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ORIGINAL ARTICLE

# Lack of Oestrogenic Inhibition of the Nuclear Factor-κB Pathway in Somatolactotroph Tumour Cells

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Activation of nuclear factor (NF)-κB promotes cell proliferation and inhibits apoptosis. We have previously shown that oestrogens sensitise normal anterior pituitary cells to the apoptotic effect of tumour necrosis factor (TNF)-α by inhibiting NF-κB nuclear translocation. In the present study, we examined whether oestrogens also modulate the NF-κB signalling pathway and apoptosis in GH3 cells, a rat somatolactotroph tumour cell line. As determined by Western blotting, 17β-oestradiol ($E_2$) ($10^{-9}$ M) increased the nuclear concentration of NF-κB/p105, p65 and p50 in GH3 cells. However, $E_2$ did not modify the expression of Bcl-xL, a NF-κB target gene. TNF-α induced apoptosis of GH3 cells incubated in either the presence or absence of $E_2$. Inhibition of the NF-kB pathway using BAY 11-7082 (BAY) (5 μM) decreased the viability of GH3 cells and increased the percentage of terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)-positive GH3 cells. BAY also increased TNF-α-induced apoptosis of GH3 cells, an effect that was further increased by an inhibitor of the c-Jun N-terminal protein kinase pathway, SP600125 (10 μM). We also analysed the role of the NF-κB signalling pathway on proliferation and apoptosis of GH3 tumours *in vivo*. The administration of BAY to nude mice bearing GH3 tumours increased the number of TUNEL-positive cells and decreased the number of proliferating GH3 cells. These findings suggest that GH3 cells lose their oestrogenic inhibitory action on the NF-κB pathway and that the pro-apoptotic effect of TNF-α on these tumour pituitary cells does not require sensitisation by oestrogens as occurs in normal pituitary cells. NF-κB was required for the survival of GH3 cells, suggesting that pharmacological inhibition of the NF-κB pathway could interfere with pituitary tumour progression.

**Key words:** NF-κB, pituitary, apoptosis, TNF-α, oestrogens

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The activity of nuclear factor (NF)-κB, an inflammatory transcription factor, is dysregulated in tumour cells (1–4). An important molecular link between inflammation and tumour promotion and progression is represented by the activation of NF-κB because it regulates the expression of many genes involved in the suppression of tumour cell death (5,6). Also, the activation of inflammatory genes by NF-κB creates an microenvironment that promotes tumour progression, angiogenesis and metastasis (7). NF-κB is a family of transcription factors that exist as homodimers or heterodimers of its members: c-Rel, RelA (p65), RelB, p105/p50 and p100/p52 (8). In the classical NF-κB pathway, binding of pro-inflammatory cytokines to their receptors activates the I-κB kinase complex, which phosphorylates the inhibitory proteins I-κBs, leading to their ubiquitination and proteasomal degradation. Subsequently, NF-κB/p65 and p50 can translocate to the nucleus thereby regulating gene transcription by binding to specific κB response elements (9,10). Although NF-κB generally inhibits cell death by inducing the expression of pro-survival proteins, it also sensitises cells to pro-apoptotic factors, depending on the cell context (11,12). Activation of this transcription factor leads to the secretion of many immunoregulatory molecules, including pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α (13,14). In turn, TNF-α exerts its biological action by triggering various signalling pathways, including the NF-κB pathway, c-Jun N-terminal protein kinase (JNK) and caspases (15–17). JNK is a group of serine/threonine kinases that turn on genes controlling several cellular functions, including stress response, cell differentiation, cell survival and apoptosis (18). Activation of NF-κB and transient activation of JNK are associated with cell survival, whereas sustained JNK activation contributes to cell death. Importantly, it has been shown that impaired NF-κB activity

results in sustained JNK activation, leading to cell death (1,19). Nevertheless, JNK activation may be required for cell survival in some biological contexts (20–22).

Oestrogen receptors (ERs) have been reported to interact with NF-κB, usually in an antagonistic way (23,24). However, an agonistic effect has also been observed (25). ERs can inhibit NF-κB transcriptional activity by several mechanisms depending on the stimulus or cell type. A reduction in nuclear DNA binding of NF-κB has been reported to occur via protein–protein interaction between ER and NF-κB or by ER-mediated inhibition of NF-κB binding to transcriptional coactivators (24,26).

We previously reported that oestrogens sensitise the anterior pituitary to pro-apoptotic stimuli such as lipopolysaccharide (LPS), TNF-α, Fas ligand (FasL) and dopamine (27–30). We also reported that oestrogens inhibit NF-κB activation in normal pituitary cells, suggesting that this action is involved in the pro-apoptotic action of oestrogens in these cells (31). Pituitary adenomas are among the most prevalent types of intracranial tumours in adults (32). To examine whether oestrogens also modulate the NF-κB signalling pathway in pituitary tumours, we investigated the effect of 17β-oestradiol ($E_2$) on nuclear translocation of NF-κB and on TNF-α-induced apoptosis in GH3 cells, a rat pituitary cell line that secretes growth hormone and prolactin. Also, to explore the involvement of NF-κB signalling pathway in the physiopathology of pituitary tumours, we examined the effect of NF-κB and JNK inhibition, using an inhibitor of the NF-κB pathway, BAY 11-7082 (BAY) and a JNK inhibitor, SP600125 (SP), respectively, on the apoptotic response of GH3 cells (33–36). Finally, we analysed the role of the NF-κB signalling pathway on proliferation and apoptosis of GH3 tumours *in vivo*. Our results indicate that somatolactotroph tumour cells lose oestrogenic modulation of the NF-kB pathway and also suggest that NF-κB would be an interesting target for the clinical management of pituitary tumours.

