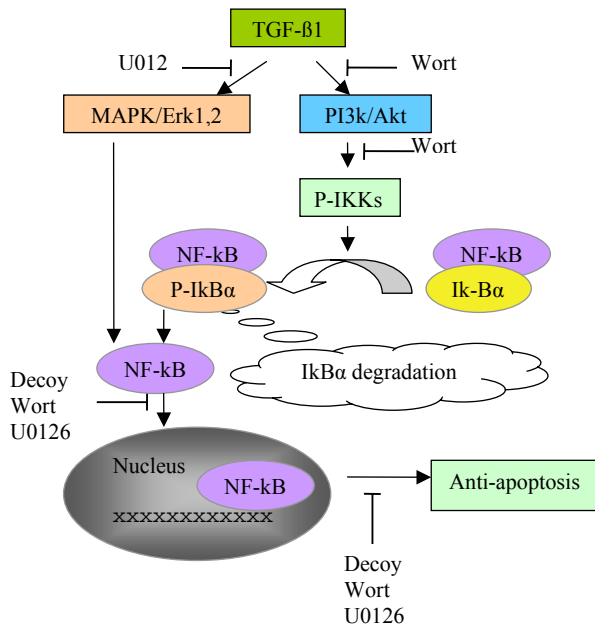
Fig. 8. Scheme summarizing the major findings of the present study. TGF-β1 causes activation of NF-κB as evidenced by: i) an increase in phosphorylation and subsequent degradation of IκBα; ii) increased nuclear translocation of p65/NF-κB; iii) enhanced transcriptional activity of NF-κB which is blocked by the specific inhibitor κB-decoy DNA. TGF-β1 is able to activate both MAPK/Erk1,2 and PI3k/Akt signaling pathways, which lead to the activation of NF-κB through distinct mechanisms. The increased phosphorylation of IκBα seems to be related to the activation of IKKs through PI3k/Akt signaling, since the increased phosphorylation of both IκBα and IKKs mediated by TGF-β1 was blocked by wortmannin (Wort), but not by U0126. Because Wort, U0126 and κB-decoy DNA blocked the antiapoptotic activity of TGF-β1, we further suggest that both MAPK/Erk1,2 and PI3k/Akt signaling pathways and the underlying target NF-κB activity are necessary for the anti-apoptotic effect of this cytokine in neurons.

vated protein kinase-1, a direct downstream kinase of Erk1,2, led to phosphorylation of Ser276 on p65/NF-κB and the subsequent transcriptional activation of NF-κB (Vermeulen et al., 2003). Nevertheless, the molecular details of how TGF-β1-mediated activation of Erk1,2 and PI3k/Akt pathways contributes to the activation of NF-κB remain to be characterized.

Finally, we showed that both PI3k/Akt and MAPK/Erk1,2 pathways are necessary for the anti-apoptotic effect of TGF-β1 in cultured hippocampal neurons (Fig. 7). The precise mechanisms for the anti-apoptotic effect of these two signaling pathways have been extensively elucidated (Brazil and Hemmings, 2001; Brunet et al., 2001; Chang et al., 2003). Based on our finding that TGF-β1-induced activation of NF-κB is associated with the activation of Akt and Erk1,2, we conclude that NF-κB is an important target of both PI3k/Akt and MAPK/Erk1,2 signaling pathways which were both triggered by TGF-β1. Because κB-decoy DNA blocked the anti-apoptotic activity of TGF-β1, we further propose that NF-κB activity plays a pivotal role in the anti-apoptotic effect of this cytokine in neurons (Fig. 8).

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