Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1642 (2003) 33–44
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

Activation of NF-κB nuclear transcription factor by flow in human endothelial cells

David C. Hay[a], Catherine Beers[b], Vicky Cameron[b], Lesley Thomson[a], Frederick W. Flitney[b,*], Ronald T. Hay[a]

[a] *Centre for Biomolecular Sciences, University of St. Andrews, St. Andrews, Fife KY16 9TS, UK*
[b] *Division of Cell and Molecular Biology, School of Biology, Bute Medical Buildings, University of St. Andrews, St. Andrews, Fife, Scotland KY16 9TS, UK*

Received 19 February 2003; received in revised form 12 June 2003; accepted 18 June 2003

Abstract

The tractive force generated by blood flow, called fluid shear stress, is an important regulator of endothelial cell gene expression. Several transcription factors are activated by shear stress, including members of the NF-κB/Rel family. The nature of the upstream-signaling components involved in the activation of NF-κB by flow has been studied in human endothelial cells. Flow rapidly increased endogenous IKK1/2 activity and transiently degraded IκBα and IκBβ$_1$, but not p105/p50. Nuclear translocation of the p65 subunit was induced by flow in wild-type (w/t) cells and in cells overexpressing w/t NIK, IKK1 or IKK2, but not in cells transiently transfected with kinase-inactive mutants of these enzymes. Nuclear translocation of p65 in response to flow was not affected by overexpressing a dominant-negative mutant of a MAPKKK related to NIK, called TPL2 kinase, nor by pretreating cells with the selective PKC inhibitor bisindoylmaleimide-1. Gel shift assays showed that the binding of p50/p65 heterodimer to radiolabeled oligonucleotide containing a shear-stress response element was increased by flow. The activity of a 3κB conA-luciferase reporter was also increased, confirming that NF-κB activated by flow was transcriptionally active. We conclude that shear stress induces gene transactivation by NF-κB (p50/p65) via the NIK-IKK1/2 pathway and proteosome-dependent degradation of IκB and that induction by flow does not involve TPL-2 kinase or PKC.

Keywords: Endothelial cell; NF-κB transcription factor; Flow mechanotransduction; Shear stress; Gene regulation; Atherogenesis

1. Introduction

Endothelial cells (EC) are exquisitely responsive to the tractive force associated with blood flow, called fluid shear stress [1,2]. Shear stress activates multiple signaling cascades [3–6], whose downstream targets include several inducible transcription factors (e.g. c-myc, c-fos, c-jun, Egr-1, AP-1, SP-1 and NF-κB/Rel) that regulate endothelial gene expression [7–14]. Lan et al. [12] showed that the NF-κB transcription factor is activated by flow in cultured bovine aortic endothelial cells. A *cis*-acting shear-stress responsive element (SSRE) was found in the promoter of the flow-sensitive PDGF-β chain gene [8] and this was later shown to act as a nonconsensus binding site for NF-κB [11]. The 6-bp core binding sequence (GAGACC) has been identified in other genes that are known to be regulated by flow (e.g. tPA, ICAM-1, TGFβ, c-fos, c-jun, MCP-1, eNOS); furthermore, insertion of the SSRE into the promoters of several flow-insensitive genes renders them inducible by flow [15].

NF-κB family members (p50/NF-kB1, p52/NF-kB2, p65/Rel A, c-Rel, and Rel B) form homo- or heterodimers that are retained in the cytoplasm in quiescent (unstimulated) cells by a family of protein inhibitors, called IκBs. The formation of a NF-κB/IκB complex masks a critical nuclear localisation signal (NLS) on a conserved region of the N terminus of NF-κB proteins, called the Rel homology domain (RHD), and this prevents translocation of NF-κB dimers into the nucleus. The IκB family of inhibitor proteins (IκBα, IκBβ, IκBε and Bcl 3) each contain multiple copies (3–7) of an amino acid sequence known as the ankyrin repeat. The genes for p50 and p52 encode larger precursor proteins, designated NF-kB1/p105 and NF-kB2/p100, respectively. Both proteins contain several ankyrin repeats in their carboxy domains and function as

* Corresponding author. Tel.: +44-1334-463490; fax: +44-1334-463600.
E-mail address: fwf@st-and.ac.uk (F.W. Flitney).

0167-4889/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0167-4889(03)00084-3

NF-κB inhibitors, retaining p50, c-Rel and Rel-A proteins within the cytoplasm.

Agonist-mediated activation of NF-κB (e.g. by TNFα) involves posttranslational modification of IκB proteins that targets them for destruction by the 26S proteasome-dependent pathway [16,17]. IκB is first phosphorylated by a serine-specific kinase called IκB kinase, or IKK. IKK comprises three subunits: IKKα/IKK1 and IKKβ/IKK2 are catalytic [18–22] and able to form homo- or heterodimers through leucine zipper domains, while the third, called IKKγ or NF-κB essential modulator (NEMO), is regulatory [23]. Phosphorylated IκB then undergo polyubiquitination. P-IκB is first recognised by the β-transducin repeat-containing protein (β-TrCP), a component of an E3 ubiquitin ligase complex that catalyses the transfer of ubiquitin to lysine residues on IκB [24–26]. Polyubiquitinated IκB is then degraded by the proteasome pathway [27–29], exposing the NLS and facilitating the entry of NF-κB into the nucleus.

IKK1 and IKK2 are themselves downstream targets for members of the MAP kinase kinase kinase (MAP3 K) family. Two of these, called NF-κB-interacting kinase (NIK) and MAPK/extracellular signal-regulated kinase kinase 1 (MEKK-1), can activate IKK1 and 2 by phosphorylating specific serine residues. NIK binds to the adaptor protein TNF-receptor-associated factor 2, or TRAF2, and preferentially phosphorylates IKK1, while MEKK-1 activates IKK2, suggesting that distinct signaling pathways may be triggered by different extracellular stimuli [30]. IKKs are also downstream targets for TAK1, or TGF-β-inducible kinase-1 [31], protein kinase B, or Akt [32], and two protein kinase C (PKC) isoforms, PKC ζ and θ [33].

Some components of the signaling pathway involved in the activation of NF-κB by flow have been identified in bovine aortic endothelial cells. Bhullar et al. [10] showed that activation of IKKs and degradation of IκBs are essential steps in the process. They further demonstrated that induction of IKK1/2 activity by shear stress is inhibited by pretreating cells with a blocking antibody (LM609) to the $\alpha_v\beta_3$ integrin, pointing to a role for this integrin in the mechanotransduction process. The nature of the upstream signaling elements involved in the activation of NF-κB by flow in human endothelial cells has not been studied, though this is of considerable interest in light of recent reports that implicate NF-κB in the pathogenesis of atherosclerosis [34–37]. The matter has now been investigated using human umbilical vein endothelial cells (HUVEC). Our results show that NIK, as well as IKK1 and IKK2, is an essential component of the flow-activated pathway. The process involves proteasome-dependent degradation of IκBα and $I\kappa B\beta_1$, facilitating nuclear translocation of p50/p65 heterodimer and resulting in NF-κB-dependent gene transactivation. Activation of NF-κB by flow does not involve TPL-2 kinase-mediated proteolysis of p105 and it is also independent of PKC.

