

Title:

The antiangiogenic factor 16K hPRL induces caspases-dependent apoptosis by a mechanism that requires activation of NF- κ B

Abbreviated title:

NF- κ B essential for 16K hPRL-induced apoptosis

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Abstract

We have previously shown that the 16-kDa N-terminal fragment of human prolactin (16K hPRL) has antiangiogenic properties, including the ability to induce apoptosis in vascular endothelial cells. Here, we examined whether the NF- κ B signaling pathway was involved in mediating the apoptotic action of 16K hPRL in bovine adrenal cortex capillary endothelial (BACE) cells. In a dose-dependent manner, treatment with 16K hPRL induced I κ B- α degradation permitting translocation of NF- κ B to the nucleus and reporter gene activation. Inhibition of NF- κ B activation by overexpression of a non-degradable I κ B- α mutant or treatment with NF- κ B inhibitors blocked 16K hPRL-induced apoptosis. Treatment with 16K hPRL activated the initiator caspases 8 and 9 and the effector caspase 3, all of which were essential for stimulation of DNA fragmentation. This activation of the caspases cascade by 16K hPRL was also NF- κ B-dependent. These findings support the conclusion that NF- κ B signaling plays a central role in 16K hPRL-induced apoptosis in vascular endothelial cells.

Introduction

Angiogenesis, the formation of new capillaries from pre-existing blood vessels, is necessary for many physiological processes, including embryonic vascular development and differentiation, wound healing, and organ regeneration (1). In addition, angiogenesis is associated with pathological conditions such as cancer, rheumatoid arthritis, diabetic retinopathy, and atherosclerosis (2). Angiogenesis is tightly regulated by both activators and inhibitors of endothelial cell proliferation, migration and organization. Many growth factors and cytokines are known to stimulate angiogenesis. Among these, the best characterized are basic fibroblast growth factor (bFGF) (3) and vascular endothelial growth factor (VEGF) (4). On the other hand, factors with antiangiogenic activity include endostatin (5), angiostatin (6), thrombospondin (7), and the 16-kDa N-terminal fragment of prolactin (16K hPRL) (8). In addition, we have shown that recombinant 16-kDa N-terminal fragments of the human PRL-family members (i.e. growth hormone (GH), variant GH (GH-v), and placental lactogen (PL)) are also antiangiogenic (9).

The use of antiangiogenic factors as therapeutic agents is now being widely tested. Biological active 16K hPRL was expressed and secreted from HCT116 human colon cancer cells stably transfected with an expression vector encoding 16K hPRL. 16K hPRL production by the transfected HCT116 cells inhibited tumor growth and neovascularization when implanted subcutaneously in Rag1 mice (10). Furthermore, using an adenovirus transfer vector, Kim et al. have shown that expression of 16K hPRL in the prostate cancer cells markedly reduced their ability to form tumors in a xenograft animal model (11). These new data strongly suggest that 16K hPRL has potential as an anti-cancer drug.

The mechanisms by which 16K hPRL inhibits angiogenesis have been partially elucidated. 16K hPRL inhibits capillary endothelial cell proliferation, migration, and

organization into microvessels (8). This antiangiogenic effect appears to be mediated, in capillary endothelial cells, by a saturable high-affinity 16K hPRL binding site that differs from the PRL receptor (12). To date, the receptor mediating 16K hPRL activity remains unknown. Formation of new microvasculature requires activation of proteases, including urokinase (uPA). Lee *et al.* have shown that 16K hPRL inhibits uPA activation by increasing the expression of type 1 plasminogen activator inhibitor (PAI-1)(13). The anti-proliferative effect of 16K hPRL appears to involve inhibition of VEGF-induced Ras activation resulting in blocking of MAPK activation (14). Recently, Martini *et al.* demonstrated that 16K hPRL induces apoptosis of bovine brain capillary endothelial (BBCE) cells and human umbilical vein endothelial (HUVE) (15) cells. Signaling events associated with 16K hPRL-induced apoptosis includes increased DNA fragmentation, activation of caspases-1 and 3, inactivation of two caspase-3 substrates: poly (ADP-ribose) polymerase (PARP) and the inhibitor of caspase-activated DNase (ICAD). Furthermore, treatment with 16K hPRL induces conversion of the antiapoptotic form of Bcl-X, Bcl-X_L, to its proapoptotic form, Bcl-X_S.