## Materials and methods

### Drugs

All drugs, media and supplements were obtained from Sigma (St Louis, MO, USA), except for foetal bovine serum (FBS) (Natocor, Córdoba, Argentina), amphotericin B, essential amino acids and gentamicin (Invitrogen, Carlsbad, CA, USA), terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) reagents (Roche Molecular Biochemicals, Mannheim, Germany), anti-BrdU (BD Bioscience, San Jose, CA, USA), anti-rabbit and anti-mouse streptavidin horseradish peroxidase (HRP) conjugated secondary antibodies and anti-guinea pig Alexa 555-conjugated secondary antibody (Chemicon International, Temecula, CA, USA), guinea pig anti-rat prolactin antiserum (Dr A. Parlow, National Hormone and Pituitary Program, Torrance, CA, USA), BAY 11-7082 (Enzo Life Sciences International, Plymouth Meeting, PA, USA), SP600125 (Calbiochem, Nottingham, UK) and the materials indicated below.

### Animals

Female adult Wistar rats (200–250 g) and N:NIH (S)-Foxlnu nude mice (25–30 g) were maintained under 12 : 12 h light/dark cycles at 20–22 °C. Animals were fed standard laboratory chow and water *ad lib.* and kept in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Rats were ovariectomised 2 weeks before the experiments under ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) anaesthesia and ketoprofen (5 mg/kg) for analgesia. Rats were killed by decapitation and mice were killed by cervical dislocation. Animal protocols were previously approved by the Ethics Committee of the School of Medicine, University of Buenos Aires (Res. No 1204/2010).

### *In vivo* experiments: xenograft model

Eight-week old nude mice were injected s.c. into the right flank with $3 \times 10^6$ GH3 cells. Tumour size was measured every 2 days with a calliper and tumour volume was estimated according to the formula: [width$^2$ × length]/2 (mm$^3$). When the tumour volume reached approximately 500 mm$^3$, mice were injected i.p. with BAY (20 mg/kg b.w.) or vehicle (2% ethanol in 0.9% NaCl) every 2 days for a week. Twenty four hours before killing, mice were injected i.p. with 5-bromo-2′-deoxyuridine (BrdU) (50 mg/kg b.w.). Tumours were removed and fixed with 4% paraformaldehyde and embedded in paraffin. Sections (4 μm) were deparaffinised in xylene, rehydrated in graded ethanol, and processed for determination of cell proliferation and apoptosis by BrdU incorporation and TUNEL methods, respectively.

### Cell culture

Rat anterior pituitary glands were washed several times with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 μl/ml MEM amino acids, 2 mM glutamine, 5.6 μg/ml amphotericin B, 100 μg/ml streptomycin (DMEM-S) and 3 mg/ml bovine serum albumin (BSA). Then, glands were cut into small fragments. Sliced fragments were dispersed enzymatically by successive incubations in DMEM-S-BSA, containing 0.75% trypsin, 10% FBS previously treated with 0.025% dextran-0.25% charcoal (FBS-DCC) to remove steroids, and 45 U/μl deoxyribonuclease type I (Invitrogen). Finally, cells were dispersed by extrusion through a Pasteur pipette in Krebs buffer without $Ca^{2+}$ and $Mg^{2+}$. Dispersed cells were washed and resuspended in DMEM-S with 10% FBS-DCC. GH3 cells were cultured in flasks containing DMEM supplemented with 10 μl/ml MEM amino acids, 2 mM glutamine, 0.56 μg/ml amphotericin B, 100 U/ml penicillin, 100 μg/ml streptomycin, 5% FBS and 5% foetal horse serum. Cells were harvested with 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA). Cell viability assessed by trypan blue exclusion was over 90%. Dispersed cells were seeded onto coverslides in 24-well tissue culture plates for the TUNEL assay ($1 \times 10^5$ cells/ml/well), Western blotting ($1 \times 10^6$ cells/ml/well) or flow cytometry ($3 \times 10^5$ cells/ml/well) or onto 96-well tissue culture plates for 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) ($2.5 \times 10^5$ cells/l/well) assays. After the culture period, cells were incubated in serum-free Dulbecco's medium without phenol red containing vehicle (ethanol; 1 μl/l) or $E_2$ ($10^{-9}$ M) for 24 h and for a further 24 h with TNF-α (50 ng/ml) or vehicle in the same media with or without $E_2$. In some experiments, GH3 cells were incubated with TNF-α in serum-free Dulbecco's medium without phenol red containing a NF-κB pathway inhibitor, BAY (2.5 μM) and/or a JNK inhibitor, SP (10 μM) or the corresponding vehicle [dimethyl sulphoxide (DMSO)] 0.5 ml/l, ethanol 0.05 ml/l] for further 24 h.

### Cell-cycle analysis by flow cytometry [fluorescence-activated cell sorting (FACS)]

Cultured GH3 cells were harvested with 0.05% trypsin-EDTA and washed in-cold phosphate-bufffered saline (PBS). Cells were fixed in ice-cold 70% ethanol and centrifuged. DNA was stained with propidium iodide (50 mg/ml) in PBS containing ribonuclease (10 mg/ml) for 20 min at 37 °C. The fluorescence intensity of ≥ 6000 gated cells/tube was analysed by FACS using a FACScan (Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA). Cells with

a propidium iodide staining intensity lower than the G0/G1 peak were considered hypodiploid. Analysis of DNA content was performed using WinMDI 98 (http://facs.scripps.edu/software.html) and Cylcherd 1.2 software (http://www.imm.fm.ul.pt/wiki/doku.php?id=facility:flowcyt:software).

