Insulin-like Growth Factor-1-mediated Neuroprotection against Oxidative Stress Is Associated with Activation of Nuclear Factor κB*

(Received for publication, September 21, 1998, and in revised form, December 24, 1998)

Stefanie Heck‡, Frank Lezoualc’h‡§, Stefanie Engert, and Christian Behl¶
From the Max Planck Institute of Psychiatry, Kraepelinstrasse 2-10, 80804 Munich, Germany

The role of insulin-like growth factor 1 (IGF-1) for the treatment of neurodegenerative disorders, such as Alzheimer’s disease, has recently gained attention. The present study demonstrates that IGF-1 promotes the survival of rat primary cerebellar neurons and of immortalized hypothalamic rat GT1–7 cells after challenge with oxidative stress induced by hydrogen peroxide (H₂O₂). Neuroprotective concentrations of IGF-1 specifically induce the transcriptional activity and the DNA binding activity of nuclear factor κB (NF-κB), a transcription factor that has been suggested to play a neuroprotective role. This induction is associated with increased nuclear translocation of the p65 subunit of NF-κB and with degradation of the NF-κB inhibitory protein IκBα. IGF-1-mediated protection of GT1–7 cells against oxidative challenges was mimicked by overexpression of the NF-κB subunit c-Rel. Partial inhibition of NF-κB baseline activity by overexpression of a dominant-negative IκBα mutant enhanced the toxicity of H₂O₂ in GT1–7 cells. The pathway by which IGF-1 promotes neuronal survival and activation of NF-κB involves the phosphoinositol (PI) 3-kinase, because both effects of IGF-1 are blocked by LY294002 and wortmannin, two specific PI 3-kinase inhibitors. Taken together, our results provide evidence for a novel molecular link between IGF-1-mediated neuroprotection and induction of NF-κB that is dependent on the PI 3-kinase pathway.

Insulin-like growth factor 1 (IGF-1)† is a pleiotropic factor with a wide spectrum of action in the central nervous system and also in the peripheral nervous system (1, 2). It belongs to a superfamily of structurally related proteins that includes insulin and IGF-2. The biological functions of the IGFs and insulin are mediated by specific membrane receptors, designated the IGF-1, IGF-2, and insulin receptors (2, 3). The IGF-1 receptor is the primary mediator of the action of IGF-1.

Recently, IGF-1 has gained increasing attention for the treatment of neurodegenerative disorders, such as the amyotrophic lateral sclerosis, which is characterized by the progressive loss of motor neurons (4, 5). Moreover, with respect to the central nervous system, IGF-1 has been found to protect hippocampal neurons against the toxicity of the Alzheimer’s disease-associated amyloid β protein (Aβ) (6). Aβ is the main component of the senile plaques in the brain of Alzheimer’s disease patients and its cytotoxic action on neurons results from oxidative damage to susceptible cells (7). Specifically, it has been shown that hydrogen peroxide (H₂O₂) is an important intermediate in Aβ neurotoxicity (7). In accordance with a neuroprotective role of IGF-1 against oxidative injury, this growth factor is also effective in protecting neurons against glutathione depletion, suggesting a more general protective potency of IGF-1 against oxidative stress (8).

The molecular signaling pathways by which IGF-1 promotes survival, in particular survival of neurons of the central nervous system, are not well characterized. Dudek et al. (9) revealed a critical function of the serine-threonine protein kinase Akt to mediate the protective effects of IGF-1 on the survival of cerebellar neurons against serum deprivation. Moreover, the inhibition of apoptosis by IGF-1 has been shown to require the activation of signaling molecules such as the phosphatidylinositol (PI) 3-kinase (9, 10).

The nuclear factor κB (NF-κB) is composed of homo- and heterodimers of members of the Rel family of related transcription factors that are well characterized for controlling the expression of numerous immune and inflammatory response genes (11, 12). Frequently, NF-κB is present as a heterodimer comprising a 50-kDa (p50) and a 60-kDa (p65) subunit that is sequestered in the cytoplasm by an inhibitory protein of the IκB family, with IκBα being the best characterized member of this family (13, 14). NF-κB-inducing agents, such as cytokines, viruses, phorbol esters, and UV light, result in the phosphorylation and degradation of the IκB inhibitory protein (15, 16) allowing free NF-κB to enter the nucleus, to bind to its cognate DNA sequences, and to induce target gene transcription.