To better understand the mechanisms by which 16K hPRL activates programmed cell death in endothelial cells, we have analyzed the effect of 16K hPRL on NF- κ B activation in BACE cells. The role of NF- κ B in apoptotic signaling is complex. It protects many cells from cell death stimuli, but in a few cases it contributes to apoptosis (16). We show here that 16K hPRL can activate NF- κ B by causing degradation of its inhibitor I κ B- α . NF- κ B is essential to 16K hPRL-induced apoptosis, since inhibition of the NF- κ B pathway prevents 16K hPRL-induced activation of caspase-3 and DNA fragmentation. The apoptotic cascade can be initiated either by death receptor activation, involving activation of initiator protease caspase-8, or by release of mitochondrial components, involving activation of initiator enzyme caspase-9. Once these caspases are activated, the cascade they have initiated leads to activation of the downstream effector protease caspase-3. We show that in BACE cells, both

pathways are essential to 16K PRL-induced apoptosis, since inhibitors of either caspase-8 or caspase-9 prevent 16K hPRL-mediated cell death. Furthermore, we show that NF- κ B plays a role in regulating this process, since NF- κ B inhibitors abolish caspase-8 and -9 activation.

RESULTS

16K hPRL Activates NF- κ B in BACE Cells

EMSA was performed in order to determine whether treatment with 16K hPRL could cause NF- κ B activation in BACE cells. Nuclear extracts from BACE cells stimulated by increasing concentrations of 16K hPRL were incubated with a 32 P-labeled κ B DNA sequence prior to gel electrophoresis (Fig. 1A). The band corresponding to the κ B/NF- κ B complex was found to increase with the 16K hPRL concentration from 2-10 nM (lanes 1-4) as compared to the band obtained with unstimulated cells (lane 1). The specificity of the κ B/NF- κ B complex detected was demonstrated by the reduced band intensity observed in the presence of a 100-fold molar excess of unlabeled κ B probe (lane 8) but not with an unlabeled mutated κ B probe (lane 9). Furthermore, the p50/p65 heterodimer was identified by its immunoreactivity towards p50- and p65- specific antibodies (lanes 6-7).

A luciferase reporter assay was used to confirm 16K hPRL-induced activation of NF- κ B. BACE cells were transiently transfected with the pElam-Luc plasmid coding for a luciferase reporter gene under the control of the Elam-1 promoter (containing three NF- κ B binding sites (17)). 16K hPRL was found to induce luciferase activity in a concentration-dependent manner in the transfected cells (Fig. 1B). Induction was maximal (8.5-fold) with 10 nM 16K hPRL. The effect of 16K hPRL on adult bovine aortic endothelial (ABAE) and brain bovine capillary endothelial (BBCE) cells was also tested, with similar results (Fig. 1C, Fig. 1D). Since I κ B- α is the most common NF- κ B inhibitor, we then examined by Western blotting the effect of 16K hPRL on the degradation of this inhibitor. When BACE cells were treated for 30 min with 16K hPRL, the level of I κ B- α protein in the cytoplasm of these cells decreased progressively as the 16K PRL concentration increased (Fig. 1E). The lowest level

of I κ B- α was observed with 10 nM 16K hPRL. I κ B- α degradation was first detected 20 min after 16K hPRL treatment (data not shown).

Since the 16K hPRL used for these studies was produced in *E.coli*, it was necessary to demonstrate that the observed effects were not due to bacterial contaminants (e.g. endotoxin). The amount of endotoxin (0.08 ng/ml) present in 10 nM 16K hPRL (Endotoxin-C lane) was found to induce only a weak response in the various NF- κ B assays. This could not account for the observed activation of NF- κ B by our 16K PRL preparation. In addition, the action of 16K hPRL was reduced when the protein was boiled for 2 min before its addition to the cells (concentration: 10 nM). Boiling for 2 min did not affect the action of endotoxin (1 ng/ml) (Fig. 1F). Furthermore, immunoneutralization with polyclonal antibody against 16K hPRL (1/250) significantly decreased the effect of 10 nM 16K hPRL, but not that of endotoxin (1 ng/ml). These results clearly demonstrate that 16K hPRL causes activation of NF- κ B in BACE cells, independent of any effect of contaminating endotoxin.