### Western blotting

Total proteins were extracted from GH3 cells in fresh lysis buffer containing 250 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1% IGEPAL, 0.02% sodium azide, 0.1% sodium dodecyl sulphate, in 50 mM Tris-HCl pH 7.4 and protease inhibitor cocktail (dilution 1 : 100). Following centrifugation, the supernatant was used for the Bcl-xL immunoblot assay. The nuclear fraction was obtained from rat anterior pituitary cells or GH3 cells using fresh lysis buffer containing10 mM KCl, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 1% IGEPAL, 0.2 mM EDTA in 10 mM Hepes, pH 7.9 and protease inhibitor cocktail (dilution 1 : 100). Following differential centrifugation as described previously (31), the supernatant was used for the NF-κB immunoblot assay. The protein concentration of each sample was determined by the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Thirty micrograms of total protein or 15 μg of protein from the nuclear fraction were size-fractionated in 12% sodium dodecyl sulphate-polyacrylamide gel, and then electrotransferred to polyvinyl difluoride membranes. Blots were blocked for 90 min in 5% non-fat dry milk-Tris-buffered saline 0.1% Tween 20 at room temperature and incubated overnight with the appropriate primary antibody in the same buffer at 4 °C. The primary antibodies used were: anti-NF-κB/p65 (Becton-Dickinson Biosciences; dilution 1 : 250), which recognises p105, p65 and p50 proteins of the NF-κB family, and Bcl-xL (Stressgen, Ann Arbor, MI, USA; dilution 1 : 500). This was followed by incubation with the corresponding HRP secondary antibody (dilution 1 : 500) for 1 h. Proteins incubated with buffer without primary antibody were used as negative controls. Immunoreactivity was detected by enhanced chemiluminescence (Productos Bio-Lógicos, Buenos Aires, Argentina). Chemiluminescence was determined by chemiluminiscence imaging system (G Box Chemi HR16; Syngene, Cambridge, UK) and bands were quantified using Gene Tools software (Syngene). Intensity data were normalised with respect to the corresponding β-actin blot. Data were expressed as relative increment versus corresponding control.

### Detection of BrdU incorporation

Tumour sections were permeabilised by microwave irradiation. Sections were incubated with anti-BrdU diluted in nuclease solution (GE Healthcare, Little Chalfont, UK) in accordance with the manufacturer's instructions, then incubated with fluorescein-conjugated anti-mouse secondary antibody (dilution 1 : 200). For identification of lactotropes, sections were incubated with 10% normal donkey serum in PBS-0.2% Triton for 90 min. Then, sections were incubated for 1 h with guinea pig anti-rat prolactin antiserum (dilution 1 : 1500) followed by 1 h of incubation with Alexa 555-conjugated anti-guinea pig secondary antibody (dilution 1 : 2000). Control sections were incubated with buffer instead of primary antibodies. Data were expressed as the number of BrdU-positive cells/field.

### TUNEL assay

Deparaffinised tumour sections or fixed GH3 cells were permeabilised by microwave irradiation. DNA strand breaks were labelled with digoxigenin-deoxyuridine triphosphate using terminal deoxynucleotidyl transferase (0.18 U/μl) in accordance with the manufacturer's instructions. After incubation with 10% donkey serum and 10% sheep serum in PBS for 90 min, sections were incubated for 60 min with guinea pig anti-rat prolactin antiserum (dilution 1 : 1500). Tissue sections or cells were incubated with 10% sheep serum and then for 1 h with fluorescein conjugated anti-digoxygenin antibody (dilution 1 : 10) to detect the incorporation of nucleotides into the 3′-OH end of damaged DNA. Tissue sections were incubated simultaneously with anti-guinea pig-Alexa 555 antibody (dilution 1 : 5000). Then, slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) containing 4,6-diamidino-2-phenylindoledihydrochloride for DNA staining and visualised under a fluorescent light microscope (Axiophot; Carl Zeiss, Jena, Germany). Data of apoptotic cells in sections were expressed as the number of TUNEL-positive cells/field. The percentage of apoptotic cultured GH3 cells was calculated as [(TUNEL+)/total GH3 cells] × 100.

### Assessment of metabolic activity of viable cells

The metabolic activity of viable cells was determined by the MTS assay (Promega, Madison, WI, USA). Twenty microlitres of reaction solution containing MTS (final concentration 333 μg/ml) and an electron coupling reagent (phenazine ethosulphate, final concentration 25 μM) were added to each well containing 100 μl of culture medium. After 4 h at 37 °C, the optical density was read at a wavelength of 490 nm. The quantity of formazan product is directly proportional to the number of living cells in culture.

### Statistical analysis

#### In vitro *experiments*

Data on cell viability and the percentage of hypodiploid cells were expressed as the mean ± SE and evaluated by Student's *t*-test or two-way ANOVA followed by Tukey's test. Normalised Western blot data were analysed by repeated measures ANOVA followed by Tukey's test (NCSS statistical software; NCSS, Kaysville, UT, USA). Apoptosis, as determined by the TUNEL method, was expressed as the percentage of apoptotic cells ± 95% confidence limits (CL) of the total number of cells counted in each specific condition. Confidence intervals for proportions were analysed by chi-squared. $P < 0.05$ was considered statistically significant. All experiments were performed at least twice.

#### In vivo *experiments*

The mean of BrdU-positive cells or TUNEL-positive cells per field from 10 to 24 fields of three sections from each mouse was considered as an individual value and data were analysed by Student's *t*-test.

