RAPID COMMUNICATION

16K Prolactin induces NF- κ B activation in pulmonary fibroblasts

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Abstract

The amino-terminal 16 kDa fragment of prolactin (16K PRL) promotes the expression of the inducible isoform of nitric oxide synthase (iNOS) accompanied by the production of nitric oxide (NO) by rat pulmonary fibroblasts. The present study was designed to elucidate whether the mechanism by which 16K PRL promotes iNOS expression involves the activation of nuclear factor-kappa B (NF- κ B), a key transcription factor for iNOS induction. 16K PRL stimulated DNA-binding activity of NF- κ B in pulmonary fibroblasts as demonstrated by gel shift assays. Likewise, fluorescence immunocytochemistry showed that

16K PRL promotes nuclear translocation of the p65 subunit of NF- κ B. Finally, treatment with 16K PRL induced the degradation of the NF- κ B inhibitor κ B-beta (I κ B- β), and such degradation was prevented by blocking I κ B- β phosphorylation. Altogether, these results show that 16K PRL activates NF- κ B nuclear translocation via the phosphorylation and degradation of I κ B- β . These findings are consistent with NF- κ B being part of the signal transduction pathway activated by 16K PRL to induce iNOS expression.

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Introduction

Nitric oxide (NO) is a gaseous biological mediator with effects on smooth muscle relaxation, neurotransmission, host defense, immunity and inflammation. NO produced by inducible nitric oxide synthase (iNOS) plays critical roles in modulating inflammation and responses to infectious and degenerative diseases. iNOS expression is induced by a variety of immunologic and inflammatory stimuli, including pro-inflammatory cytokines (e.g., interleukin-1 β [IL-1 β], tumor necrosis factor- α [TNF- α], interferon- γ [IFN- γ]) and lipopolysaccharides (LPS). The expression of iNOS appears to be species and cell-type specific and involves a complex interplay of various transcriptional factors, including NF- κ B (Xie *et al.* 1994). Inactive NF- κ B is normally bound to a family of cytoplasmic proteins called inhibitors of kappa B (I κ B), which mask the NF- κ B nuclear localization signal and prevent its translocation from the cytoplasm to the nucleus. Cytokine-mediated NF- κ B activation requires the phosphorylation and degradation of the I κ B proteins, followed by NF- κ B

nuclear translocation. Once in the nucleus, NF- κ B binds to specific DNA sequences on the iNOS gene and activates iNOS transcription (Karin & Ben-Neriah 2000, Xie *et al.* 1994).

Recently, a specific fragment of the hormone prolactin (PRL) was found to induce iNOS expression (Corbacho *et al.* 2000). PRL is a pleiotropic protein that acts as both a circulating hormone and cytokine on physiological processes that range from reproduction and osmoregulation to immunomodulation (Bole-Feysot *et al.* 1998). PRL can be proteolytically cleaved to form a 16 kDa N-terminal fragment (16K PRL). This proteolytic cleavage adds diversity to the already varied functions of PRL, since 16K PRL has unique effects not shared with the parent molecule. 16K PRL, but not PRL, acts as a potent inhibitor of angiogenesis *in vivo* (Clapp *et al.* 1993, Dueñas *et al.* 1999) and *in vitro*, inhibiting endothelial cell proliferation (Clapp *et al.* 1993) and stimulating type 1 plasminogen activator inhibitor expression (Lee *et al.* 1998) and endothelial cell apoptosis (Martini *et al.* 2000). In addition, 16K PRL, but not PRL, promotes the expression of iNOS

and NO production by pulmonary fibroblasts, acting as a pro-inflammatory cytokine. Finally, the unique effects of 16K PRL appear to occur through a receptor distinct from known PRL receptors, as specific, high affinity, saturable binding sites for 16K PRL are found in endothelial cells and fibroblasts (Clapp *et al.* 1992, Corbacho *et al.* 2000). However, the molecular identity of 16K PRL receptors and their associated signaling pathways remain to be elucidated. Here, we demonstrate that 16K PRL activates the NF- κ B signaling pathway in pulmonary fibroblasts. This finding is consistent with our previous observations demonstrating that 16K PRL stimulates the expression of iNOS in the same cell type.