16K hPRL Induces Apoptosis of BACE Cells

Fig. 2A shows that an 18-h treatment of BACE cells with 16K hPRL resulted in the appearance of DNA fragments in the cytoplasm of the treated cells, and that the effect was dose-dependent. The highest concentration of 16K hPRL (5 nM) caused an 8.5-fold increase in DNA fragmentation as compared with untreated cells. Endotoxin-C caused only a 2-fold increase in DNA fragmentation. Heat-denaturation and immunoneutralization treatments significantly reduced the effect observed with the 16K hPRL preparation but not the action of 1 ng/ml endotoxin (Fig. 2B). These results show that the DNA fragmentation elicited by the recombinant 16K hPRL preparation is due to 16K hPRL itself and not to endotoxin contamination.

To confirm that 16K hPRL induces apoptosis in BACE cells, we studied activation of the caspase cascade. Activation of the effector protease caspase-3 is one of the most common events of the apoptotic signaling pathway. When used to treat BACE cells for 6 hours, 16K hPRL was found to induce caspase-3 activation in a concentration-dependent manner (Fig 2C). Maximal activation (20-fold) was achieved with 10 nM 16K hPRL. Endotoxin-C induced only a slight increase in activity (3-fold) as compared to untreated cells. In a time-course assay with 10 nM 16K hPRL, caspase-3 activation was visible already after a 2-h incubation and maximal after 3 h (Fig. 2D).

A Non-degradable Mutant of I κ B- α Blocks 16K hPRL-induced Apoptosis.

Given the dual effect of NF- κ B on apoptosis, we further examined the role of this factor in 16K hPRL-induced apoptosis. BACE cells were co-transfected with the pElam-Luc reporter plasmid and either the empty pcDNA-3 plasmid (control) or plasmid pmutI κ B, coding for a mutant I κ B- α (mutI κ B- α) characterized by serine-to-alanine substitutions at residue positions 32 and 36. These mutations are known to prevent I κ B- α phosphorylation and subsequent degradation. In this experiment, 16K hPRL-induced enhancement of luciferase activity was reduced in cells expressing pmutI κ B as compared to cells transfected with the empty vector (Fig. 3A). These data confirm that expression of mutI κ B- α can inhibit NF- κ B activation by 16K hPRL in BACE cells.

We then asked if overexpression of mutI κ B- α would inhibit the activation of caspase 3 by treatment with 16K hPRL (5 or 10 nM 16K hPRL for 6 hrs). In treated cells expressing mutI κ B- α , caspase-3 activity was lower than in cells transfected with the control plasmid (Fig. 3b). In four independent experiments overexpression of mutI κ B- α inhibited 16K hPRL-induced caspase 3 activation by 29% (Figure 3 shows a representative experiment).

Chemical Inhibitors of NF- κ B Block 16K hPRL-induced Apoptosis

To confirm the hypothesis that NF- κ B plays a role in 16K hPRL-induced programmed cell death, we used different pharmacological inhibitors of NF- κ B (BAY 11-7082, BAY 11-7085, and gliotoxin), known to interfere with NF- κ B activation at various levels (18, 19). In a luciferase reporter-gene expression assay, we showed that all these inhibitors block 16K hPRL-induced activation of NF- κ B. Treatment for 1 h with BAY 11-7082 (1 μ M), BAY 11-7085 (1 μ M), or gliotoxin (300 nM) prior to addition of 5 nM 16K hPRL to the cells strongly reduced the luciferase activity increase (Fig. 4A). For each compound, we have used the maximal non-toxic concentration. The action of gliotoxin was specific, since the inactive analog, methylgliotoxin (300 nM) had no effect (19).

Pretreatment for 1 h with BAY 11-7082 (1 μ M), BAY 11-7085 (1 μ M), or gliotoxin (300 nM) significantly reduced 16K hPRL-induced DNA fragmentation in BACE cells (Fig. 4B). Methylgliotoxin (300 nM) failed to block 16K hPRL-induced DNA fragmentation. Likewise, a 1-h pretreatment with either of the three inhibitors strongly reduced caspase-3 activation by 5 nM 16K hPRL, while methylgliotoxin again had no effect (Fig. 4C).

16K hPRL induces Caspase-8 and Caspase-9 Activation via NF- κ B

To investigate the initial events leading to caspase-3 activation, we monitored caspase-8 and -9 activities. The highest concentration of 16K hPRL (10 nM) caused a significant 2-fold increase in caspase-8 activity in BACE cells, as compared to untreated cells (Fig 5A). A stronger 10-fold activation of caspase-9 was observed following treatment with 16K hPRL (Fig. 5B). Activation of both caspases was detected after 2 h and peaked at 3 h. Endotoxin-C treatment led to only slightly increased caspase-8 and caspase-9 activities.