## Results

### Effect of $E_2$ on TNF-α-induced activation of the NF-κB pathway in GH3 cells

We previously reported that, in normal anterior pituitary cells, $E_2$ decreases TNF-α-induced NF-κB nuclear translocation (31) and reduces Bcl-xL expression, an anti-apoptotic protein target for this transcriptional factor (37). To evaluate whether oestrogens also modulate NF-κB activity in pituitary tumour cells, we examined the effect of $E_2$ on basal and TNF-α-induced nuclear translocation of NF-κB and on Bcl-xL expression in GH3 pituitary tumour cells. We detected NF-κB/p50 and p65 in the nucleus of these cells (Fig. 1A,B). In addition, we also detected nuclear NF-κB/p105 (Fig. 1C), a NF-κB family member that was absent in normal pituitary cells (Fig 1D). $E_2$ ($10^{-9}$ M) increased the nuclear concentration of NF-κB/p50, p65 and p105 in GH3 cells (Fig. 1A–C). TNF-α (50 ng/ml) increased

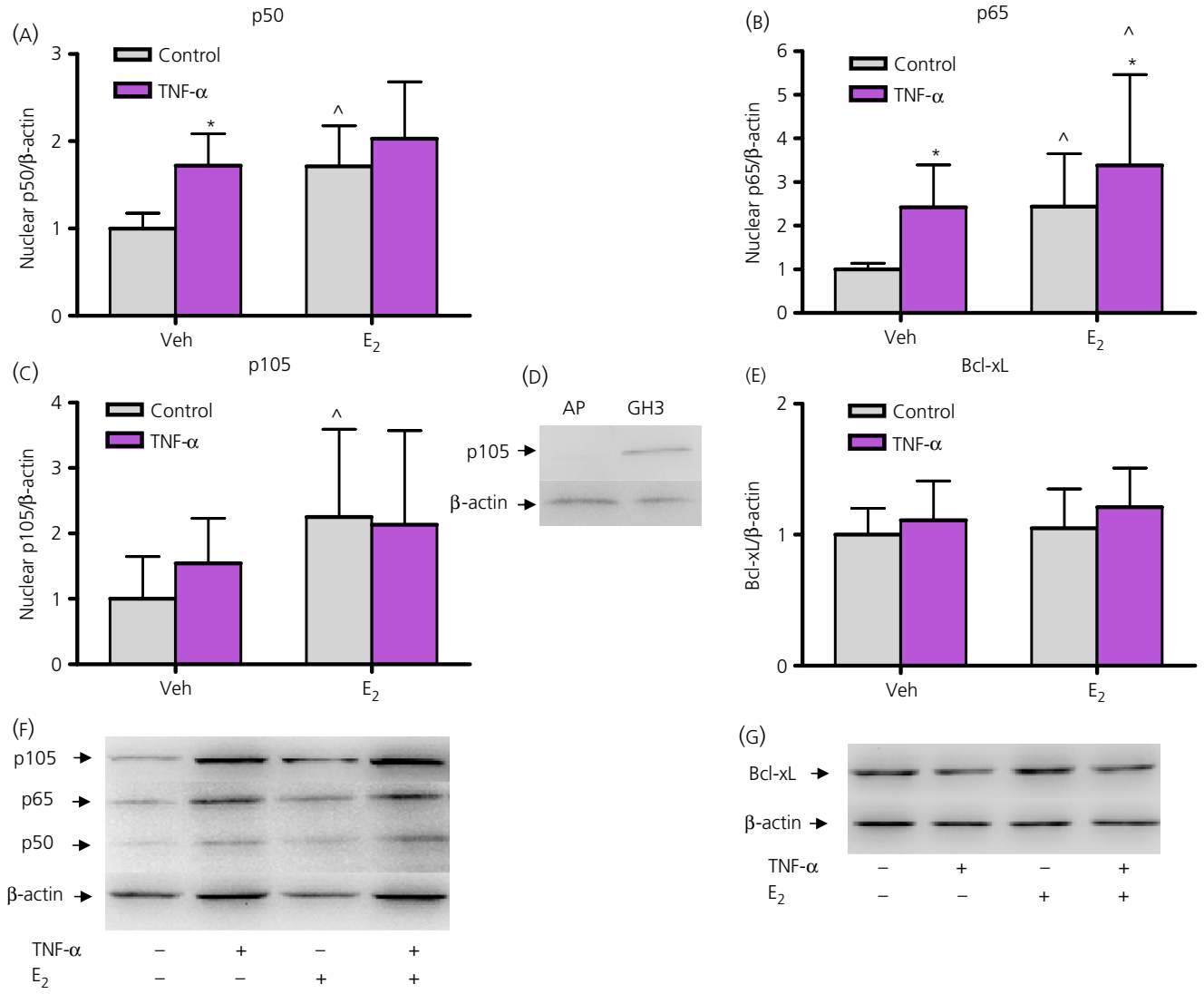


**Fig. 1.** Effect of oestrogens on tumour necrosis factor (TNF)-α-induced nuclear concentration of nuclear factor (NF)-κB/p105, p65 and p50 and total expression of Bcl-xL in somatolactotroph tumour cells. GH3 cells were incubated with 17β-oestradiol ($E_2$) ($10^{-9}$ M) or vehicle (Veh; ethanol, 1 μl/l) for 24 h, then with TNF-α (50 ng/ml) in the same media for further 24 h. The nuclear concentration of NF-κB/p50 (A), p65 (B), p105 (C) and total levels of Bcl-xL (E) were determined by Western blotting. Densitometric data from three or four experiments were normalised by the corresponding β-actin value and analysed by repeated measures ANOVA followed by Tukey's test. Each column represents the mean ± SE. *$P < 0.05$ versus respective control without TNF-α. ^$P < 0.05$ versus the respective control without $E_2$. (D) Representative western blot of nuclear NF-κB/p105 in cultured anterior pituitary cells from ovariectomised rats (AP) and GH3 cells (F, G). Representative western blots for NF-κB proteins and Bcl-xL, respectively, using β-actin as loading control.

the nuclear levels of NF-κB/p50 and NF-κB/p65 (Fig. 1A,B). However, neither $E_2$, nor TNF-α modified Bcl-xL expression (Fig. 1E), suggesting that this NF-κB transcriptional activity may be absent in GH3 cells.