As for IGF-1, an important role for NF-κB during cell death has been suggested (17). Several reports have shown that NF-κB counteracts the induction of apoptosis by the cytokine tumor necrosis factor-α (18–20), by ionizing radiation, and by the cancer chemotherapeutic agent daunorubicin (21). Recently, we have demonstrated that constitutively increased NF-κB activity mediates the protection of neuronal cells against oxidative stress (22). This prompted us to investigate whether IGF-1 offers neuroprotection against oxidative insult by affecting the activity of NF-κB as downstream target in neurons. For this study, two IGF-1 receptor-expressing neuronal cell systems were employed, the gonadotropin-releasing hormone-secreting neuronal cell line GT1–7 (23) and primary cultures of rat cerebellar granule neurons (24, 25). We demonstrate that IGF-1 exerts a neuroprotective effect against oxidative...
IGF-1 Activation of NF-κB

**EXPERIMENTAL PROCEDURES**

**Reagents**—All media, sera, and media supplements were from Life Technologies, Inc. IGF-1 was purchased from Promega (Heidelberg, Germany) and Sigma. IGF-1 stock solutions were dissolved in 0.1 M acetic acid at a concentration of 250 μg/ml and, subsequently, dissolved in water. The inhibitors wortmannin and LY294002 were from Calbiochem (Bad Soden, Germany). All other chemicals were from Sigma unless otherwise stated.

**Plasmids**—NF-κB-Luc was a plasmid construct containing six NF-κB-binding DNA consensus sites linked to a luciferase reporter gene. Tk-Luc was an empty cassette construct used as a negative control for NF-κB-Luc. Both plasmids were provided by Dr. P. Baueerle (Tularik Inc., San Francisco, CA). The c-Rel-expression plasmid was a kind gift from Dr. P. Jalinot (CNRS UMR 48, Lyon, France), and the dominant-negative IκBα-expression plasmid was from Dr. D. W. Ballard (Vanderbilt University, Nashville, TN).

**Cell Cultures**—Primary cultures of rat cerebellar granule neurons were prepared from 8-day-old Sprague-Dawley (Charles River, Sulzdorf, Germany) rat pups, as described previously (26). Neurons dissociated from (rat brain were cultured at different densities (see below) on plastic dishes coated with poly-L-lysine (10 μg/ml) and grown in basal modified Eagle’s medium containing 10% heat-inactivated fetal calf serum, 25 mM KCl, 2 mM glutamine, and 100 μg/ml gentamicin. Cultures were placed in a humidified incubator at 37 °C under 95% air/5% CO2 atmosphere. Experiments were performed after 5–8 days in vitro as indicated. Glial proliferation was prevented by addition of 10 μg/ml cytosine arabinofuranoside, an inhibitor of cell proliferation, 16 h after plating. GT1–7 cells (kindly provided by Dr. R. Weiner, University of California, San Francisco, CA) were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum under standard culture conditions.

**Cell Survival Analysis**—Cell viability was assessed using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay described for the first time by Mosmann (27). Cultures were treated with H2O2 (60 μM), a mediator of the oxidative toxicity of the stressor H2O2, the cell viability was dose-dependently increased in both neuronal systems (Fig. 2). This was also evident when inhibitors received control vehicle (dimethyl sulfoxide for LY294002, ethanol for wortmannin). Luciferase activity was monitored as previously described (29). Each transfection experiment was done in triplicate, repeated at least four times, and normalized for identical amounts of protein using the Bio-Rad protein reagent to determine protein concentrations of the samples (Bio-Rad).

**Western Blotting**—Western blotting was performed as described previously (31, 22) with minor modifications. Briefly, medium of subconfluent GT1–7 cells was exchanged to serum-free medium, the cells were treated with IGF-1 as indicated, and cytoplasmic and nuclear extracts were prepared by a mini-extraction protocol (30). Protein concentrations were determined using the Bio-Rad protein reagent (Bio-Rad). Aliquots (10 μg) were mixed with SDS sample buffer containing 4% β-mercaptoethanol and resolved on a 10% SDS-polyacrylamide gel. Proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, United Kingdom) and reacted with an anti-IκBα antibody (C-21, 1:500 dilution, Santa Cruz Biotechnology) or an anti-p65 antibody (C-20, 1:200 dilution, Santa Cruz Biotechnology). The primary antibody was detected by counterstaining with a horseradish peroxidase-linked antibody and visualized by the ECL detection kit (Amersham). A part of the SDS gel was stained with Coomassie Blue to verify whether equal amounts of proteins had been used. Densitometer reading of the autoradiograph of the Western blot was performed using a Beckmann photometer.