To determine whether caspase-3 activation is dependent on caspase-8, caspase-9, or both, we examined caspase-3 activation in the presence of either a caspase-9 inhibitor (Z-LETD-FMK) or a caspase-8 inhibitor (Z-IETD-FMK). Both inhibitors were found to abolish 16K hPRL-induced caspase-3 activity almost totally in BACE cells (Fig. 5C). This indicates that both initiator caspases are necessary for caspase-3 activation.

To establish whether activation of caspase-8 and caspase-9 is NF- κ B dependent, we then measured caspase-8 and caspase-9 activities in the presence of two NF- κ B inhibitors. A 1-h pretreatment with BAY 11-7082 (1 μ m) or BAY 11-7085 (1 μ M) efficiently blocked caspase-8 and caspase-9 activation by 10 nM 16K hPRL (Fig. 5D and Fig 5E).

Discussion

To better understand the signaling mechanisms involved in activation of endothelial-cell apoptosis by 16K hPRL, we have investigated the effect of 16K hPRL on the caspase signaling cascade and on activation of the NF- κ B signaling pathway. We show that, in endothelial cells, 16K hPRL can induce translocation to the nucleus and activation of NF- κ B. In BACE cells, this activation appears to be mediated by I κ B- α degradation. Degradation of I κ B- α permits translocation of NF- κ B to the nucleus. Recently, rat 16K PRL has been shown to induce NF- κ B translocation in fibroblasts. However, in this non-endothelial cell type, this translocation requires I κ B- β but not I κ B- α degradation (20).

To determine the role of NF- κ B in 16K hPRL-induced apoptosis, we first used the non-degradable I κ B- α serine-32 and serine-36 mutant. In BACE cells, phosphorylations on serine 32 and 36 of I κ B- α are required for 16K hPRL-triggered NF- κ B activation, since no activation occurs when these serines are mutated to alanine. This is consistent with the previous finding that phosphorylation of these serines triggers I κ B- α degradation (21). Transfection with the pmutI κ B- α (S32-S36) plasmid totally inhibits NF- κ B activation. On the contrary, transfection with the pmutI κ B- α (S32-S36) plasmid reduces 16K hPRL-induced caspase-3 activation by only 29%. This discrepancy can be explained by the fact that all cells that harbor the pElam-Luc plasmid also harbor the pmutI κ B- α (S32-S36) plasmid, leading to a complete repression. On the other side, the caspase activity assay is performed on all cells while only a minor fraction of them have been transfected by the pmutI κ B- α (S32-S36) plasmid. In this assay therefore, the repression level is restricted by the transfection efficiency. According to a common transfection rate obtained with primary endothelial cells

(roughly 30%,(22)), we have observed a repression of about 29%, which is the maximal we could expect. To confirm our results, we used drugs that inhibit NF- κ B activation: BAY 11-7082, BAY 11-7085 or gliotoxin. Using these chemical inhibitors, the repression levels are similar in both NF- κ B luciferase reporter- and caspase-3 activity- assays. These two last experiments provide evidence that 16K hPRL-induced apoptosis is fully NF- κ B dependent.

The antiapoptotic action of NF- κ B is well documented (23). In contrast, this study shows that NF- κ B participates in a signaling cascade leading to apoptosis of BACE cells. Induction of NF- κ B appears to be a general mechanism of 16K hPRL-induced endothelial cell apoptosis, since 16K hPRL can activate NF- κ B in two other endothelial cell types (ABAE and BBCE). Recent reports have shown that NF- κ B activation is essential for p53-induced (24) and glutamate-induced programmed cell death (25). In endothelial cells, Aoki *et al.* have demonstrated that oxidative stress can induce apoptosis through NF- κ B activation (26). In addition, NF- κ B is reported to play a proapoptotic role in growth-factor-withdrawal-induced apoptosis of B-lymphocytes (27). To date, the mechanisms underlying the pro-apoptotic action of NF- κ B remain unclear. Several findings suggest that the ability of NF- κ B to induce programmed cell death is due to its capacity to activate genes encoding Fas, Fas ligand (FasL), TRAIL, p53, or c-Myc (28-31). The Fas/FasL pathway has been shown to mediate induction of apoptosis by two other antiangiogenic factors: thrombospondin-1 and pigment-epithelium-derived factor (32). Triggering of apoptosis by activation of cell-surface death receptors involves subsequent activation of caspase-8. As NF- κ B is known to activate death ligand or death receptor expression, we speculate that 16K hPRL might induce caspase 8 activation through a similar extrinsic pathway. This hypothesis is consistent with our results, since NF- κ B inhibition prevents caspase-8 activation and caspase-8 inhibition suppresses 16K hPRL-induced caspase-3 activation. We also show that inhibition of caspase-9, known to play