## Effect of TNF-α on apoptosis of GH3 cells

We previously reported that TNF-α-induced apoptosis of normal pituitary cells is oestrogen-dependent (28). To explore whether oestrogens modulate TNF-α-induced apoptosis in pituitary tumour cells, GH3 cells were incubated with TNF-α in the presence of $E_2$ ($10^{-9}$ M). TNF-α (50 ng/ml) increased the percentage of hypodiploid GH3 cells incubated either with or without $E_2$ (Fig. 2A), indicating that the pro-apoptotic action of TNF-α in pituitary tumour cells is oestrogen-independent. Indeed, TNF-α increased the percentage of TUNEL-positive GH3 cells incubated without $E_2$ (Fig. 2B).

## Role of the NF-κB signalling pathway in TNF-α-induced apoptosis of GH3 cells

The fact that NF-κB nuclear accumulation did not suppress TNF-α-induced apoptosis in GH3 cells raised the question of whether the NF-κB pathway is necessary for GH3 cell survival. Therefore, we explored the effect of the NF-κB pathway inhibitor BAY on GH3

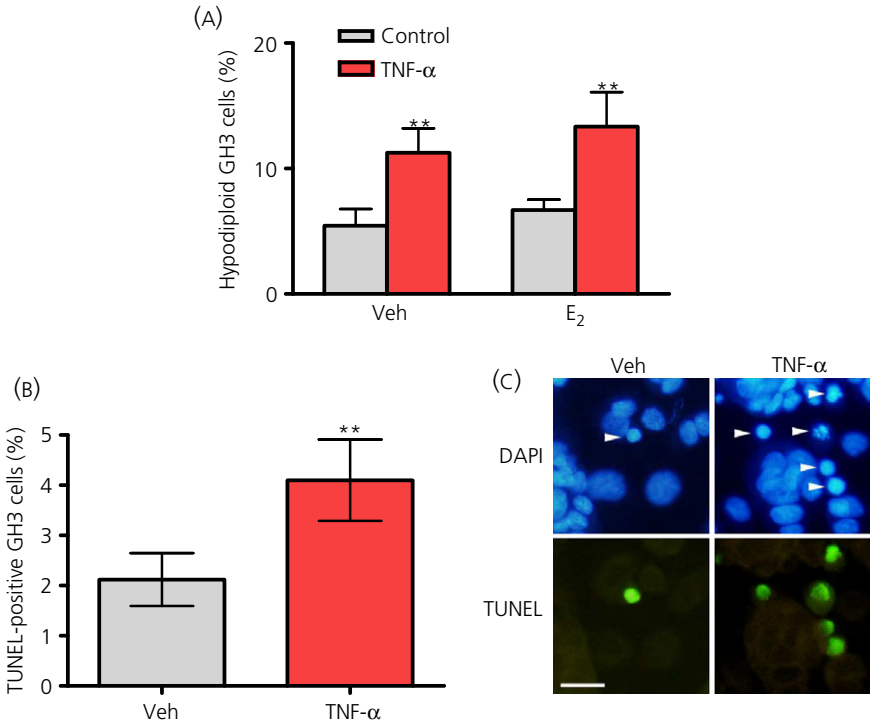


**Fig. 2.** Effect of tumour necrosis factor (TNF)-α on GH3 cell apoptosis. (A) GH3 cells were incubated with 17β-oestradiol ($E_2$) ($10^{-9}$ M) or vehicle (Veh; ethanol, 1 μl/l) for 24 h, then with TNF-α (50 ng/ml) in the same media for further 24 h. The percentage of hypodiploid cells was determined by flow cytometry using propidium iodide (PI). Each column represents the mean ± SE of the percentage of Sub G0/G1-phase cells in three wells per group. **P < 0.01; two-way ANOVA followed by Tukey's test. (B) GH3 cells were incubated with TNF-α (50 ng/ml) for 24 h. The percentage of apoptotic cells was determined by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL). Each column represents the percentage ± confidence limit (CL) of TUNEL-positive GH3 cells (n = 2500–3000 GH3 cells/group from two independent experiments). **P < 0.01; chi-square. (C) Representative microphotographs show TUNEL-positive GH3 cells. Arrowheads indicate apoptotic GH3 cells. Scale bar = 20 μm. DAPI, 4,6-diamidino-2-phenylindoledihydrochloride.

cell apoptosis. BAY irreversibly inhibits cytokine-induced IκB-α phosphorylation, thus blocking its ubiquitination and proteasomal degradation (33). BAY alone at a concentration of 5 μM decreased the metabolic activity of viable GH3 cells (Fig. 3A) and increased the percentage of TUNEL-positive GH3 cells (Fig. 3B). We then incubated GH3 cells in the presence of 2.5 μM BAY, the highest concentration without effect *per se*, and TNF-α. Although TNF-α alone induced apoptosis of GH3 cells, the presence of BAY further increased this pro-apoptotic effect (Fig. 4). These data suggest that, although NF-κB activation is not sufficient to restrain the pro-apoptotic effect of TNF-α in GH3 cells, NF-κB may still be functional in these cells.

### Involvement of the JNK pathway on TNF-α-induced apoptosis of GH3 cells

Considering that the NF-κB pathway provides a survival signal for GH3 cells and that JNK activity is regulated by NF-κB, we studied the effect of a JNK inhibitor, SP (36), on TNF-α-induced apoptosis of GH3 cells. SP (10 μM) did not modify the pro-apoptotic action of TNF-α in pituitary tumour cells (Fig. 5A). However, when the NF-κB pathway was inhibited by BAY (2.5 μM), SP increased TNF-α-induced apoptosis of GH3 cells (Fig. 5B) suggesting that, when NF-κB activity is reduced, JNK can inhibit cell death in pituitary tumour cells.