**Statistical Analysis**—An unpaired Student’s t test was used to calculate differences between means; differences were considered significant at p < 0.05.

**RESULTS**

**IGF-1 Protects Primary Cerebellar Neurons and GT1–7 Cells against Oxidative Stress Induced by H2O2**—The treatment of primary postmitotic cerebellar granular neurons and GT1–7 cells with H2O2 (60 μM), a mediator of the oxidative toxicity of Aβ and other neurotoxins, reduced neuronal viability as assessed by trypan blue exclusion/cell countings using phase-contrast microscopy and by MTT assays (Figs. 1B and 2). When the cells were preincubated for 24 h with different concentrations of IGF-1 and were challenged for additional 24 h with the indicated concentration of H2O2, the cell viability was dose-dependently increased in both neuronal systems (Fig. 2). This was also evident in the maintenance of morphology and integrity of GT1–7 cells after incubation with 50 ng/ml IGF-1 (Fig. 1C). Higher concentrations of IGF-1 did not further increase cell survival (data not shown). These data demonstrated that IGF-1 can exert a neuroprotective function against oxidative stress.

**IGF-1 Activates NF-κB DNA Binding Activity in Neuronal Cells**—To assess whether IGF-1 has any effect on the activity of NF-κB in neuronal cells, we first analyzed the influence of IGF-1 on the DNA binding activity of this transcription factor. We carried out EMSAs with nuclear extracts from primary cerebellar neurons and GT1–7 cells, employing a DNA probe

**EXPERIMENTAL PROCEDURES**

**Reagents**—All media, sera, and media supplements were from Life Technologies, Inc. IGF-1 was purchased from Promega (Heidelberg, Germany) and Sigma. IGF-1 stock solutions were dissolved in 0.1 M acetic acid at a concentration of 250 μg/ml and, subsequently, dissolved in water. The inhibitors wortmannin and LY294002 were from Calbiochem (Bad Soden, Germany). All other chemicals were from Sigma unless otherwise stated.

**Plasmids**—NF-κB-Luc was a plasmid construct containing six NF-κB-binding DNA consensus sites linked to a luciferase reporter gene. Tk-Luc was an empty cassette construct used as a negative control for NF-κB-Luc. Both plasmids were provided by Dr. P. Baueerle (Tularik Inc., San Francisco, CA). The c-Rel-expression plasmid was a kind gift from Dr. P. Jalinot (CNRS UMR 48, Lyon, France), and the dominant-negative IκBα-expression plasmid was from Dr. D. W. Ballard (Vanderbilt University, Nashville, TN).

**Cell Cultures**—Primary cultures of rat cerebellar granule neurons were prepared from 8-day-old Sprague-Dawley (Charles River, Sulzdorf, Germany) rat pups, as described previously (26). Neurons dissociated from (rat brain were cultured at different densities (see below) on plastic dishes coated with poly-L-lysine (10 μg/ml) and grown in basal modified Eagle’s medium containing 10% heat-inactivated fetal calf serum, 25 mM KCl, 2 mM glutamine, and 100 μg/ml gentamicin. Cultures were placed in a humidified incubator at 37 °C under 95% air/5% CO2 atmosphere. Experiments were performed after 5–8 days in vitro as indicated. Glial proliferation was prevented by addition of 10 μg/ml cytosine arabinofuranoside, an inhibitor of cell proliferation, 16 h after plating. GT1–7 cells (kindly provided by Dr. R. Weiner, University of California, San Francisco, CA) were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum under standard culture conditions.

**Cell Survival Analysis**—Cell viability was assessed using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay described for the first time by Mosmann (27). Cultures were treated with H2O2 (60 μM), a mediator of the oxidative toxicity of the stressor H2O2, the cell viability was dose-dependently increased in both neuronal systems (Fig. 2). This was also evident when inhibitors received control vehicle (dimethyl sulfoxide for LY294002, ethanol for wortmannin). Luciferase activity was monitored as previously described (29). Each transfection experiment was done in triplicate, repeated at least four times, and normalized for identical amounts of protein using the Bio-Rad protein reagent to determine protein concentrations of the samples (Bio-Rad).