2. Materials and methods

2.1. Cell culture and treatment

HUVEC were used in all experiments (Cell Tech, Slough, UK). They were maintained in EBM-2 medium supplemented with 5% foetal bovine serum and Bullet™ kit reagents (Clonetics) and grown in a humidified 5% CO_2 and 95% air incubator at 37 °C. Approximately 4.5×10^5 cells were seeded onto uncoated glass coverslips (22 × 22 mm) for immunofluoresence and $\sim 1.5 \times 10^6$ cells seeded on glass microscope slides (75 × 25 mm) for preparing cell lysates (Western blotting, DNA binding assay, kinase assay and luciferase assay). In some experiments, cells were grown on coverslips and pretreated with MG132 (20 μM; Biomol), leptomycin B (LMB; 20 nM; Novatex) or bisindolylmaleimide (bisin; 100 nM; Calbiochem) for 30 min before subjecting them to flow. Cells cultured in six-well plates and on glass microscope slides were also stimulated with tumour necrosis factor α (TNFα; 30 ng/ml; Insight) before immunoblotting and kinase assays. NF-κB and PKC were activated in response to treatment with a combination of phorbol myristyl acetate (PMA; 25 ng ml^{-1}) and ionomycin (Sigma Ltd., 1 μg ml^{-1}).

2.2. Flow apparatus

Cells were exposed to uniform laminar shear stress in one of two parallel plate flow chambers [38], one of which accommodated cells grown on cover slips for morphological studies, while the other held cells grown on microscope slides for biochemical analyses. The chambers were incorporated into a closed loop perfusion system containing culture medium, driven by an adjustable peristaltic pump (Cole Parmer Masterflex® type 7521–47, CP Instruments Ltd, Herts). Culture medium was equilibrated with 5% CO_2/95% air and the temperature was maintained at 37 °C.

We determined that each of the chambers generated uniform laminar (two dimensional) flow by measuring shear stresses generated at different flow rates and comparing the values obtained (τ) with those calculated on theoretical grounds (τ^*). Shear stress was determined experimentally from measurements of the absolute pressure gradient along the flow channel, using the relation:

$$\tau = \rho g a h / l \quad (1)$$

where ρ = medium density (g cm^{-3}); g = acceleration due to gravity (cm s^{-2}); h = difference in pressure (cm water) along chamber; a = chamber half-height (cm); l = distance between pressure measuring ports (cm). Theoretical values were calculated from:

$$\tau^* = 3Qv/2a^2W \quad (2)$$

where Q = volume flow rate (ml s^{-1}); v = medium viscosity (Poise); a = chamber half-height (cm); and W = channel

width (cm). The values obtained for τ and $\tau*$ were within $\pm$ 15% over a 100-fold range of flow rates. In the experiments to be described, the flow rate was adjusted to generate a shear stress of 15 dyn cm^{-2}, a value within the normal physiological range encountered in medium size arteries [7,39].

2.3. DNA plasmids and transient transfection procedures

HUVEC were transfected with expression plasmids containing cDNAs for TPL2 (provided by Dr. Steve Ley, NIMR, London, UK), NIK (provided by Dr. David Wallach, Israel) and IKK1 and 2 (provided by Dr John Taylor, Pfizer Central Research, Sandwich, Kent, UK) or, alternatively, with cDNAs encoding the corresponding kinase inactive mutants. As a control, cells were transfected with pcDNA 3 empty vector. After transfection, the cells were grown to confluence, exposed to shear stress for different times and then processed for immunofluoresence [40].

Gene reporter experiments were performed using HUVEC transfected with 10 μg of a 3-κB enhancer conA-luciferase plasmid, or with a con A-luciferase plasmid that lacked NF-κB binding sites as a negative control. They were grown to confluence and then exposed to shear stress for 12 h. After shearing, cells were maintained in EBM-2 for a further 24 h and then assayed for luciferase activity (RLU/mg protein) as described elsewhere [40].

cDNA plasmids were transfected into HUVEC at ~ 80% confluence using Lipofectamine™ reagent (GIBCO-BRL). After incubating the cells for 4 h, they were washed with EBM-2 culture medium (Clonetics) and maintained in EBM-2 until a confluent monolayer had formed. Cells were subjected to shear stress within 36 h of transfection.

2.4. Western immunoblotting

HUVEC were subjected to flow and immediately afterwards extracted with lysis buffer (composition: 20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.25% Triton-X 100, 1 mM DTT, 10 mM β-glycerophosphate, 300 μM sodium orthovanadate, 2 μM PMSF, 10 mM sodium fluoride and a protease inhibitor cocktail tablet). Cell extracts were cleared by centrifugation at 14,000 rpm for 10 min at 4 °C and the protein concentration in the supernatants determined using the BCA (Pierce) assay reagent. Proteins were separated by electrophoresis in 10% polyacrylamide gels containing SDS, then transferred to polyvinylidene difluoride (PVDF) membrane and subjected to ECL™ (Amersham, UK) Western blotting. Monoclonal antibody to IKK1 (B8) was used at a dilution of 1:100. Rabbit polyclonal antibodies to IκBα (C-21) and IκBβ (C-20) were used at a dilution of 1:250 (Santa Cruz Biotechnology Inc.). Antibody to p105/p50 was raised in sheep, antigen affinity purified and used at a dilution of 1:500. The secondary antibodies used were anti-sheep HRP (Dako), anti-mouse HRP (Amersham) and anti-rabbit HRP (Amersham), all used at a dilution of 1:10,000.

2.5. Immunostaining and fluorescence microscopy

Nuclear translocation of NF-κB was monitored by indirect immunofluorescence using monoclonal antibody to p65 (F-6; Santa Cruz Biotechnology). Confluent monolayers of HUVEC were fixed in 3% paraformaldehyde dissolved in phosphate buffered saline (PBS; 10 min, room temperature), then quenched by immersion in PBS containing 0.1 M glycine (2 × 10 min). Following permeabilisation in PBS containing 0.1% Triton X-100 (10 min), cells were immersed in PBS containing 0.2% BSA (10 min). Rabbit polyclonal antibody to TPL-2 (TSP-3; courtesy Dr Steve Ley, NIMR, London, UK) was used at a dilution of 1:250. Rabbit polyclonals to NIK (H-248), IKK1 (H-744) and IKK2 (H-470) (Santa Cruz Biotechnology) and antibody to p65 (F-6) were all used at a concentration of 1:100. Antibody to hnRNP1 (4B10 monoclonal), courtesy Dr. Gideon Dreyfuss (University of Pennsylvania), was used at 1:100 dilution and antibody to PML (5E10), provided by Professor R. van Driel (Amsterdam), was used at 1:10 dilution. Secondary antibodies were goat anti-mouse FITC and goat anti-rabbit Texas Red (1:200). Cells were examined using a Nikon Microphot fluorescence microscope.