a role in the mitochondria-dependent apoptosis pathway, suppresses caspase-3 activation. The requirement for both caspase-8 and caspase-9 is not contradictory, as caspase-9 might act to amplify the caspase cascade in situations where only a small amount of caspase-8 is activated (33). Indeed, this is the case here as 16K hPRL activates caspase-8 only slightly.

That an antiangiogenic factor induces apoptosis via NF- κ B activation is intriguing. In all previous studies, NF- κ B is described as a proangiogenic factor, since it can activate expression of VEGF and interleukin 8, two angiogenesis-promoting molecules (34). In addition, Shono *et al.* have demonstrated that inhibition of NF- κ B abolishes induction of angiogenesis by H₂O₂ (35). Here we propose an antiangiogenic role for NF- κ B, linked to its ability to induce apoptosis of 16K hPRL-treated endothelial cells.

The blockade of apoptosis by the constitutive activation of NF- κ B signaling pathways in cancer cells limits the efficacy of chemotherapy and radiotherapy (36). Based on this observation, treatment with NF- κ B inhibitors was proposed to improve the apoptotic response to radiotherapy or chemotherapy (37). Here we show that in highly differentiated, non-transformed, capillary endothelial cells, the apoptotic process requires NF- κ B signaling pathways. If similar mechanisms occur *in vivo*, treatment with NF- κ B inhibitors might increase apoptosis of cancer cells but might also inhibit the activity of naturally occurring antiangiogenic factors. This blocking of antiangiogenic factors could result in increased angiogenesis, thus countering the proapoptotic action of the inhibitors on the tumor cells.

In conclusion, we have demonstrated that 16K hPRL induces NF- κ B activation in endothelial cells. This induction leads, by the activation of two upstream caspases, caspase-8 and caspase-9, to the cell apoptosis. This finding reveals a novel important mechanism involved in the antiangiogenic action of 16K hPRL. Indeed, this article presents detail functional evidence that NF- κ B activation is fundamental for 16K hPRL-induced apoptosis.

Materials and Methods

Production of Recombinant Proteins and Antibodies

Recombinant human 16-KDa PRL (16K hPRL) was produced in *E. coli*. Briefly, plasmid pT7L containing 23K hPRL cDNA was altered by site-directed mutagenesis: Cys 58 (TGG) of the construct was mutated to Ser (TCC) and the GAT codon corresponding to Glu 140 was mutated to TAA so as to generate a premature stop codon. Recombinant human 16K hPRL (16K hPRL) was produced in *E.coli*. The 16K hPRL coding sequence were obtained by site-directed mutagenesis performed on the cDNA encoding hPRL minus the corresponding signal peptide inserted into the pT7L expression vector (38). An ATG was genetically engineered 5' to the first codon, Cys 58 (TGC) was mutated to serine (TCC) to prevent incorrect disulfite bonds, and Glu 140 (GAA) was mutated to TAA to generate a premature stop codon. In brief, after induction of protein expression by treatment with IPTG, the cells (*E. coli* BL21 DE3) were disrupted and the inclusion bodies were isolated. After washing, the inclusion bodies were solubilized in 20 mM Ethanolamine-HCl, pH 9, containing 8M deionized urea, 1% β -mercaptoethanol (β -ME), heated at 55°C for 10 min and incubated overnight at RT. Denatured proteins were purified by anion exchange chromatography (Hitrap Q, Amersham-Pharmacia). Elution was performed in 20 mM Ethanolamine-HCl, pH 9, 6M deionized urea, with a gradient of 0 to 1M NaCl. The renaturation was performed by a two-step dialysis during 72 hours. For the first 6 hours, a dialysis against 10 volumes of 20 mM Ethanolamine-HCl, pH 9, 6M deionized urea was performed. Then, urea from this bath was removed by buffer exchange dialysis against 500 volumes of 20 mM Ethanolamine-HCl, pH9. Purification was then performed by molecular sieve chromatography (Sephadex G100, Amersham-Pharmacia) performed in 50 mM NH_4HCO_3 , pH 7.5, 0.1M NaCl. Purified proteins were dialyzed against 100 mM Ethanolamine-HCl, pH9, and stored at -20°C with 0.1 mg/ml of bovine serum albumin (BSA).