**Western Blotting**—Western blotting was performed as described previously (31, 22) with minor modifications. Briefly, medium of subconfluent GT1–7 cells was exchanged to serum-free medium, the cells were treated with IGF-1 as indicated, and cytoplasmic and nuclear extracts were prepared by a mini-extraction protocol (30). Protein concentrations were determined using the Bio-Rad protein reagent (Bio-Rad). Aliquots (10 μg) were mixed with SDS sample buffer containing 4% β-mercaptoethanol and resolved on a 10% SDS-polyacrylamide gel. Proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, United Kingdom) and reacted with an anti-IκBα antibody (C-21, 1:500 dilution, Santa Cruz Biotechnology) or an anti-p65 antibody (C-20, 1:200 dilution, Santa Cruz Biotechnology). The primary antibody was detected by counterstaining with a horseradish peroxidase-linked antibody and visualized by the ECL detection kit (Amersham). A part of the SDS gel was stained with Coomassie Blue to verify whether equal amounts of proteins had been used. Densitometer reading of the autoradiograph of the Western blot was performed using a Beckmann photometer.

**Statistical Analysis**—An unpaired Student’s t test was used to calculate differences between means; differences were considered significant at p < 0.05.

**RESULTS**

**IGF-1 Protects Primary Cerebellar Neurons and GT1–7 Cells against Oxidative Stress Induced by H2O2**—The treatment of primary postmitotic cerebellar granular neurons and GT1–7 cells with H2O2 (60 μM), a mediator of the oxidative toxicity of Aβ and other neurotoxins, reduced neuronal viability as assessed by trypan blue exclusion/cell countings using phase-contrast microscopy and by MTT assays (Figs. 1B and 2). When the cells were preincubated for 24 h with different concentrations of IGF-1 and were challenged for additional 24 h with the indicated concentration of H2O2, the cell viability was dose-dependently increased in both neuronal systems (Fig. 2). This was also evident in the maintenance of morphology and integrity of GT1–7 cells after incubation with 50 ng/ml IGF-1 (Fig. 1C). Higher concentrations of IGF-1 did not further increase cell survival (data not shown). These data demonstrated that IGF-1 can exert a neuroprotective function against oxidative stress.

**IGF-1 Activates NF-κB DNA Binding Activity in Neuronal Cells**—To assess whether IGF-1 has any effect on the activity of NF-κB in neuronal cells, we first analyzed the influence of IGF-1 on the DNA binding activity of this transcription factor. We carried out EMSAs with nuclear extracts from primary cerebellar neurons and GT1–7 cells, employing a DNA probe
that represents the NF-κB DNA-binding motif. Because IGF-1 produced a considerable neuroprotective effect at a concentration of 50 ng/ml in our neuronal systems (Fig. 2), we used this concentration to study its influence on NF-κB DNA binding.

Upon treatment with IGF-1, DNA binding activity of NF-κB was increased in cerebellar neurons after 1, 4, and 6 h and in GT1–7 cells after 4 and 6 h (Fig. 3, A (lanes 2–4) and B (lanes 3 and 4)). A longer treatment of the cells with IGF-1 did not further increase the binding of NF-κB to DNA (data not shown).

The specificity of NF-κB DNA binding was demonstrated by the addition of a 100-fold excess of unlabeled NF-κB oligonucleotide probe that acts as a competitor for the binding of NF-κB (Fig. 3, A (lane 5) and B (lane 5)). To further characterize the DNA-protein complexes, nuclear extracts from IGF-1-treated cells (6 h, 50 ng/ml) were incubated with antibodies against the p65 and p50 subunits of NF-κB in a separate set of experiments. Both antibodies markedly reduced the specific DNA-protein complexes, with the anti-p50 antibody creating a supershift as shown for GT1–7 cells (Fig. 3C, lanes 3 and 4). This suggests the presence of both p65 and p50 in the protein complexes that bind to the NF-κB oligonucleotide. These results are consistent with previous observations in other cellular systems (22, 32, 33).

To demonstrate that IGF-1 does not lead to a general nonspecific increase of various transcription factors, the DNA binding activity of the transcription factor Oct-1 was investigated as control. No increase in Oct-1 DNA binding activity could be observed after treatment of GT1–7 cells with IGF-1 under the same experimental conditions (Fig. 3D).

**NF-κB-dependent Gene Transcription Is Increased by IGF-1**—To determine whether IGF-1-induced DNA binding of NF-κB results in activation of NF-κB-mediated gene transcription, cerebellar granule neurons and GT1–7 cells were transiently transfected with a plasmid construct containing a promoter composed of six NF-κB-binding DNA consensus sites linked to a luciferase reporter gene (NF-κB-Luc). Upon stimulation with neuroprotective doses of IGF-1 (25 and 50 ng/ml) for 12–14 h, luciferase activities were significantly increased in cerebellar neurons and in GT1–7 cells (Fig. 4). Lowering the IGF-1 concentration to 10 ng/ml could not significantly enhance NF-κB-dependent reporter-gene activity. Transcription of the plasmid construct Tk-Luc, which served as a control, was not altered by the growth factor neither in cerebellar neurons nor in GT1–7 cells (Fig. 4), indicating the specificity of NF-κB activation by IGF-1.