2.6. IKK activity assay

HUVEC were grown on microscope slides and either exposed to flow or activated with TNFα. They were lysed immediately afterwards in 1 ml of lysis buffer (composition as above). The resulting lysate was cleared by high-speed centrifugation (80,000 rpm, 4 °C, 30 min) and incubated for 30 min at 4 °C with 10 μl of protein G beads conjugated to an affinity-purified sheep antibody raised against the C-terminus of IKK1 (residues 734–745). The immunoprecipitate was washed twice with lysis buffer, twice with pulldown buffer (composition: 40 mM Tris–HCl, pH 8.0, 500 mM NaCl, 6 mM EDTA, 6 mM EGTA, 0.1% NP-40, 1 mM DTT, 10 mM β-glycerophosphate, 300 μM sodium orthovanadate, 2 μM PMSF, 10 mM sodium fluoride, containing a protease inhibitor cocktail tablet), then once with kinase assay buffer (composition: 20 mM HEPES, pH 7.7, 2 mM $MgCl_2$, 1 mM DTT, 10 mM β-glycerophosphate, 300 μM sodium orthovanadate, 10 mM NaF, containing a protease inhibitor cocktail tablet). The immunoprecipitate was resuspended in 20 μl kinase assay buffer containing 3 μCi of [γ-^{32}P] ATP, 10 μM ATP and 1 μg of either wild-type (w/t) GST-N terminal IκBα (amino acids 1–70) or a GST-N terminal IκBα mutant (S32E/S36E) for 1 h at 30 °C. The reaction was terminated by adding 3 × SDS to the sample and boiling for 5 min. Proteins were separated on a 12.5% SDS polyacrylamide

gel and radioactivity detected using a phoshorimager (Fuji Bas 1500).

2.7. Protein–DNA binding assay

Nuclear extracts were analysed in a gel electrophoresis DNA binding assay with a ^{32}P-labelled ds oligonucleotide (top strand: 5′ GTCCTCAGAGACCCCCTAAGC 3′) containing a 6-mer shear stress response element (underlined) that was previously shown to bind NF-κB [11]. The procedure was carried out and controlled as described elsewhere [41]. Radioactivity was detected using a phoshorimager (Fuji Bas 1500).

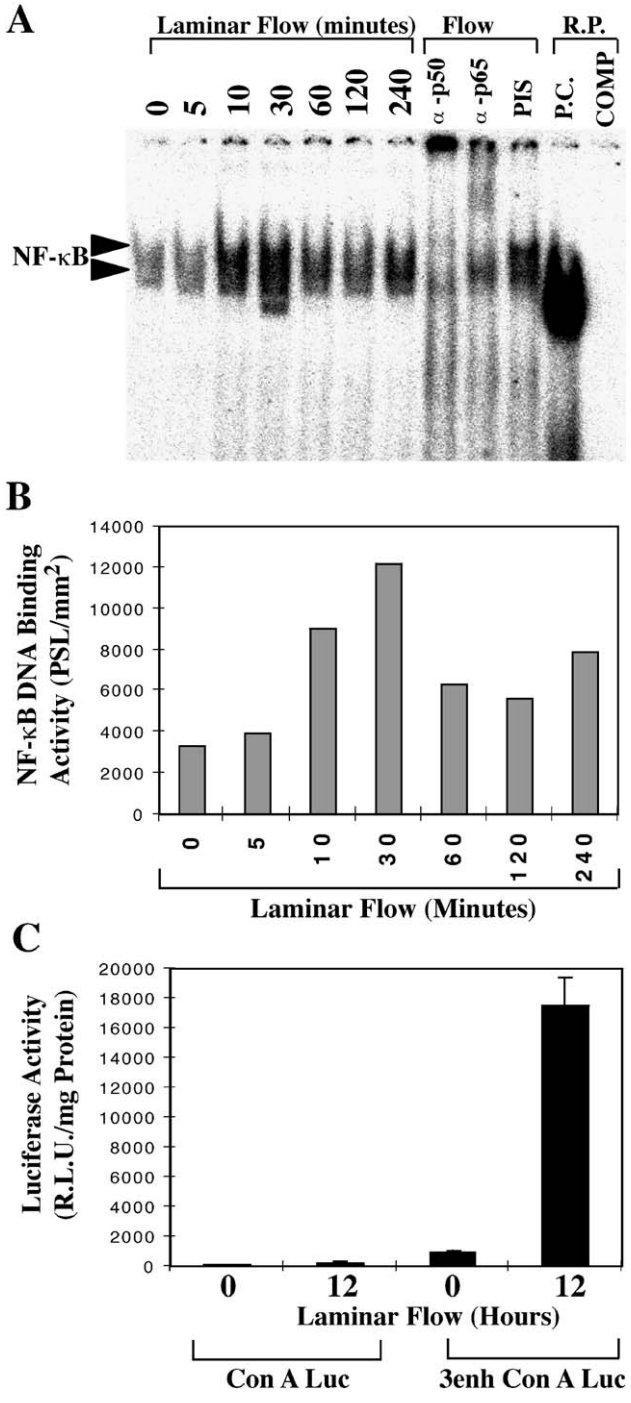

Fig. 1. Flow increases p50/p65 binding to an SSRE and upregulates an NF-κB luciferase reporter. (A) EMSA of nuclear extracts obtained from cells sheared at 15 dyn cm^{-2} for the times indicated and incubated with labeled oligonucleotide containing a known NF-κB-binding SSRE. Pretreating extracts with antibodies to p50 and p65, but not PIS, produced a supershift, indicating binding of flow-activated p50/p65 heterodimer to the SSRE. Control experiments (R.C.) showed that labeled SSRE bound to recombinant p50/p65 heterodimer (positive control; P.C.) and that this was prevented by the addition of excess unlabelled oligonucleotide (COMP). (B) Binding of labeled oligonucleotide was transient, reaching a maximum (ca. 3 × control levels) after 30-min flow. (C) Gene reporter assay using a conA-luciferase reporter containing 3 κB bindings sites. Cells exposed to flow (12 h) showed a 19-fold increase in luciferase reporter activity when compared to cells maintained in static culture. Results are mean ± S.E. of three experiments.