Materials and Methods

Materials

Reagents were obtained from the following sources: recombinant murine TNF- α from R&D Systems (Minneapolis, MN); Inhibitor of the I κ B kinases (BAY 11-7085 (E)3-[(4-*t*-Butylphenyl) sulfonyl]-2-propenenitrile) from Calbiochem (Merck KGaA, Darmstadt, Germany); anti-p65, anti-I κ B α and anti-I κ B β polyclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); goat anti-rabbit antibodies conjugated to fluorescein isothiocyanate (FITC) and to horseradish peroxidase (HP) from Jackson ImmunoResearch Laboratories (West Grove, PA); and polymyxin B from Sigma (St Louis, MO). 16K PRL was generated after the enzymatic proteolysis of PRL with a particulate fraction from rat mammary glands, gel filtration, and carbamidomethylation, as described (Clapp 1987).

Cell culture

Rat lung fibroblasts were isolated from Sprague-Dawley rat fetuses of 19–20 days of gestational age (Cannigia *et al.* 1991). Cells were cultured on Ham's F12 medium with 10% heat-inactivated fetal bovine serum. Cells grown to confluence were incubated with Ham's F12 medium–0.5% FBS and treated with 16K PRL (160–1600 ng/ml) in the presence or absence of polymyxin B (10 μ g/ml). TNF- α (50 ng/ml) was used as a positive control of NF- κ B activation.

Preparation of nuclear extracts and electrophoretic mobility shift assays (EMSAs)

Cells were centrifuged at 800 *g* for 5 min, pellets were frozen, resuspended in hypotonic buffer (Hepes 10 mM, pH 7.9; KCl 10 mM; MgCl₂ 1.5 mM; DTT 1 mM) and centrifuged after a 5 min incubation. The nuclear pellets were resuspended in extraction buffer (Hepes 20 mM, pH 7.9; NaCl 400 mM; MgCl₂ 1.5 mM; PMSF 0.5 mM;

glycerol 25%, DTT 1 mM), incubated 30 min at 4 °C with gentle mixing, centrifuged at 1500 *g* and the supernatants diluted with an equal volume of nuclear extraction buffer (Hepes 20 mM, pH 7.9; KCl 50 mM; EDTA 0.2 mM; PMSF 0.5 mM; DTT 1 mM). EMSA was performed as described previously (Reyes *et al.* 2001). Briefly, 10 μ g of nuclear protein extract was incubated with 1 μ g of poly dI-dC and 5000–10 000 c.p.m. of a ³²P-labeled oligonucleotide probe (AGTTGAGGGGACTTTCCTCCCA GG; the NF- κ B consensus sequence appears underlined; Santa Cruz Biotechnology). After a 20 min incubation at RT, unbound and complexed probe were separated using a native 4% polyacrylamide gel.

Immunofluorescence

Pulmonary fibroblasts grown on glass cover slips (10⁵ cells per well in 15 mm well plates) were incubated in the absence (control) or presence of TNF- α (50 ng/ml) for 20 min, or of 16K PRL (160 ng/ml) for 2 h, the latter in the absence or presence of polymyxin B (10 μ g/ml). After treatment, cells were fixed in 4% paraformaldehyde, washed and incubated with 10% normal goat serum in 0.6% Triton-PBS for 2 h, followed by an overnight incubation at RT with 2 μ g/ml of anti-p65 NF- κ B subunit antibodies. Preparations were then incubated with a 1:100 dilution of goat anti-rabbit antibodies conjugated to FITC for 1 hr at RT. Omission of primary antibodies or substitution with rabbit pre-immune antibodies confirmed the specificity of the reaction.

Western blot

Fibroblasts were homogenized in lysis buffer (0.01 M Tris-HCl, 0.14 M NaCl, 1% Triton X-100, pH 8, with aprotinin at 0.2 U/ml and 1 mM PMSF). Homogenates corrected for their protein concentration were subjected to reducing SDS-PAGE (12%) and the proteins were electroblotted onto nitrocellulose and detected by using enhanced chemiluminescence (Pierce, Rockford IL) with 1 μ g/ml of anti-I κ B- α or anti-I κ B- β polyclonal antibodies, followed by a 1:15 000 dilution of anti-HP conjugates. Optical density values were determined using the 1D image analysis software v3.5 (Eastman Kodak company, Rochester NY).

Data analysis

All results were replicated in three or more independent experiments. Values were compared with a one way analysis of variance, followed by a Duncan range test.