**IGF-1 Increases the Amount of Nuclear p65 and Decreases**

---

**Fig. 1.** Morphological changes of GT1–7 cells in response to H$_2$O$_2$ and neuroprotective effect of IGF-1. Phase contrast photomicrographs of GT1–7 cells treated with vehicle only (A), treated for 24 h with 60 μM H$_2$O$_2$ (B), or treated for 24 h with 60 μM H$_2$O$_2$ after a 24-h preincubation with IGF-1 (50 ng/ml) (C). D, subsequently to these experimental treatments, 0.12% trypan blue was added to the cell cultures, and the viable cells were determined per low magnification field in five optical fields. The data are expressed as the mean ± S.E. of viable cells relative to control cultures (defined as 100%). (*, p < 0.001 compared with cells not treated with IGF-1.) Scale bar = 50 μm.

**Fig. 2.** IGF-1 protects cerebellar granule neurons and GT1–7 cells against H$_2$O$_2$ in a dose-dependent manner. Cerebellar granule neurons (A) and GT1–7 cells (B) were preincubated for 24 h with different concentrations of IGF-1 (1–50 ng/ml) and were challenged for another 24 h with 60 μM H$_2$O$_2$. Thereafter, MTT assays were performed, and MTT reduction was expressed as percentage of cell survival compared with control cells that received no H$_2$O$_2$. The viability of control cells was defined as 100%. Experiments were repeated four times in triplicate with comparable results. The presented data are means ± S.E. of one representative triplicate determination. (*, p < 0.05 and **, p < 0.001 compared with cells that received no IGF-1).
**FIG. 3.** IGF-1 increases the DNA binding activity of NF-κB in cerebellar granule neurons and in GT1-7 cells. Nuclear extracts from cerebellar granule neurons cultured for 5 days in vitro (A) and from GT1-7 cells (B-D) were analyzed by EMSAs. Cell cultures were left untreated (control (CT)) or were treated for the indicated time points with 50 ng/ml IGF-1. The EMSAs were performed with labeled oligonucleotides representing an NF-κB (A–C) or Oct-1 (D) DNA-binding site. Binding of nuclear extracts after reaction with a 100-fold excess of unlabeled probe is also shown (competitor). In C, lanes 3 and 4, nuclear extracts from GT1-7 cells treated for 6 h with IGF-1 (50 ng/ml) were preincubated for 16 h at 4 °C with 2 μl of either anti-p65 (sc-109X) or anti-p50 (sc-114X) antibody (Santa Cruz Biotechnology) prior to the binding reaction. Filled arrowheads represent the position of specific NF-κB/DNA bound complexes, circles depict the position of nonspecific complexes, and open arrowheads indicate the position of the free DNA probe. The arrow in C indicates the position of a supershift after anti-p50 pretreatment (lane 4).

**the Level of Inhibitory IκBa Protein—**Because it is known that NF-κB inducing stimuli result in degradation of the inhibitory protein IκBa and subsequent nuclear translocation of p65, we investigated whether neuroprotective doses of IGF-1 have the same effect. In nuclear extracts from GT1–7 cells treated with 50 ng/ml IGF-1 for 1–6 h, p65 expression was already increased after 1 h. This increase correlated with the rapid degradation of IκBa in the cytoplasmic portion of these cells as shown by Western blotting (Fig. 5). After 6 h of IGF-1 treatment, the amount of translocated nuclear p65 was increased 2.3-fold, whereas the level of IκBa protein was reduced approximately 60% as compared with extracts from untreated cells (Fig. 5, A and B, compare the left lane with the 6 h lane in each). Taken together, the above results indicate that following degradation of the inhibitory protein IκBa and nuclear translocation of p65, neuroprotective concentrations of IGF-1 can specifically induce the DNA binding activity and the transcriptional activity of NF-κB in neuronal cells.