### *In vivo* effect of NF-κB pathway inhibition on apoptosis and proliferation of pituitary tumour cells

To explore the *in vivo* effect of NF-κB pathway inhibition on apoptosis and proliferation of pituitary tumour cells, we administered BAY to nude mice bearing GH3 tumours. When the tumour volume reached approximately 500 $mm^3$, mice were injected with BAY (20 mg/kg b.w.) every 2 days for 1 week and with BrdU 24 h before killing. BAY treatment did not affect the general health of the mice. Tumours were removed and processed for determination of cell proliferation by BrdU incorporation and apoptosis by the TUNEL method. Although tumour volume was not significantly modified by BAY treatment (vehicle: 710.00 $mm^3$ ± 57.50, BAY 622.22 ± 25.60), administration of BAY increased the number of TUNEL-positive cells (Fig. 6A,C), whereas it decreased the number of proliferating GH3 cells in tumour xenografts (Fig. 6B,D). Our findings suggest that inhibition of the NF-κB pathway could delay pituitary tumour progression after long-term treatment.

## Discussion

Oestrogens have been shown to modulate apoptosis of normal anterior pituitary cells through different mechanisms involving both extrinsic and intrinsic apoptotic pathways (37,38). Apoptotic actions

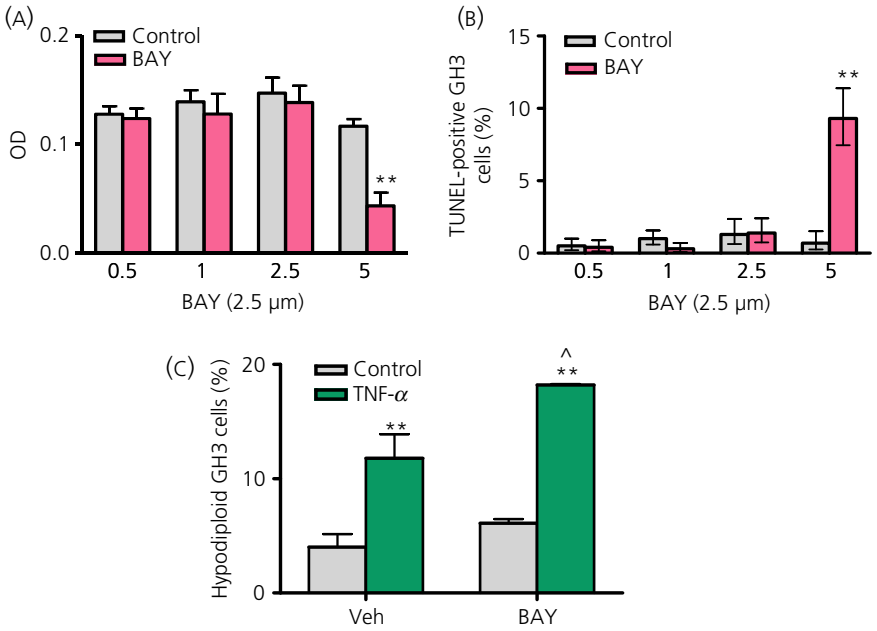


**Fig. 3.** Effect of nuclear factor (NF)-κB pathway inhibition of nuclear translocation on somatolactotroph tumour cell apoptosis. GH3 cells were incubated with different concentrations of BAY 11-7082 (BAY) or vehicle (Veh; ethanol 0.05 ml/l) for 24 h. (A) Cell viability was assessed by the MTS assay. Each column represents the mean ± SE of eight wells. **P < 0.01 versus corresponding control without BAY; Student's *t*-test. (B) The percentage of apoptotic cells was determined by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL). Each column represents the percentage ± confidence limits (CL) of TUNEL-positive GH3 cells (n = 2000–2500 GH3 cells/group from two independent experiments). **P < 0.01; chi-square. (C) GH3 cells were incubated with TNF-α (50 ng/ml) in the presence of BAY (2.5 μM) or vehicle (Veh; ethanol, 0.05 ml/l) for 24 h. The percentage of hypodiploid GH3 cells was determined by flow cytometry using propidium iodide (PI). Each column represents the mean ± SE of the percentage of Sub G0/G1 cells (n = 3 wells/group, representative of three independent experiments). **P < 0.01 versus the respective control without TNF-α; ^P < 0.05 versus respective control without BAY. Two-way ANOVA followed by Tukey's test. OD, optical density.

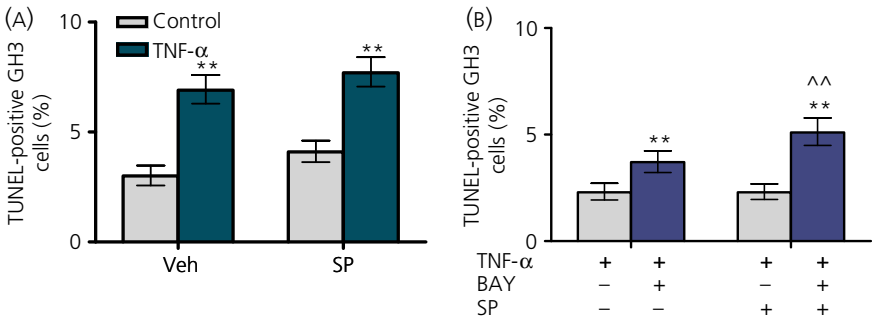


**Fig. 4.** Effect of c-Jun N-terminal protein kinase (JNK) pathway inhibition with SP600125 (SP) on tumour necrosis factor (TNF)-α-induced apoptosis of somatolactotroph tumour cells. (A) GH3 cells were incubated with TNF-α (50 ng/ml) in the presence of SP (10 μM) or vehicle [Veh; dimethyl sulphoxide (DMSO), 0.5 ml/l] for 24 h. The percentage of apoptotic cells was determined by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL). Each column represents the percentage ± confidence limits (CL) of TUNEL-positive GH3 cells (n = 3000–6000 GH3 cells/group from two independent experiments). **P < 0.01 versus corresponding control without TNF-α; chi-square. (B) GH3 cells were incubated with TNF-α (50 ng/ml) in the presence of vehicle (Veh; DMSO 0.5 ml/l, ethanol, 0.05 ml/l), BAY 11-7082 (BAY) (2.5 μM), SP (10 μM) or BAY plus SP for 24 h. The percentage of apoptotic cells was determined by TUNEL. Each column represents the percentage ± CL of TUNEL-positive GH3 cells (n = 3000–6000 GH3 cells/group from two independent experiments). **P < 0.01 versus respective control without BAY; ^^P < 0.01 versus respective control without SP; chi-square.