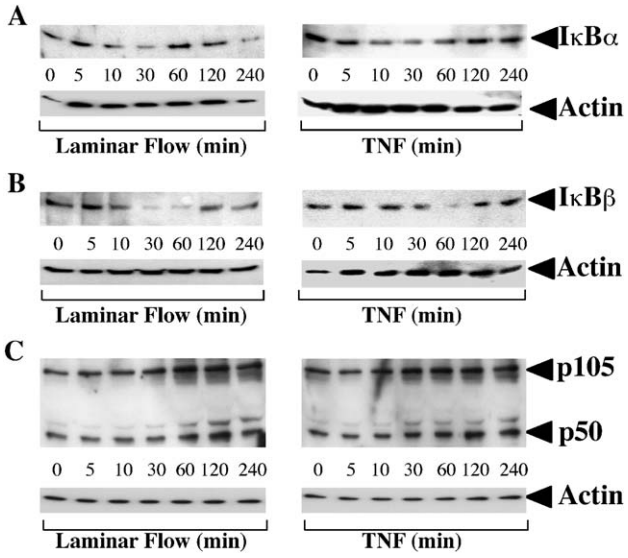

Fig. 2. Flow and TNFα treatment induces degradation of IκBα and β, but not p105/p50. Western immunoblots of whole cell extracts from monolayers exposed to flow (15 dyn cm^{-2}; left panels) or to TNF-α (right panels) for the times indicated. Membranes were probed with antibodies to IκBα, IκBβ1 and p50/p105 and detected by ECL using HRP-tagged secondary antibodies. Flow and TNF-α caused transient reductions in both IκBα and IκBβ1 levels. Immunoreactivity to p50 and p105 was increased after 60–120 min flow. Membranes were stripped and reprobed with antibody to actin to check uniformity of loading. Result of a single experiment using same cell lysates that were later assayed for IKK activity (see Fig. 3).

3. Results

3.1. Fluid shear stress stimulates NF-κB-dependent gene transcription

HUVEC were exposed to uniform shear stress (15 dyn cm^{-2}) for periods ranging from 5 to 240 min. Nuclear extracts were prepared and subjected to a DNA binding assay (Fig. 1A) using ^{32}P-labelled oligonucleotide contain-

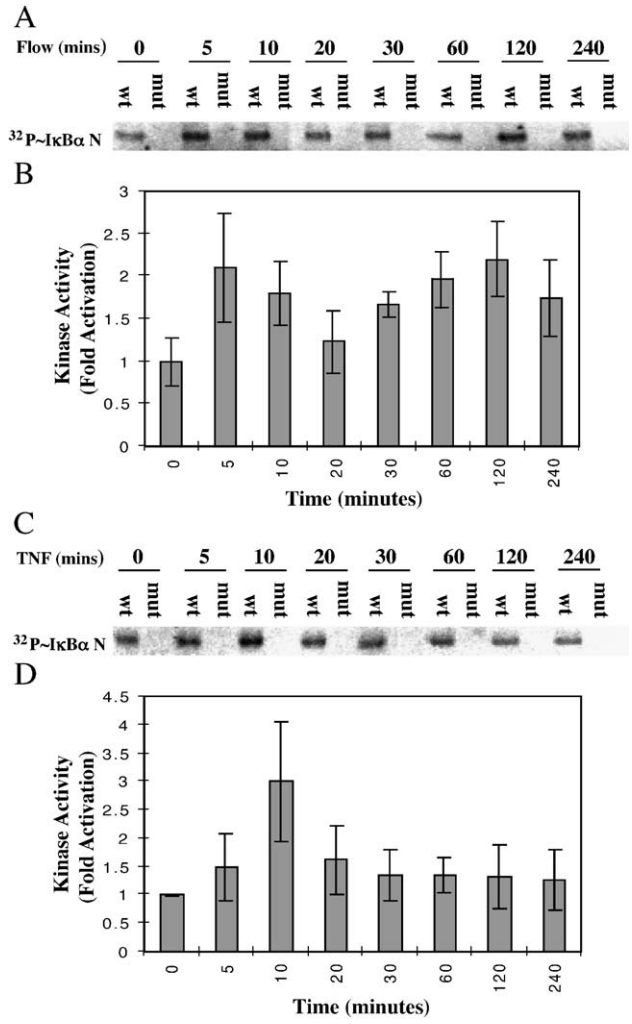

Fig. 3. Flow activates endogenous IKKs. (A,B) Assay of IKK1 and 2 activities in response to flow (15 dyn cm^{-2}) for the times indicated. Flow produced a biphasic pattern of ^{32}P incorporation into a GST-tagged IκBα (1–70) fusion protein. Mutant substrate lacking serines 32 and 36 did not incorporate radioactive label. (C,D) IKK1 and 2 activities in cells treated with TNF-α for the times indicated. TNF-α produced a monophasic pattern of kinase activity. Results are mean ± S.E. of three experiments.

ing a SSRE. Flow produced a transient increase in DNA binding that peaked after ~30 min, then decreased to around 50% of its maximum value in cells that were sheared for 60–240 min (Fig. 1B). Pretreating nuclear extracts with anti-p65 or anti-p50 caused a supershift of labelled oligonucleotide, while the addition of pre-immune serum (PIS) had no effect on DNA binding. These results show that flow causes activation of the p65/p50 heterodimer. Binding of recombinant p50/p65 is shown as a positive control (PC). A competition asssay (COMP) using excess nonradioactive oligonucleotide (20×) abolished retention of ^{32}P-labelled material on the gel.

NF-κB-dependent gene transcription was measured in cells transfected with the NF-κB-dependent luciferase reporter (3κB Con A-Luc). Cells expressing a version lacking NF-κB consensus binding sites (Con A-Luc) were used as a negative control. Confluent monolayers of HUVEC were first exposed to shear stress (15 dyn cm^{-2}) for 12 h and then cultured for a further 24 h before preparing nuclear extracts and measuring luciferase activity. The results are shown in Fig. 1C. Cells transfected with reporter incorporating the 3 κB enhancer showed a ~19-fold increase in luciferase activity, as compared to only a 2.6-fold increase in cells expressing the NF-κB independent reporter (Con A Luc).