Results

16K PRL stimulates DNA binding activity of NF- κ B

The DNA binding activity of NF- κ B in pulmonary fibroblasts was investigated after treatment with 16K PRL

Figure 1 Gel shift assay (EMSA) showing that 16K PRL stimulates DNA binding activity of nuclear factor kappa B (NF- κ B) in primary fetal lung fibroblasts. Cells were incubated in the absence (C) or presence of TNF- α (50 ng/ml) or 16K PRL (160 ng/ml) with or without polymyxin B (PMB) (10 μ g/ml), for the indicated times before preparation of nuclear extracts for gel retardation analysis with 32 P-labeled consensus DNA sequence oligonucleotide for NF- κ B. Specificity of the reaction was assessed using a 100-fold excess of unlabeled NF- κ B oligonucleotide as competitor (not shown). Specific complex is indicated (arrow).

and compared with that resulting from incubation with TNF- α , a known activator of NF- κ B (Baud & Karin 2001) (Fig. 1). Nuclear extracts prepared from replicate cultures of pulmonary fibroblasts treated for various times with either peptide were assayed for NF- κ B binding in an EMSA of DNA binding factors using a radiolabeled consensus sequence κ B probe containing the core κ B site. Both TNF- α and 16K PRL induced the formation of a similar nuclear complex, albeit with different kinetics. With TNF- α the complex was formed rapidly (≤ 20 min) and declined after 60 min of treatment (Fig. 1). In contrast, with 16K PRL the complex was initially detected at 60 min, was stronger at 120 min, and declined by the 180 min time point (Fig. 1). Specificity of the DNA-protein interaction for NF- κ B was demonstrated by competition with a 100-fold excess of unlabeled oligonucleotide (not shown). Because LPS is a known stimulator of NF- κ B activation (Xie *et al.* 1994), we ascertained the effect of 16K PRL in the presence of polymyxin B (PMB), an inhibitor of endotoxin activity. PMB did not interfere

with the formation of the nuclear complex elicited by 16K PRL (Fig. 1), suggesting that 16K PRL alone is an effective activator of NF- κ B.

16K PRL induces the nuclear translocation of NF- κ B

The cellular distribution of NF- κ B was studied in pulmonary fibroblasts using fluorescence immunocytochemistry and polyclonal antibodies against the p65 subunit of NF- κ B (Fig. 2). In the absence of treatment, p65 immunoreactivity was homogeneously distributed throughout the cytoplasm and nuclei of cells. Treatment with 16K PRL induced the specific localization of p65 immunostaining in the cell nucleus and this redistribution was similar to that induced by TNF α (Fig. 2).

16K PRL induces I κ B- β degradation

The I κ B family consists of three members, I κ B- α , I κ B- β , and I κ B- ϵ (Karin & Ben-Neriah 2000) which can respond differentially to NF- κ B-inducing signals and inhibit NF- κ B with different efficiencies (Simeonidis *et al.* 1999). We have measured the intracellular degradation kinetics of I κ B isoforms α and β in pulmonary fibroblasts treated with TNF- α or 16K PRL for the indicated time periods (Fig. 3). The degradation of the individual I κ B proteins was followed in cellular lysates by Western blots with isoform-specific antibodies. 16K PRL did not alter I κ B- α levels at the times analyzed. However, 16K PRL induced a gradual degradation of I κ B- β that was maximal by the 120 min time point (Fig. 3A). This kinetics of I κ B- β degradation was similar to that after TNF- α treatment (Fig. 3B). Nevertheless, in contrast to 16K PRL, TNF- α also induced a rapid (≤ 10 min) and transient degradation of I κ B- α , with the protein beginning to be recovered by the 60 min time point.

16K PRL induces degradation of I κ B- β through the activation of I κ B kinases

Degradation of I κ B proteins requires their phosphorylation at Ser residues by I κ B kinases (IKK). Phosphorylated I κ Bs

Figure 2 16K PRL induces nuclear translocation of NF- κ B in fibroblasts. Immunofluorescence detection of NF- κ B (p65) in fibroblasts cultured in the absence (control) or presence of TNF- α (50 ng/ml) for 20 min or of 16K PRL (160 ng/ml) for 2 hrs.