of TNF-α/TNFR1 and Fas/FasL systems, LPS and dopamine in the normal anterior pituitary were shown to be oestrogen-dependent (27–30). Oestrogens were reported to exert part of their regulatory action on gene transcription by interacting with NF-κB. Several studies in physiological and tumour models have demonstrated that mutual trans-repression occurs between ER and NF-κB, resulting in ER-dependent repression of NF-κB-mediated gene transcription (24) and NF-κB repression of ER-induced gene expression (39). In normal pituitary cells, oestrogens inhibit the NF-κB pathway, probably by controlling NF-κB intracellular localisation as reported for

 

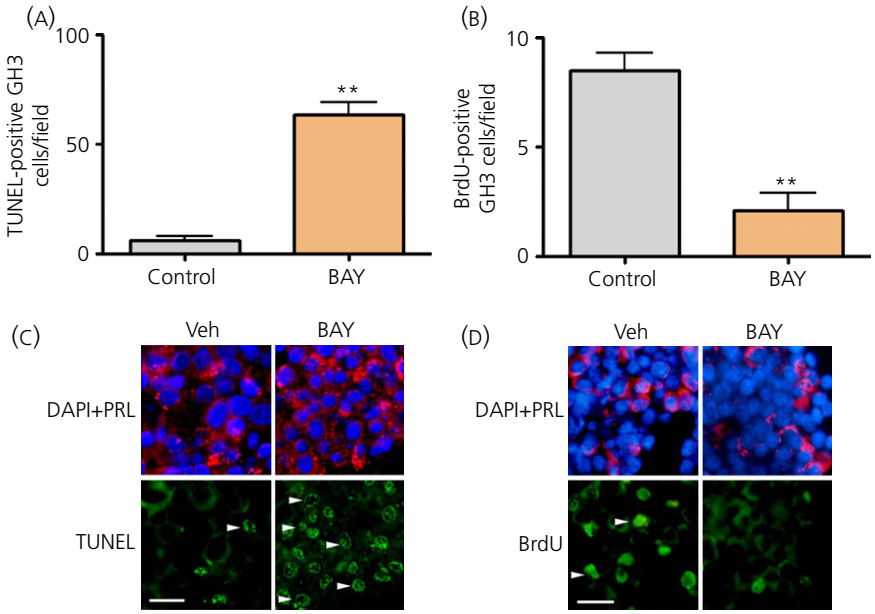


**Fig. 5.** *In vivo* effect of inhibition of nuclear translocation of NF-κB in pituitary tumour cell xenografts. Nude mice were implanted with GH3 cells. When tumour volume reached 500 $mm^3$, mice were injected i.p. with BAY 11-7082 (BAY) (20 mg/kg b.w.) or vehicle (Veh; 2% ethanol in 0.9% NaCl) every 2 days for 1 week. Twenty four hours before killing, mice were injected i.p. with 5-bromo-2′-deoxyuridine (BrdU) (50 mg/kg b.w.). Tissue sections from GH3 tumours were analysed for detection of (A) apoptosis by TUNEL or (B) cell proliferation by incorporation of BrdU. Each column represents the mean ± SE of TUNEL-positive or BrdU-positive cells per field (n = 4 animals per group). **P < 0.01 versus respective control without BAY; Student's *t*-test. Lower panels: representative microphotographs of GH3 cell xenografts in mice treated with or without BAY. (C) Arrowheads indicate TUNEL-positive GH3 cells and (D) BrdU-positive GH3 cells. Scale bar = 20 μm. DAPI, 4,6-diamidino-2-phenylindoledihydrochloride; PRL, prolactin.