3.2. Fluid shear stress and TNFα stimulate IκBα and IκBβ degradation, but not p105

HUVEC were exposed to laminar shear stress (15 dyn cm^{-2}) or treated with TNFα (30 ng/ml) for periods of

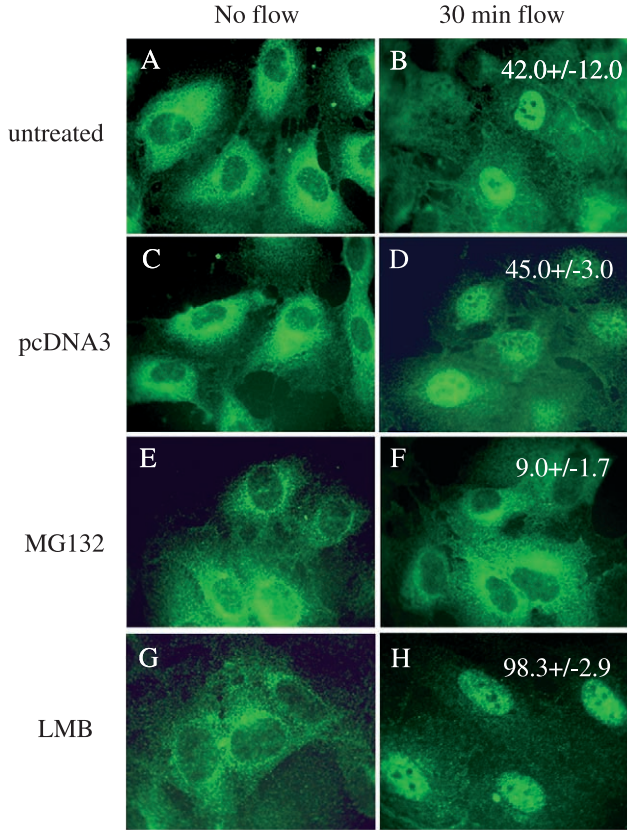

Fig. 4. Flow stimulates nuclear translocation of p65 subunit. Immunofluorescence images of cells stained with rabbit polyclonal antibody to recombinant p65 (C-20) and goat anti-rabbit secondary antibody tagged with FITC. The numbers shown in panels B, D, F and H indicate percentage of cells exhibiting nuclear p65 immunoreactivity. Staining was largely confined to the cytoplasm in cells maintained in static culture (A, C, E and G). Flow (15 dyn cm^{-2} for 30 min; right panels) caused p65 to move from the cytoplasm (A) into the nucleus (B). Nuclear translocation of p65 in response to flow was unaffected by transfecting cells with empty vector (C, D) but prevented in cells treated with MG132, which blocks nuclear import (E, F). The proportion of cells that stained for p65 decreased after ~120 min flow (not shown) due to CRM-1-dependent nuclear export of p50/p65 complexed with newly synthesised IκB. Leptomycin B (LMB) blocks nuclear export and therefore resulted in nearly all cells exhibiting nuclear p65 (G, H).

5–240 min (Fig. 2). Whole-cell lysates were prepared and analysed by ECL Western blotting, using rabbit polyclonal antibodies to IκBα and IκBβ$_1$ and an affinity-purified sheep polyclonal antibody to p105/p50. Protein levels were quantified by gel densitometry and the values obtained were normalised with respect to those of actin.

Flow resulted in a transient reduction of IκBα. IκBα levels were minimal after ~30 min, falling to ~32% of the amount present in cells maintained in static culture, but then recovered fully in cells that were sheared for 60 and 120 min (Fig. 2A). Restoration of IκBα to control levels is due to activation of IκBα gene transcription by NF-κB and the subsequent expression of new protein [41]. Significantly, however, after 240 min shearing, the levels of IκBα had fallen again, to ~35% of the levels in unsheared cells, suggestive of a biphasic pattern of degradation. The levels of IκBβ$_1$ were also reduced by flow, to 6–7% of unsheared control cells after 30–60 min (Fig. 2B). Recovery of control IκBβ$_1$ levels was complete in cells that had been sheared for 120–240 min.

The time course of the degradation of IκB proteins was also studied after the same periods of TNFα (30 ng ml^{-1}) stimulation. IκBα was maximally degraded (48% of unsheared control) after treating cells with TNFα for 30 min (Fig. 2A). Some recovery was seen after 60-min treatment and this was complete in cells treated for 120 and 240 min. IκBβ$_1$ was degraded maximally after 60 min exposure to TNFα (11% unsheared control cells) and returned to pretreatment levels in cells treated for 120 min (Fig. 2B).

The levels of p105/p50 were not reduced by flow or by TNFα, but instead showed a slight increase at the 60-, 120- and 240-min time points (Fig. 2C).