The purity of the recombinant protein exceeded 95% (as estimated by Coomassie Blue staining), and the endotoxin level was 0.0005 ng/ng recombinant protein, as quantified with “the rapid endo test” of the European endotoxin testing service (Biowithaker Europe). The anti-16K hPRL antibody used (602) is a rabbit polyclonal antibody recognizing 16K hPRL. *E. coli* (serotype 055:B5) endotoxin was purchased from Sigma. In experiments where the action of 16K hPRL was compared with that of endotoxin, the amount of endotoxin used was the amount present in the highest 16K hPRL concentration used in the assay.

Cell Cultures

BACE (bovine adrenal cortex capillary endothelial), ABAE (adult bovine aortic endothelial), and BBCE (bovine brain capillary endothelial) cells were isolated as previously described(37). The cells were grown and serially passaged in low-glucose Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS) and 100 U/ml penicillin/streptomycin per ml (10% FCS medium). Recombinant human basic FGF (bFGF, Sigma) was added (1 ng/ml) to the culture every other day. Confluent cells corresponding to passages 7 to 12 were used in the experiments.

Preparation of Cell Extracts

BACE cells were treated or not for 40 min with various concentrations of 16K hPRL or endotoxin. The cells were scraped into PBS, centrifuged at 300g for 5 min and the pellet resuspended in 80 µl of ice-cold hypotonic buffer (10 mM HEPES, pH 7,9; 2 mM MgCl₂; 10 mM KCl; 0,1 mM EDTA; 1 mM PMSF; 1 mM DTT; 0,2% NP-40; 10 µg/ml Leupeptine (Sigma); 0,7 µg/ml Aprotinine (Roche); 0,1 µg/ml Pefabloc (Boehringer)) for 30 sec just before centrifugation at 300g for 5 min. Aliquots of supernatant containing the cytoplasmic proteins were quickly frozen and stored at -80°C. The nuclear pellet was washed in PBS before being resuspended in 40 µl of extraction Buffer (20 mM HEPES, pH 7,9; 1.5 mM

MgCl₂; 10 mM KCl; 0,2 mM EDTA; 1 mM PMSF; 0.5 mM DTT; 630 mM NaCl) and left 30 min at 4° C. After centrifugation (30 min at 2500g), aliquots of supernatant containing the nuclear proteins were stored at -80°C.

Protein concentrations were determined by the Bradford method using the Bio-Rad protein assay reagent.

Western Blot Analysis

Thirty micrograms of cytoplasmic cell lysates were resolved by SDS/PAGE (12%) and transferred to a Polyvinilidene fluoride membrane (Millipore). The blots were blocked for 1 h with 8% milk in TBS with 0,1 % Tween-20 (TBST) and were probed for 1 h with rabbit anti-human IκB-α pAb (C-21, Santa-Cruz) at 1:600 dilution (333 ng/ml). After three washing with TBST, the antigen-antibody complexes were detected with goat peroxidase conjugated secondary antibody and an enhanced fluoro-chemiluminescent system (ECL-plus; Amersham Pharmacia). Finally, the blots were visualized on a Molecular Imager Fx (Biorad).

Electrophoretic Mobility Shift Assays (EMSAs)

The double-stranded oligonucleotide containing a palindromic variant of the κB enhancer of the interleukin-2 receptor α-chain binding site (39) has the following sequence: 5'-TTGGCAACGGCAGGGGAATCCCCCTCTCCTTA-3' (the core of the NF-κB binding site is underlined). This κB sequence (Eurogentec, Seraing, Belgium) was labeled using the Klenow fragment of *E. coli* DNA polymerase (Gibco), α-³²P dCTP, and α-³²P dATP.