Figure 3 16K PRL induces IκB-β degradation in fibroblasts. Cells were treated with or without 16K PRL (160 ng/ml) (A) or TNF-α (50 ng/ml) (B) for the times indicated and cellular lysates subjected to Western blot. Degradation of the IκB isoforms was visualized with anti-IκB-α (○) or anti-IκB-β (●) antibodies. Membranes were re-blotted with anti-actin antibodies. IκB values were quantitated densitometrically, normalized with respect to the actin signal and plotted as percent of value at 0 min. Values are mean ± S.E.M. * $P < 0.05$ vs. 0 min.

are polyubiquitinated and subsequently degraded by the proteasome (Karin & Ben-Neriah 2000). An inhibitor of IKKs (BAY 117085) that blocks IκB phosphorylation (Pierce *et al.* 1997) prevented the degradation of IκB-β induced by 16K PRL (Fig. 4). Likewise, BAY 117085 interfered with the degradation of both IκB-α and IκB-β induced by TNF-α (Fig. 4).

Figure 4 Inhibitor of IκB kinases (BAY 11-7085) blocks 16K PRL-induced degradation of IκB-β. (A) Western blot analysis of untreated cells (C) or of cells treated with TNF-α (50 ng/ml) for 20 min or 16K PRL (160 ng/ml) for 2 h with or without a 30 min pre-treatment with 1 mM of BAY11-7085. Degradation of the IκB isoforms was visualized with anti-IκB-α and anti-IκB-β antibodies. (B) Density of IκB bands was plotted as percent of untreated control. Values are mean ± S.E.M. * $P < 0.05$ vs untreated control.

Discussion

The N-terminal 16 kDa fragment of PRL may function as a new regulatory cytokine. It inhibits angiogenesis via direct effects on endothelial cells (Corbacho *et al.* 2002), and has pro-inflammatory actions via the stimulation of iNOS expression and NO production in pulmonary fibroblasts (Corbacho *et al.* 2000). Although these effects are clear, the molecular identity of the 16K PRL receptor is unknown, and its associated signaling pathways remain to be elucidated.

The current study shows that 16K PRL is able to stimulate the signaling cascade leading to NF-κB activation in pulmonary fibroblasts. This conclusion is based on the use of four independent methods. First, EMSA analysis detected NF-κB binding activity in nuclear extracts from cells treated with 16K PRL. Second, fluorescence immunocytochemistry demonstrated the accumulation of NF-κB-like immunoreactivity in the nuclei of 16K PRL treated cells. Third, 16K PRL induced the degradation of the IκB-β protein, a degradation known to be required for NF-κB activation. Finally, treatment with an inhibitor of IκB phosphorylation prevented the degradation of IκB induced by 16K PRL. Therefore, these observations indicate that 16K PRL can stimulate the nuclear translocation of NF-κB and promote its DNA-binding activity via the phosphorylation and subsequent degradation of IκB-β.

Along with 16K PRL treatment, parallel studies were carried out with TNFα as a control for NF-κB activation. The results from these comparative studies indicated that the timescale of nuclear translocation and the IκB isoforms recruited during NF-κB activation differed between the two peptides. 16K PRL-dependent NF-κB activation was detected by 60 min and reached a maximal level by

120 min. This behavior resembles the activation of NF- κ B in response to growth factors, such as platelet derived growth factor (PDGF). In fibroblasts treated with PDGF, NF- κ B translocation is detected 60 min after stimulation and reaches a maximal level by 180 min (Romanshkov & Madarov 1999). In contrast, TNF- α dependent NF- κ B activation was fast and reached a maximal level by 20 min consistent with previous reports for TNF- α (Ozes *et al.* 1999). In addition, 16K PRL-dependent NF- κ B activation was linked to degradation of the I κ B- β isoform, while for TNF- α both I κ B- α and I κ B- β were degraded. Interestingly, degradation only of the I κ B- β isoform has been reported in persistent or biphasic NF- κ B activation (Thompson *et al.* 1995). The functional implications of these differences are unknown. However, it has been suggested that variations in the kinetics of NF- κ B activation and I κ B degradation vary among cytokines and cell types and may have an impact on NF- κ B regulated genes at later times (Johnson *et al.* 1996). Altogether, it appears that the 16K PRL-dependent NF- κ B activation resembles a more persistent effect of growth and surviving factors such as PDGF rather than the transient effect of proinflammatory cytokines such as TNF- α .