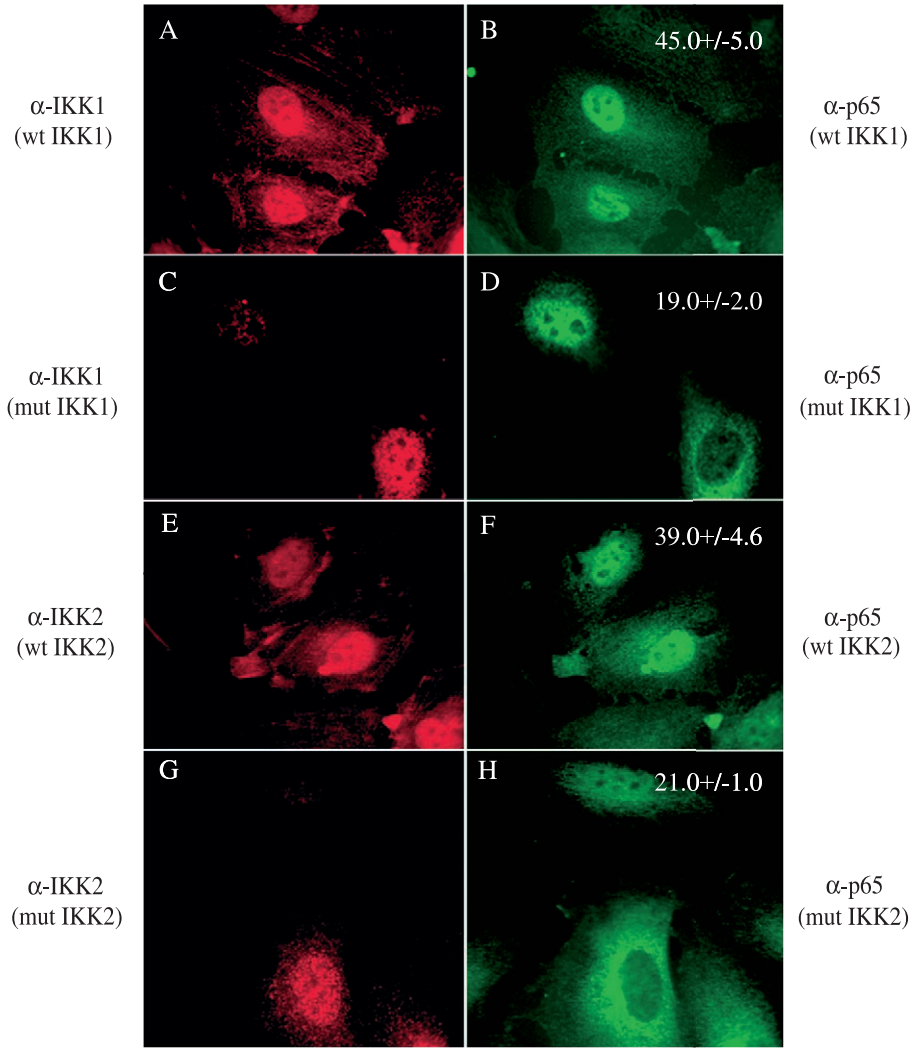

Fig. 5. Catalytically inactive mutants of IKK1 and 2 block nuclear translocation of p65. Double indirect immunofluorescence of IKK1/2 (red) and p65 (green). Paired images of cells exposed to 15 dyn cm^{-2} for 30 min. Nuclear translocation of p65 in response to flow was normal in cells overexpressing w/t IKK1 (A,B) and IKK2 (E,F) but prevented in cells expressing mutant forms of IKK1 (C,D) and 2 (G,H). p65 staining was present in nuclei of individual cells not transfected with mutant IKK1 and 2 but absent in those that were. Numbers in panels B,D,F and H show percentage of nuclei containing p65.

3.3. Fluid shear stress and TNFα activate endogenous IKK in endothelial cells

Cells were first exposed to flow (15 dyn cm^{-2}) or to TNFα α (30 ng/ml), for periods ranging from 5 to 240 min, then extracted immediately afterwards in lysis buffer. The IKK complex was then immunoprecipitated with a sheep polyclonal antibody raised against the C-terminus of IKK1. This antibody also recognises IKK2. IKK1/2 activity was measured by monitoring the incorporation of ^{32}P into a GST-IκBα (residues 1–70) w/t fusion protein. The radiolabel is incorporated into serine residues 32 and 36. A mutant form of the substrate, in which S32 and S36 were replaced with glutamic acids (GST-IκBα 1–70 S/E), served as a negative control. IKK recovery was monitored by immunoblotting using antibody to IKK1 (not illustrated).

Shear stress caused rapid activation of endogenous IKK. Kinase activity peaked after 5 min flow, declined to control levels in cells exposed to flow for 20 min, and then rose again, reaching a secondary maximum after 120 min flow (Fig. 3A,B). The mutant substrate (GST-IκBα 1–70 S/E) was not phosphorylated confirming the specificity of the assay (Fig. 3A).

IKK1/2 were stimulated in HUVEC treated with TNFα. Activity was maximal after 10-min treatment (Fig. 3C,D) and then fell back to control levels in cells exposed to TNFα for 30 min or more. Again, the mutant form of the substrate (GST-IκBα 1–70 S/E) was not phosphorylated.

3.4. Fluid shear stress activation of NF-κB in single cells

NF-κB activation under flow was studied by monitoring nuclear translocation of the p65 subunit by indirect immunofluorescence. Untransfected HUVEC (Fig. 4A,B) and HUVEC transfected with empty pcDNA 3 (Fig. 4C,D) were exposed to shear stress (15 dyn cm^{-2}) for 30 or 120 min. The number of cells showing nuclear staining with anti-p65 was measured from photographic enlargements of

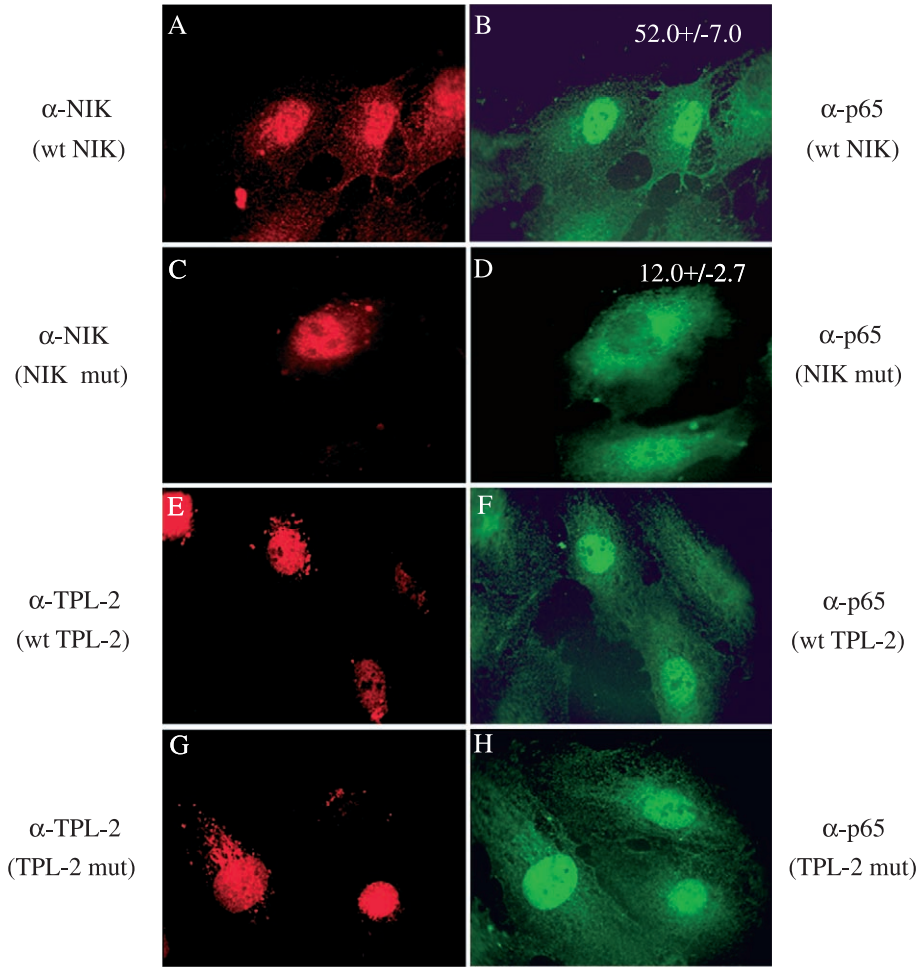

Fig. 6. A catalytically inactive mutant of NIK, but not mutant TPL-2 kinase, blocks nuclear translocation of p65. Double indirect immunofluorescence of NIK or TPL-2 (red) and p65 (green). Paired images of cells exposed to 15 dyn cm^{-2} for 30 min. Numbers indicate percentage of cells showing nuclear staining for p65. Nuclear translocation of p65 in response to flow was normal in cells overexpressing w/t NIK (A,B) but not in those overexpressing the catalytically inactive form of the enzyme (C,D). In contrast to this, nuclear translocation was seen both in cells overexpressing w/t TPL-2 kinase (E,F) and in those overexpressing the inactive mutant (G,H), indicating that TPL-2 kinase is not involved in flow-mediated activation of NF-κB.

randomly selected areas of the cover slip and expressed as a percentage of the total number of cells examined ($n = 100-150$ cells).