**Overexpression of c-Rel Protects GT1-7 Cells against H2O2—**Several previous reports suggest a role for NF-κB in cell survival. In view of these findings, we investigated whether the activation of this transcription factor is directly involved in IGF-1-mediated neuroprotection. Therefore, GT1–7 cells were transiently transfected with an expression plasmid coding for
c-Rel, a subunit of NF-κB, the expression of which leads to the induction of an NF-κB-dependent luciferase reporter gene (Fig. 6B). Whereas cells transfected with an empty control vector showed a decreased survival upon challenge with H₂O₂, c-Rel-overexpressing cells were completely protected against the oxidative challenge as determined by the MTT assay performed subsequently to transfection (Fig. 6A).

To further ascertain the involvement of NF-κB in protection of neuronal cells, we transiently transfected GT1–7 cells with an expression plasmid coding for a mutant form of IκBα, which is resistant to both phosphorylation and proteolytic degradation and therefore prevents nuclear translocation of NF-κB (34). This dominant-negative IκBα, the overexpression of which significantly reduced basal NF-κB-mediated reporter gene activity (Fig. 6B), indeed decreased cell survival after oxidative challenge when compared with cells expressing an empty control vector (Fig. 6A).

These results show that neuroprotection by IGF-1 can be mimicked by NF-κB activation, and cell viability is decreased by NF-κB inhibition, indicating that NF-κB might at least in part be involved in the mediation of the neuroprotective function of IGF-1.

Inhibition of the PI 3-Kinase Does Block IGF-1-mediated Neuroprotection and NF-κB Activation—Recent data from various groups suggested that the molecular signaling pathway by which IGF-1 exerts its neuroprotective function might involve

**Fig. 4.** IGF-1 increases the transcriptionsal activity of NF-κB in cerebellar granule neurons and in GT1–7 cells. Cerebellar granule neurons cultured for 8 days in vitro (A) and GT1–7 cells (B) were transfected with either 2 μg of the indicator plasmid NF-κB-Luc (gray bars) or 2 μg of Tk-Luc control vector (black bars) and were incubated for 16 h with increasing concentrations of IGF-1 (1–50 ng/ml). Thereafter cells were harvested and luciferase activity was determined. Results are expressed in arbitrary units of luciferase activity (relative luciferase activity) corrected for identical amounts of protein. Experiments were repeated four times in triplicate. The presented data are the means ± S.E. of one representative triplicate determination (*, p < 0.05 and **, p < 0.001 compared with control values).

**Fig. 5.** IGF-1 increases the amount of nuclear p65 and decreases cytoplasmic IκBα protein levels in GT1–7 cells. GT1–7 cells were treated with vehicle only or with 50 ng/ml IGF-1 for the indicated time. Cytoplasmic and nuclear extracts were prepared, and equal amounts of protein were analyzed for the presence of nuclear p65 and cytoplasmic IκBα by Western blotting. Intensity of the signals was analyzed by densitometer reading of the autoradiographs of the Western blots and is presented as relative protein expression. The expression in untreated cells was defined arbitrarily as 1.
PI 3-kinase (9, 10). This is confirmed in our cellular system, because in MTT assays, the potent PI 3-kinase inhibitors LY294002 (35) and wortmannin (36) did indeed block IGF-1-mediated protection of GT1–7 cells against hydrogen peroxide induced cell death (Fig. 7).

To investigate whether the PI 3-kinase is also involved in the activation of NF-κB by IGF-1, we examined the influence of these inhibitors on IGF-1 induced transcription of an NF-κB-dependent luciferase gene in transiently transfected GT1–7 cells. In this assay, the inhibition of PI 3-kinase by LY294002 (10 μM) and wortmannin (100 nM) completely abolished the stimulation of NF-κB transcriptional activity by IGF-1 (Fig. 8), consistent with their ability to block IGF-1-mediated neuroprotection (Fig. 7). These results strongly support a novel pathway for NF-κB activation exerted by IGF-1 that involves the PI 3-kinase and mediates IGF-1 neuroprotection.

**DISCUSSION**

The aim of the present study was to investigate the ability of IGF-1 to protect neurons against H₂O₂-induced neuronal cell death and to examine a possible involvement of NF-κB in this function. We employed GT1–7 hypothalamic neuronal cells and mature primary cerebellar granule neurons that have been shown to express functional IGF-1 receptors (9, 37). As previously reported for other oxidative stressors, such as the Alzheimer’s disease-associated Aβ and the glutathione depleting agent buthionine sulfoximine (6, 8), we found that IGF-1 is effective in protecting neuronal cells against oxidative injury induced by H₂O₂. A 24-h pretreatment with 50 ng/ml of IGF-1 caused an enhanced cell survival of about 30% after H₂O₂ challenge in GT1–7 cells and primary mature postmitotic neurons of 5 days in vitro. IGF-1 did not significantly affect GT1–7