In the supershift assay, 1 μl antibody against p50 or p65 (Santa Cruz Biotechnology: sc 114X, sc 109X) was added to the extract (30 min on ice) before incubation with the labeled κB

probe. In the competition assay, a 100-fold molar excess of unlabeled wild-type or mutant κ B was added to the binding reaction mixture 15 min before incubation with the nuclear extract. The gel was dried for 1 h at 80°C before visualization with a Molecular Imager Fx (Biorad).

DNA Fragmentation ELISA

BACE cells were treated or not with various concentrations of 16K hPRL or endotoxin for the indicated time. DNA fragmentation was measured with the Cell Death^{plus} Detection ELISA kit (Roche Molecular Biochemicals) according to the manufacturer's instructions.

Caspase Activity Assay

BACE cells were treated or not with various concentrations of 16K hPRL or endotoxin. After the indicated incubation time, caspase-3 activity was measured with the CaspACE Assay System Fluorometric (Promega), caspase-8 with the Caspase-8 Assay kit Fluorimetric (Sigma), and caspase-9 with the Caspase-9 Fluorimetric Assay (RD systems) according to the manufacturer's instructions. Z-IETD-FMK and Z-LEHD-FMK were purchased from Calbiochem.

Transient Transfections

BACE cells were transfected with 0.4 μ g pElam-Luc reporter plasmid alone or in combination with 0.2 μ g pmutI κ B- α (S32-S36) plasmid or 0.2 μ g of pcDNA-3 plasmid (Luciferase assay). In the caspase-3 assay, cells were transfected with 0.4 μ g pmutI κ B- α (S32-S36) plasmid or with the same quantity of plasmid pcDNA-3. Transfections were performed with Fugene 6 liposomes (Roche) according to the manufacturer's instruction. The pElam-Luc plasmid contains, upstream from the luciferase gene (17), the -730 to +52 region of the E-Selectin

promoter, containing 3 copies of the NF- κ B binding site. Plasmid pmutI κ B- α (S32-S36) was kindly provided by Dr C. Jobin (University of North Carolina, Chapel Hill, NC) (40).

Luciferase activity

Cells were transfected as described above and treated or not for 6 h with various concentrations of 16K hPRL or endotoxin. They were washed once with cold PBS before addition of 200 μ l lysis buffer (25 mM Tris-P; 8 mM MgCl₂; 1 mM EDTA; 1% Triton; 15% Glycerol; 1 mM DTT; 0.2 mM PMSF) and incubation for 20 min. Luciferase activity was measured in 100 μ l supernatant with a 96-well plate reader (Wallac Victor², PerkinElmer).

Statistical analysis

All values are expressed as means \pm SD. All experiments were performed in triplicate at least 3 times. Comparisons between different treatments were assessed with Student's T-test. The statistical significance limit was set at $p < 0.05$.

Figure 1: 16K hPRL induces NF- κ B activation in endothelial cells.

A) EMSAs were performed with BACE-cell nuclear extracts treated or not for 40 min with various concentrations of 16K hPRL or Endotoxin-C (0.08 ng/ml) and pre-incubated either without (lanes 1-5) or with addition of p50-specific antibody (lane 6) or p65-specific antibody (lane 7). In competition assays, extracts were pre-incubated with a 100-fold molar excess of unlabeled oligonucleotide containing either wild-type (wt) (lane 8) or mutated (mut) (lane 9) NF- κ B binding sites.

B, C, and D) Luciferase activity was measured in BACE (B), ABAE (C), and BBCE (D) cells transfected with the pElam-Luc reporter gene vector after incubation for 6 h with increasing concentrations of 16K hPRL. E) Western-blot detection of I κ B- α in cytoplasmic extracts of BACE cells treated for 30 min with increasing concentrations of 16K hPRL. F) Transfected BACE cells were treated for 6h with 5 nM 16K hPRL or 1 ng/ml endotoxin, these preparations being or not being boiled beforehand for 2 min or pre-incubated with a polyclonal antibody (602) (1/250) recognizing 16K hPRL. All luciferase activities, normalized with respect to the total protein content, are expressed as enhancement factors (activity measured divided by the activity of untreated cells). Each bar represents the mean \pm SD, n = 3. Endotoxin-C (0.08 ng/ml) was added as a control for endotoxin contamination.

Figure 2: 16K hPRL induces apoptosis in BACE cells.