Nuclear translocation of NF-κB was maximal after 30 min of flow. The percentage of activated cells in untransfected and pcDNA3-transfected cells was similar, 42 ± 12% and 45 ± 3%, respectively (Fig. 4B,D). This correlates well with electrophoretic mobility shift assay (EMSA) and Western blotting data, showing that DNA binding and IκBα and IκBβ$_1$ degradation were also maximal after 30 min of flow (Fig. 2). The percentage of nuclei staining for p65 was reduced in cells exposed to 120 min flow (not illustrated), to 33 ± 5.5% (untransfected cells) and 39 ± 7.8% (pcDNA3 transfected cells).

Nuclear staining for p65 was also studied in cells that had been treated with either MG132 or with leptomycin B (LMB) for 30 min before subjecting them to flow. MG132 specifically inhibits proteasome-mediated degradation of IκBs. This blocks nuclear translocation of NF-κB and results in the accumulation of polyubiquitinated complexes in the cytoplasm [16,24]. LMB targets CRM1/exportin-1 and prevents complexes of NF-κB and newly synthesised IκBα from leaving the nucleus [42,43]. Pretreating cells with MG132 prevented nuclear translocation of p65 in response to flow, as anticipated. Only 9 ± 1.9% of MG132-treated cells showed nuclear staining after 30 min of flow (Fig. 4E,F) and 6 ± 2% after shearing cells for 120 min. In contrast, virtually all of the cells exhibited nuclear staining for p65 after treatment with LMB: 97 ± 1% after 30 min (Fig. 4G,H) and 98 ± 2% after 120 min of flow (not shown).

Shear stress had no effect on the cellular distribution of the shuttling protein hnRNPA1 [44], nor did it alter the subnuclear distribution of promyelocytic leukemia protein [45,46] (data not shown), confirming that flow has a selective effect on nuclear translocation of NF-κB.

3.5. Catalytically inactive mutants of IKK1 and IKK2 block p65 nuclear translocation

HUVEC were transfected with plasmids encoding w/t IKK1 and IKK2 proteins or with the corresponding kinase inactive mutants (K44A) and then exposed to shear stress (15 dyn cm^{-2}) to determine whether flow-induced activation of NF-κB requires IKK1 and/or 2 (Fig. 5). Double indirect immunofluorescence was used to monitor NF-κB nuclear translocation, using a mouse monoclonal antibody to p65 in combination with rabbit polyclonal antibodies to IKK1 or IKK2. Under the conditions of our experiments, endogenous IKK1/2 levels were below the threshold of detection by immunofluorescence and therefore only the cells that overexpressed w/t or mutant kinases were stained. The results show that p65 was present in the nuclei of cells overexpressing w/t IKK1 (Fig. 5B) and w/t IKK2 (Fig. 5F) after 30 min flow. However, there was no nuclear staining in cells that were transfected with mut IKK1 (Fig. 5D) or mut IKK2 (Fig. 5H). Translocation of p65 was normal in cells that escaped transfection with IKK1 mut or IKK2 mut

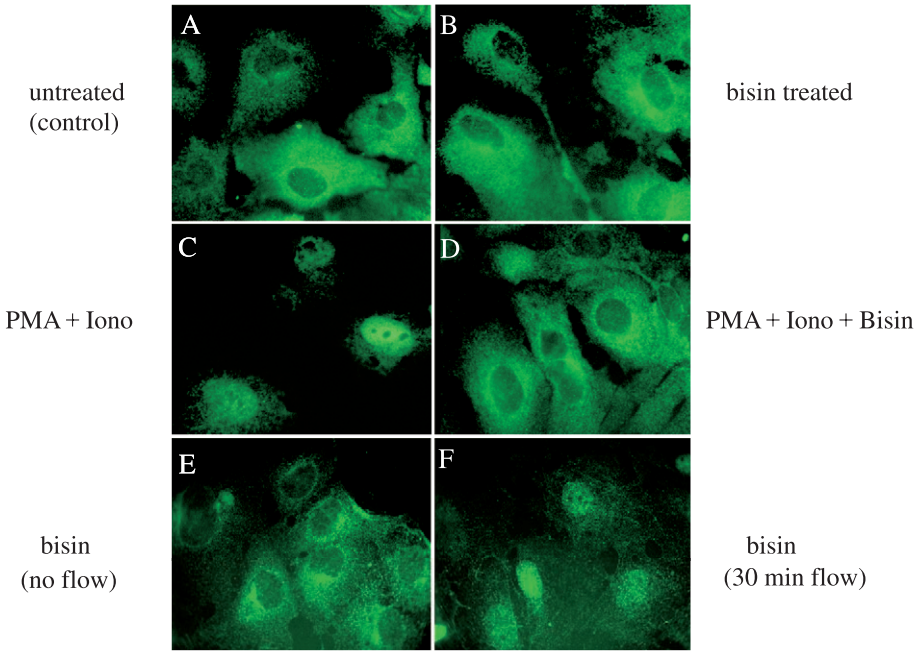

Fig. 7. Bisindolymaleimide 1 blocks agonist-induced, but not flow-induced, nuclear translocation of p65. Bisindoylmaleimide 1 (bisin), a selective inhibitor of PKC isoforms, has no effect on the distribution of p65 immunoreactivity in static cultures (A,B). Nuclear translocation of p65 caused by treating cells with PMA and ionomycin (C) was blocked by bisin (100 nM, for 30 min; D) whereas flow-dependent nuclear translocation was not (E,F). Percentage of cells showing nuclear staining for p65 shown in panel F.

constructs (Fig. 5D,H, respectively). These data demonstrate that both IKKs are required for activation of NF-κB by flow.

3.6. A catalytically inactive mutant of NIK, but not TPL2, blocks p65 nuclear translocation

HUVEC were transfected with plasmids encoding w/t NIK or the corresponding catalytically inactive mutant (KK429-430AA) and then subjected to flow. Nuclear translocation of NF-κB was monitored by immunofluorescence, using mouse monoclonal antibody to p65 and a rabbit polyclonal antibody to NIK. Again, endogenous NIK was below the limit of detection so that only those cells that were overexpressing w/t or mut NIK were stained. Fig. 6A,B shows that shear stress induced nuclear translocation of NF-κB in cells expressing w/t NIK, but that this was prevented in cells expressing mut NIK (Fig. 6C,D). Here again, nuclear translocation was seen in those cells that escaped transfection with the NIK mut. These results show that NIK is an essential component of the upstream signaling pathway activated by flow.