---

**FIG. 6.** Effect of c-Rel and dominant-negative IκBα expression on cell survival and basal NF-κB transcriptional activity. A, GT1–7 cells were transfected with 2 μg of either an expression plasmid for c-Rel or for mutant IκBα or with an empty control plasmid. 5 h after transfection cells were challenged with 60 μM H₂O₂ for 24 h and MTT assay was performed subsequently. MTT reduction was expressed as percentage of cell survival compared with cells that received no H₂O₂ (defined as 100%). Experiments were repeated three times in triplicate with comparable results. The presented data are means ± S.E. of one representative triplicate determination. (*p < 0.05 compared with cells transfected with the empty expression vector.) B, GT1–7 cells were cotransfected with 1 μg of either an empty expression vector (control), an expression plasmid for c-Rel, or an expression plasmid for mutant IκBα. After 16 h, cells were harvested, and luciferase activity was determined. Results are expressed in arbitrary units of luciferase activity (relative luciferase activity) corrected for identical amounts of protein. Experiments were repeated three times in triplicate. The presented data are the means ± S.E. of one representative triplicate determination. (*, p < 0.01 compared with control values.)

**FIG. 7.** LY294002 and wortmannin inhibit IGF-1-mediated cell survival. GT1–7 cells were left untreated or were incubated with 50 ng/ml IGF-1 in the presence or absence of 10 μM LY294002 (A) or 100 nM wortmannin (B). After 24 h, cells were challenged for another 24 h with 120 μM H₂O₂. Thereafter, MTT assays were performed and MTT reduction was expressed as percentage of cell survival compared with control cells that received no H₂O₂. The viability of control cells was defined as 100%. Experiments were repeated three times in triplicate with comparable results. The presented data are means ± S.E. of one representative triplicate determination. (**, p < 0.001 and ***p < 0.0005; IGF-1 stimulated cells compared with cells that were treated with inhibitor in addition to IGF-1.)
cell growth in serum-free conditions (data not shown), confirming that the increased cell survival is not due to a proliferative effect of IGF-1 in this neuronal system.

The fact that IGF-1 is a potent neuroprotective agent against oxidative stress induced by the lipid peroxidizing agent H$_2$O$_2$ may offer a new therapeutic avenue for the treatment of neurodegenerative disorders in which free radicals have been implicated. For instance, IGF-1 has been shown to reduce neuronal cell loss observed in vivo following ischemic insults and is beneficial in the treatment of amyotrophic lateral sclerosis (38, 39). With respect to its potency to rescue neurons against Aβ toxicity (38), one can likewise imagine that the development of IGF-1-related compounds could be a promising strategy toward the treatment of Alzheimer’s disease.

The PI 3-kinase pathway has been shown to be implicated in the signaling of IGF-1-mediated cell survival, including survival of neurons (9, 40). PI 3-kinase phosphorylates inositol lipids that act as second messengers for several targets, such as the serine-threonine kinase Akt (41), which has been shown accordingly to be involved in anti-apoptotic signaling (42–44). Experiments with pharmacological inhibitors demonstrated that IGF-1-mediated neuroprotection against serum deprivation in neuronal cells is dependent on PI 3-kinase activity (9, 10).

The data obtained in the present study extend this observation showing that the PI 3-kinase pathway is also involved in the protective effect of IGF-1 against H$_2$O$_2$-induced cell death. Two potent PI 3-kinase inhibitors, LY294002 (35) and wortmannin (36), were able to block the cellular protection afforded by IGF-1 against oxidative challenge in GT1–7 cells. The data from Parizzas et al. (10) additionally suggest a role of the mitogen-activated protein kinase pathway in IGF-1-mediated neuroprotection because the anti-apoptotic function of IGF-1 could not completely be blocked by inhibition of the PI 3-kinase pathway in the PC12 cells employed in their study. In our cellular system, the neuroprotective effect of IGF-1 was entirely prevented by blocking PI 3-kinase, arguing against the involvement of other signal transduction pathways in IGF-1-mediated neuroprotection.