DNA fragmentation induced by 16K hPRL: A) Cells were treated for 16 h with increasing concentrations of 16K hPRL. Endotoxin-C (0.04 ng/ml) was added as a control for endotoxin contamination. B) Cells were treated for 16 h with 5 nM 16K hPRL or 1 ng/ml endotoxin. 16K hPRL and endotoxin samples were also boiled for 2 min or pre-incubated with a polyclonal antibody (602) (final dilution 1/250) recognizing 16K hPRL. DNA fragmentation was measured using the Cell Death^{plus} Detection ELISA kit assay (Roche Molecular Biochemicals) and results are expressed as enhancement factors (treated vs. untreated cells).

Caspase-3 activation by 16K hPRL: C) BACE cells were treated for 6 h with increasing concentrations of 16K hPRL or with 0.04 ng/ml endotoxin (Endotoxin-C). D) BACE cells were treated with 10 nM 16K hPRL for the indicated time. Caspase-3 activation was measured with the CaspACE Assay System Fluorometric (Promega). Each result is expressed as an enhancement factor (treated vs. untreated cells).

Each bar represents the mean \pm SD, n = 3.

Figure 3: Expression of mutant *I κ B- α* decreases 16K hPRL-induced apoptosis.

A) BACE cells were co-transfected with the pElam-Luc reporter gene vector and either an expression vector for mutant *I κ B- α* (pmut*I κ B- α*) or an empty vector (pcDNA-3). Twenty-four hours after transfection, the cells were treated with 5 nM or 10 nM 16K hPRL. Luciferase activity was measured 6 h later. Luciferase activities, normalized with respect to the total protein content, are expressed as enhancement factors (treated versus untreated cells).

B) BACE cells were transfected with an expression vector for mutant *I κ B- α* (pmut*I κ B- α*) or with an empty vector (pcDNA-3). Twenty-four hours after transfection, the cells were treated with 5 nM or 10 nM 16K hPRL for 6 h. Caspase-3 activation was measured using the CaspACE Assay System Fluorometric (Promega). Results are expressed as enhancement factors (treated vs. untreated cells). The asterisk denotes a P value <0.05 vs. the corresponding pcDNA-3-transfected cells. Each bar represents the mean \pm SD, n = 3. The experiment was repeated three times with similar results.

Figure 4: Chemical inhibitors of NF- κ B reduce 16K hPRL-induced apoptosis.

A) BACE cells transfected with the pElam-Luc reporter gene vector were pretreated or not for 1 h with BAY 11-7082 (1 μ M), BAY 11-7085 (1 μ M), gliotoxin (300 nM), or methylgliotoxin (300 nM) before stimulation for 6 h with 5 nM 16K hPRL. Luciferase activities, normalized with respect to the total protein content, are expressed as enhancement factors (treated versus untreated cells).

BACE cells were treated as above. B) DNA fragmentation as estimated with the Cell Death^{plus} Detection ELISA kit assay (Roche Molecular Biochemicals). Results are expressed as enhancement factors (treated vs. untreated cells). C) Caspase-3 activity measured by means of the CaspACE Assay System Fluorometric (Promega). Results are expressed as enhancement factors (treated vs. untreated cells). Each bar represents the mean \pm SD, n = 3.

Figure 5: 16K hPRL induces NF- κ B-dependent caspase 8 and caspase 9 activation

A and B) BACE cells were treated for 3 h with 5 nM or 10 nM 16K hPRL or with 0.08 ng/ml endotoxin (Endotoxin-C). In the time-course experiments, BACE cells were treated with 10 nM 16K hPRL for the indicated time. Caspase-8 activation was measured with the Caspase-8 Assay kit Fluorimetric (Sigma). Caspase-9 activation was measured using the Caspase-9 Fluorimetric Assay (RD Systems). C) BACE cells were pretreated or not with Z-IETD-FMK (10 μ M), Z-LEHD-FMK (10 μ M), or DMSO (0.5% v/v - control) prior to stimulation for 3 h with 10 nM 16K hPRL. D and E): BACE cells were pretreated or not for 1 h with BAY 11-7082 (1 μ M), BAY 11-7085 (1 μ M), gliotoxin (300 nM), or methylgliotoxin (300 nM) before stimulation for 3 h with 10 nM 16K hPRL. Caspase-8 activation (D) and caspase-9 activation (E) were measured as describe above. Results are expressed as enhancement factors (treated vs. untreated cells). Each bar represents the mean \pm SD, n = 3.

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