The activation of NF- κ B by 16K PRL may relate to its pro-inflammatory effects. NF- κ B orchestrates the activation of numerous genes involved in the control of a variety of inflammatory responses. These include those genes encoding cytokines, chemokines, growth factors, immunoreceptors, cell adhesion molecules, and regulators of apoptosis and inflammation (Pahl 1999). One such target gene is the iNOS gene. Activation of NF- κ B is required for cytokine induction of the iNOS gene (Xie *et al.* 1994) and the iNOS promoter contains NF- κ B binding sites that are functionally important for the transcriptional activation of iNOS (Taylor *et al.* 1998). Moreover, various second messenger pathways that lead to iNOS expression converge in the activation of NF- κ B (Taylor *et al.* 1998). Therefore, the mechanism by which 16K PRL promotes iNOS expression in pulmonary fibroblasts is likely to involve NF- κ B activation. Nevertheless, expression of other genes may also result from NF- κ B activation by 16K PRL. In this regard the noted similarities between 16K PRL and PDGF kinetics of NF- κ B activation allow us to speculate on survival-related genes as putative candidates. In addition, these similarities may suggest that 16K PRL-dependent NF- κ B activation can act to sustain the inflammatory response initiated by stimuli such as TNF- α and IL-1 β .

Further work is needed to completely elucidate the signal transduction pathway that leads to the activation of NF- κ B by 16K PRL. The signaling pathway activated by TNF- α and IL-1 β for NF- κ B activation has been described. It involves two adaptor proteins, TNF- α receptor-associated factor 2 (TRAF-2) and factor 6 (TRAF-6), that function for TNF- α and IL-1 β signaling, respectively. Both TRAF-2 and TRAF-6 can lead to the

activation of NF- κ B-inducing kinase (NIK), a serine/threonine protein kinase that can form a complex with and activates the I κ B kinase signalosome complex (Karin & Ben-Neriah 2000). The activated I κ B kinase signalosome complex phosphorylates I κ B, directing it for proteasome-mediated degradation and allowing NF- κ B to translocate to the nucleus and activate gene expression.

Other second messenger pathways can converge in the activation of NF- κ B to induce iNOS expression. For example, protein kinase C-stimulating agents or cyclic AMP (cAMP) elevating agents can activate NF- κ B and are efficient inducers of iNOS expression (Kleinert *et al.* 1996). However, while NF- κ B appears to be a key control element for the induction of iNOS in response to these second messenger systems (Kleinert *et al.* 1996), cAMP can also upregulate iNOS directly via cAMP regulatory elements within the iNOS promoter (Eberhardt *et al.* 1996). Recent data suggest that activation of cAMP dependent signaling can contribute to upregulation of iNOS by 16K PRL. Accordingly, treatment of pulmonary fibroblasts with H89, a specific inhibitor of protein kinase A (PKA), reduced the stimulatory effect of 16K PRL on NO production (Macotela and Clapp, unpublished observation).

In summary, 16K PRL activates NF- κ B in primary pulmonary fibroblasts. This effect may lead to upregulation of the iNOS gene but also of other NF- κ B target genes. Because pulmonary fibroblasts produce PRL and can locally process PRL into 16K PRL (Corbacho *et al.* 2000), these actions of 16K PRL may involve autocrine mechanisms. This study helps support the proposed role of 16K PRL as a putative cytokine involved in inflammatory and host defense processes and warrants further investigation.

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References

- Baud V & Karin M 2001 Signal transduction by tumor necrosis factor and its relatives. *Trends in Cell Biology* **11** 372–377.
- Bole-Feysot C, Goffin V, Edery M, Binart N & Kelly PA 1998 Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocrine Review* **19** 225–268.