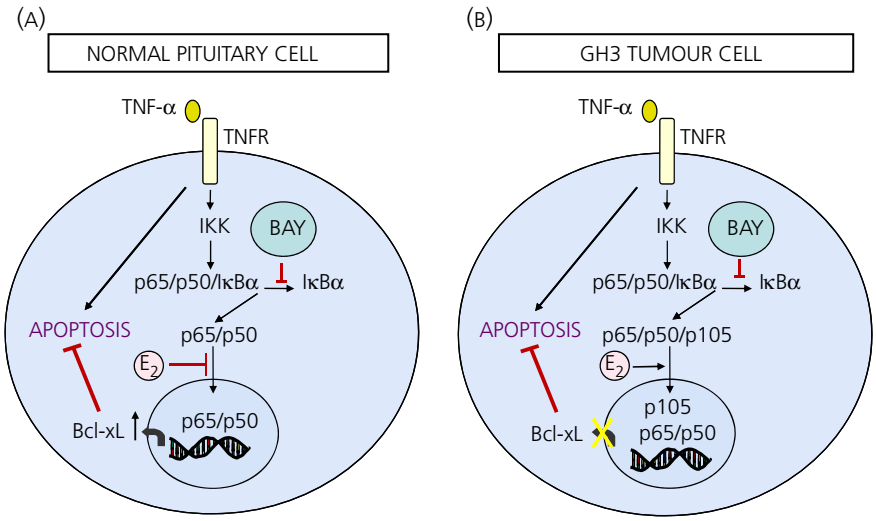


**Fig. 6.** Schematic illustration showing differences between oestrogenic effects on nuclear factor (NF)-κB pathway in normal pituitary and tumour GH3 cells. (A) Tumour necrosis factor (TNF)-α either induces apoptosis or activates the NF-κB pathway through increasing p50/p65 nuclear translocation, which in turn increases the expression of anti-apoptotic genes, such as Bcl-xL. In normal pituitary cells, oestrogens inhibit NF-κB nuclear translocation, thus decreasing Bcl-xL expresion and sensitising anterior pituitary cells to the pro-apoptotic action of TNF-α. The inhibition of the NF-κB pathway with BAY 11-7082 (BAY) further increases TNF-α-induced apoptosis (31).(B) In GH3 tumour cells, oestrogens increase nuclear levels of NF-κB but fail to affect the expression of Bcl-xL, probbaly because they also increase nuclear levels of NF-κB/p105. TNF-α induces apoptosis regardless of the presence of $E_2$ and its pro-apoptotic action is further increased by BAY.

other cell types (40). Indeed, we previously reported that ER activation blocks LPS- and TNF-α-induced nuclear translocation of NF-κB/p65 and p50 (31). In the present study, we found that oestrogens have the opposite effect in GH3 cells because they increased nuclear levels of NF-κB/105, p65 and p50. The nuclear localisation of NF-κB/105, the precursor of NF-κB/50 that is absent

in the nucleus of normal pituitary cells, suggests that NF-κB/p105 may play a role in pituitary tumour cells. NF-κB/p105 interacts with all Rel proteins (41) and was suggested to inhibit the transcriptional activity of NF-κB (42). Binding of complexes containing NF-κB/p105 may exclude certain transcriptionally active NF-κB dimers from κB sites, thereby affecting NF-κB activity (43). The increase in oestrogen-induced nuclear translocation of NF-κB/p105 suggests that oestrogens could inhibit NF-κB transcriptional activity in pituitary tumour GH3 cells by changing the subcellular localisation of NF-κB/p105. Although oestrogens increased NF-κB/p65 and p50 nuclear translocation in GH3 cells, $E_2$ failed to affect the expression of Bcl-xL, a NF-κB target gene, probably because oestrogens also increased the nuclear translocation of NF-κB/p105.

In the present study, TNF-α induced apoptosis of rat pituitary tumour GH3 cells incubated either in the presence or absence of oestrogens. These findings indicate that the pro-apoptotic effect of TNF-α in these tumour pituitary cells does not require sensitisation by oestrogens as occurs in normal pituitary cells (28,44). Also, in normal pituitary cells, we reported that TNF-α had no apoptotic effect unless the NF-κB pathway was inhibited (31). Although, in GH3 tumour cells, TNF-α induced nuclear accumulation of NF-κB, as occurs in normal pituitary cells (31), this cytokine induced apoptosis of GH3 cells even when used as a single agent. Our present data suggest that, although TNF-α induces the activation of NF-κB, this pathway may be altered in tumour cells. Indeed, TNF-α was unable to induce the expression of Bcl-xL, an anti-apoptotic protein belonging to the Bcl2 family, in GH3 cells. Consistent with our results, Bcl-xL expression was shown to remain unchanged during TNF-α-induced apoptosis in other tumour cells, such as MCF-7 breast cancer cells (45).

Nevertheless, inhibition of the NF-κB pathway with BAY increased GH3 cell apoptosis, suggesting that NF-κB activation still promotes the survival of pituitary tumour cells. The activity of NF-κB in GH3 cells appears to be considerable because 5 μM of BAY was required to induce apoptosis, whereas 2.5 μM BAY was sufficient to kill normal pituitary cells (31). Other NF-κB pathway inhibitors were reported to reduce GH3 cell proliferation, suggesting that NF-κB may promote pituitary tumour growth (2). Schaaf *et al.* (46) reported that curcumin, an inhibitor of NF-κB signalling, decreases GH3 tumour size. The present study shows that *in vivo* inhibition of the NF-κB pathway not only reduced cell proliferation, but also increased apoptosis in GH3 tumours growing in nude mice. NFκB inhibitors were suggested to reduce tumour progression by their suppressive effects on tumour neovascularisation (47,48). Therefore, we cannot rule out that the effect of BAY on GH3 cell turnover *in vivo* is additionally mediated through other mechanisms (i.e. reducing angiogenesis). Although it is difficult to extrapolate data obtained from cell lines to primary pituitary adenomas, our findings suggest that inhibition of NF-κB signalling could be effective as an additional treatment for pituitary tumours, especially in adenomas that are resistant to routine clinical treatment with dopamine analogues or in invasive adenocarcinomas.

TNF-α can activate JNK signalling by a mechanism tightly controlled by NF-κB (1,49,50). The role of the JNK pathway in cell fate is cell context-dependent, relying on differences in NF-κB levels or inductive stimuli, potentially explaining why JNK promotes either cell survival or death (51). Several lines of evidence support the idea that transient and modest activation of JNK is associated with cell survival, whereas prolonged activation of JNK plays an important role in TNF-α-induced cell death (15,52). However, in cells lacking active NF-κB, inhibition of JNK enhanced the apoptotic response to TNF-α (51,53). We found that, although inhibition of the JNK pathway in GH3 cells did not affect TNF-α-induced apoptosis, when the NF-κB pathway was also blocked using BAY, it increased the apoptotic response to TNF-α, suggesting that, in pituitary tumour cells, the JNK pathway may be involved in cell survival.

In conclusion, our present data demonstrate that NF-κB is involved in survival of GH3 cells and that these pituitary tumour cells lack the inhibitory action of oestrogens on NF-κB activity (Fig. 6). Given that NF-κB activation typically protects cells from cancer therapy-induced cell death and inhibition of NF-κB pathway gives an anti-apoptotic nature to JNK signalling, administration of NF-κB inhibitors together with JNK inhibitors could contribute to conventional therapies for pituitary tumours.

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