Because the transcription factor NF-κB can modulate neuronal vulnerability and has recently gained great interest for its potential role in neuroprotection (45–47), we tested the ability of IGF-1 to induce NF-κB activation. We found that increasing concentrations of IGF-1 in GT1–7 cells stimulate the NF-κB transcriptional activity with a substantial effect at 50 ng/ml. These results were also confirmed in postmitotic cerebellar granule neurons, indicating that the enhancement of NF-κB activity by IGF-1 is not dependent on the cell cycle. Consistent with this increase in NF-κB transcriptional activity, NF-κB DNA binding activity could also be enhanced upon stimulation with 50 ng/ml IGF-1 in both neuronal systems. In agreement with these findings, we observed increased nuclear translocation of the p65-subunit of NF-κB and a parallel decrease in the amount of the NF-κB cytoplasmic inhibitory protein, IκBα. Thus, the induction of NF-κB-driven protective transcriptional programs by IGF-1 could be one way for the neurons to protect themselves against exogenous insults. In support of this hypothesis, we have recently shown that in a clone of the sympathetic precursor-like cell line PC12 selected for its resistance against Aβ and H$_2$O$_2$, constitutively increased NF-κB activity mediates this resistance. The suppression of NF-κB activation in these cells reverses the oxidative stress resistance phenotype (22). Consistently, the inhibition of NF-κB activation results in apoptosis in PC12 cells, and nerve growth factor did not protect from apoptosis when NF-κB activation is blocked (32).

To confirm the role of NF-κB in IGF-1-mediated neuroprotection, we (i) induced an activation of NF-κB by transient expression of the c-Rel subunit of this transcription factor, and (ii) inhibited NF-κB activity by expression of a dominant-negative form of IκBα. The expression of c-Rel in GT1–7 cells, which mimicked IGF-1 induction of NF-κB activity, protected these cells completely against oxidative stress, consistent with findings from other investigators showing that the c-Rel expression is able to reduce apoptosis in nonneuronal cells (17, 19). On the other hand, the dominant-negative IκBα decreased the intrinsic survival of GT1–7 cells against oxidative insult, most likely by reducing the high basal NF-κB activity in these cells. However, this decrease in survival was not significant but rather indicative, which might be due to the fact that either the dominant-negative IκBα was not able to completely abrogate NF-κB activity (supported by the luciferase assay done in parallel) or additional factors, other than NF-κB, are involved in the protection of neuronal cells against oxidative stress. Together, these results suggest that the protection of neuronal
cells by IGF-1 may be mediated, at least in part, through the activation of NF-κB.

In view of the idea that the activation of NF-κB represents an intermediary step in IGF-1-mediated neuroprotection, one would expect that both processes are mediated by a common pathway. Upon investigating the effect of LY294002 and wortmannin on the IGF-1-induced transcriptional activity of NF-κB, it indeed turned out that these PI 3-kinase inhibitors completely blocked the IGF-1-induced increase in NF-κB mediated luciferase activity, indicating that the IGF-1 activation of NF-κB involves the PI 3-kinase pathway. In contrast to these findings, Bertrand et al. (48) reported that an insulin activation of NF-κB in Chinese hamster ovary cells does not involve the PI 3-kinase, but rather occurs via the Raf-1-mediated signal transduction pathway. However, these authors argued that the anti-apoptotic role of insulin in Chinese hamster ovary cells, besides NF-κB activation, additionally requires independent processes that involve PI 3-kinase activity (48). The reasons of these apparent discrepancies remain to be investigated, but might be due to the different cell type and the specific stimuli applied to the cells. So far, besides Raf-1 (33), various kinases have been reported to be involved in the pathway leading to activation of NF-κB, such as mitogen-activated protein kinase/extracellular signal-regulated protein kinase (ERK) kinase kinase 1 (49) and the kinase finally phosphorylating IκBα, conserved helix-loop-helix ubiquitous kinase (50), or IκBα kinase (51). Here we report for the first time that also the PI 3-kinase pathway may exist.

The mechanisms by which NF-κB may prevent neuronal cell death upon stimulation with IGF-1 remain unknown. The activity of this transcription factor may drive defense programs that afford the protection against oxidative insult. Therefore, in future, it will be important to identify neuroprotective target genes of NF-κB and to investigate their inducibility by exogenous factors, such as IGF-1, to increase the survival of neurons under oxidative stress.

Acknowledgments—We thank Drs. R. Weiner for providing the GT1–7 cell line, P. Baueuerle for the NF-κB-luciferase and TK-luciferase constructs, P. Jalinit for the c-Rel construct, and D. W. Ballard for the mutant IκBα construct.

REFERENCES

1. de Pablo, F., and de la Rosa, E. J. (1995) Trends Neurosci. 18, 143–150
2. LeRoith, D., Werner, H., Beinert-Johnson, D., and Roberts, C. T. (1995) Endocrinology 136, 1418–1422