- Caniggia I, Tseu I, Han RN, Smith BT, Transwell K & Post M 1991 Spatial and temporal differences in fibroblast behavior in fetal rat lung. *American Journal of Physiology* **261** L424–L433.
- Clapp C 1987 Analysis of the proteolytic cleavage of prolactin by the mammary gland and liver of the rat: characterization of the cleaved and 16K forms. *Endocrinology* **121** 2055–2064.
- Clapp C & Weiner RI 1992 A specific, high affinity, saturable binding site for the 16-kilodalton fragment of prolactin on capillary endothelial cells. *Endocrinology* **130** 1380–1386.
- Clapp C, Martial JA, Guzman RC, Rentier-Delrue F & Weiner RI 1993 The 16-kilodalton N-terminal fragment of human prolactin is a potent inhibitor of angiogenesis. *Endocrinology* **133** 1292–1299.
- Corbacho AM, Nava G, Eiserich JP, Noris G, Macotela Y, Struman I, Martí nez de la Escalera G, Freeman BA & Clapp C 2000 Proteolytic cleavage confers nitric oxide synthase inducing activity upon prolactin. *The Journal of Biological Chemistry* **275** 13183–13186.
- Corbacho AM, Martí nez de la Escalera G & Clapp C 2002 Roles of prolactin and related members of the prolactin/growth hormone/placental lactogen family in angiogenesis. *Journal of Endocrinology* **173** 219–238.
- Dueñas Z, Torner L, Corbacho A, Ochoa A, Gutierrez-Ospina G, Lopez-Barrera F, Barrios FA, Berger P, Martí nez de la Escalera G & Clapp C 1999 Inhibition of rat corneal angiogenesis by 16k-Da prolactin and by endogenous prolactin-like molecules. *Investigative Ophthalmology and Visual Science* **40** 1–8.
- Eberhardt W, Kunz D, Hummel R & Pfeilschifter J 1996 Molecular cloning of the rat inducible nitric oxide synthase gene promoter. *Biochemical and Biophysical Research Communications* **223** 752–756.
- Johnson DR, Douglas I, Jahnke A, Ghosh S & Pober JS 1996 A sustained reduction in I κ B- β may contribute to persistent NF- κ B activation in human endothelial cells. *The Journal of Biological Chemistry* **271** 16317–16322.
- Karin M & Ben-Neriah Y 2000 Phosphorylation meets ubiquitination: The control of NF- κ B activity. *Annual Review of Immunology* **18** 621–663.
- Kleinert H, Euchenhofer C, Ihrig-Biedert I & Forstermann U 1996 In murine 3T3 fibroblasts, different second messenger pathways resulting in the induction of NO synthase II (iNOS) converge in the activation of transcription factor NF- κ B. *The Journal of Biological Chemistry* **271** 6039–6044.
- Lee H, Struman I, Clapp C, Martial J & Weiner RI 1998 Inhibition of urokinase activity by the antiangiogenic factor 16K prolactin: Activation of plasminogen activator inhibitor expression. *Endocrinology* **139** 3696–3703.
- Martini JF, Piot C, Humeau LM, Struman I, Martial JA & Weiner RI 2000 The anti-angiogenic factor 16K PRL induces programmed cell death in endothelial cells by caspase activation. *Molecular Endocrinology* **14** 1536–1549.
- Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM & Donner DB 1999 NF- κ B activation by tumor necrosis factor requires the Akt serine-threonine kinase. *Nature* **401** 82–85.
- Pahl HL 1999 Activators and targets genes of Rel/NF- κ B transcription factors. *Oncogene* **18** 6853–6866.
- Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Tucker C & Gerritsen ME 1997 Novel inhibitors of cytokine-induced I κ B α phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects *in vivo*. *The Journal of Biological Chemistry* **272** 21096–21103.
- Reyes-Reyes M, Mora N, Zentella A & Rosales C 2001 Phosphatidylinositol 3-kinase mediates integrin-dependent NF- κ B and MAPK activation through separate signaling pathways. *Journal of Cell Science* **114** 1579–1589.
- Romashkova JA & Makarov SS 1999 NF- κ B is a target of AKT in anti-apoptotic PDGF signaling. *Nature* **401** 86–90.
- Simeonidis S, Stauber D, Chen G, Hendrickson WA & Thanos D 1999 Mechanisms by which I κ B proteins control NF- κ B activity. *PNAS* **96** 49–54.
- Taylor BS, De Vera ME, Ganster RW, Wang O, Shapiro RA, Morris Jr RM, Billiar TR & Geller DA 1998 Multiple NF- κ B Enhancer Elements Regulate Cytokine Induction of the Human Inducible Nitric Oxide Synthase Gene. *The Journal of Biological Chemistry* **273** 15148–15156.
- Thompson JE, Phillips RJ, Erdjument-Bromage H, Tempst P & Ghosh S 1995 I κ B- β regulates the persistent response in a biphasic activation of NF- κ B. *Cell* **80** 573–582.
- Xie Q, Kashiwabara Y & Nathan C 1994 Role of transcription factor NF- κ B/Rel in induction of nitric oxide synthase. *The Journal of Biological Chemistry* **269** 4705–4708.