HUVEC were also transfected with plasmids encoding the w/t TPL2 protein or the corresponding catalytically inactive mutant form (TPL-2 mut, A270) and then exposed to shear stress (15 dyn cm^{-2}) for 30 min. Mouse monoclonal antibody to p65 and a rabbit polyclonal antibody to TPL2 were used to monitor nuclear translocation of p65. Endogenous TPL2 levels were once again below the limit of detection by immunostaining. The results showed that nuclear translocation of p65 in response to flow occurred normally in cells transfected with either TPL2 w/t (Fig. 6E,F) or with the kinase inactive TPL2 mut (Fig. 6G,H), demonstrating that TPL-2 is not involved in the activation pathway.

3.7. Activation of NF-κB by flow is independent of PKC

Treating cells with PMA and ionomycin together activated NF-κB and induced nuclear translocation of p65 in 100% of cells (Fig. 7C). This was prevented by pretreating cells with bisindoylmaleimide-1 (bisin; 100 nM) for 30 min immediately before the addition of PMA+ ionomycin (Fig. 7C,D): under these conditions, fewer than 5% of nuclei stained positively for p65. In contrast, pretreating cells with bisin before subjecting them to flow did not prevent nuclear translocation of p65: around 47% of cells showed nuclear p65 immunoreactivity after 30 min (Fig. 7F), a figure similar to that obtained after shearing untreated cells (Fig. 4B). These experiments show that the PKC isoforms found in HUVEC (viz: PKC-α, -ε and ζ) are not involved in shear stress induced activation of NF-κB.

4. Discussion

There is growing evidence to implicate NF-κB-dependent gene regulation in atherogenesis [34]. NF-κB is activated in advanced atherosclerotic lesions [35], where many of the genes that are expressed are known to be regulated by NF-κB [36]. Furthermore, several components of the NF-κB signaling pathway (e.g. RelA, IκBα, IκBβ) are upregulated in areas of the vasculature that are at high risk of developing atheroma in the future but are free of disease for the present. This suggests that local haemodynamic forces may predispose (or 'prime') the endothelium to respond to systemic risk factors at some time in the future [37].

The cells used in this study were exposed to uniform (spatially and temporally) laminar flow, generating a unidirectional shear stress of 15 dyn cm^{-2}, maintained for up to 4 h. This treatment resulted in a transient reduction in IκBα and $IκBβ_1$ levels: both started to decrease within 5–10 min of commencing flow and were maximally depressed after ca. 30–60 min. The binding of p50/p65 heterodimer to the SSRE and nuclear translocation of p65 were also maximal at this time, as shown by EMSA and immunofluorescence microscopy, respectively.

The levels of IκBα and $IκBβ_1$ were fully restored in cells that were sheared for ca. 60–120 min, respectively. This was accompanied by reduced binding of p50/p65 to the SSRE. The proportion of cells showing nuclear staining for p65 was also reduced while cytoplasmic staining for p65 increased correspondingly. The recovery of IκBα was anticipated because its gene is activated by NF-κB [47–49]. Newly synthesised IκBα is transported from the cytoplasm into the nucleus where it binds to NF-κB, causing it to dissociate from DNA, thereby terminating gene transcription [41]. The IκBα–NF-κB complex is then exported from the nucleus into the cytoplasm in a CRM-1 protein-dependent process [40] that can be blocked by LMB. Nuclear export of p65 under flow was blocked in our experiments by treating cells with LMB, showing that autoregulation of NF-κB gene transcription also occurs when NF-κB is induced by flow.

Nuclear translocation of p65 in response to flow was prevented in cells that were transfected with kinase-inactive mutants of NIK, IKK1 or IKK2. However, cells overexpressing a dominant-negative form of TPL-2 kinase responded normally. TPL-2 has been implicated in the posttranslational processing of p105 [50]. The failure of flow to reduce p105 levels is consistent with the lack of involvement of TPL-2 in the activation process. Phosphorylation of p105 on serines 927 and 932 by IKK results in βTrCP-mediated ubiquitination of the protein and triggers its destruction by the proteasome pathway [51]. The fact that we did not see any degradation of p105 in our experiments suggests that flow does not activate IKK-dependent phosphorylation of p105. Furthermore, the PKC inhibitor bisin, while able to fully block PMA + ionomycin-induced activation of NF-κB, did not prevent nuclear translocation of p65 in response to flow. These results taken together indicate that NIK, IKK1 and IKK2, but not PKC or TPL-2, are required for activation of NF-κB by shear stress. The ability of MG132 to block nuclear translocation of p65 shows that

NF-κB activation by flow requires proteolysis of IκBs via the 26S proteasome pathway.

The activation of IKK1/2 under flow was the earliest event detected in our experiments. Bhullar et al. [10] reported that flow activated IKKs in bovine endothelial cells, but the time course was different from that seen here. IKK activity was monophasic in their study, requiring ca. 30 min to attain a maximum value of $\sim 3.5\times$ control levels, before returning to control levels after a further 1.5 h. Activation of IKK was more rapid in our experiments, reaching a maximum of $\sim 2\times$ control levels after only 5 min, then declining to near basal levels after 20 min, before increasing once again to reach a secondary peak after 120 min. This result is suggestive of a biphasic pattern of IKK activation, though additional measurements on cells exposed to flow for longer periods (>120 min) will be needed to confirm or refute this notion. However, it is of interest to note in this context that activation of the AP-1 transcription factor by flow is reported to be biphasic [12]. The discrepancy between our kinase assays and those of Bhullar et al. might be due to differences in methodology: their study was performed on bovine endothelial cells that were overexpressing IKK1 and IKK2, whereas the results reported here were obtained using human cells and we measured endogenous IKK activity.

The induction of NF-κB-dependent transcription was maximal after ca. 30 min of flow, as shown by gel shift assay, yet anti-p65 nuclear staining indicated that fewer than 50% of cells were activated at this time. The flow chambers used in this study were carefully tested to ensure that they generated steady, two-dimensional flow. However, the testing procedure employed measured shear forces exerted on a uniformly flat surface (glass coverslip) and did not take account of local variations in the surface topography of real cell monolayers. Barbee et al. [52] studied living endothelial monolayers by atomic force microscopy and used their measurements to estimate the spatial gradients of shear stress due to surface undulations. Their analysis showed a $\sim$ 6-μm range of cell heights with a mean slope of 11°. In a later study [53], they calculated that for a nominal shear stress of 12 dyn cm^{-2} the actual shear stress on the steepest part of a cell would change at the rate of ± 4 dyn cm^{-2} μm^{-1}. Thus, even if the steep region were only 2-μm long, shear stress could vary from as much as 20 dyn cm^{-2} on the upstream surface of a cell to as little as 4 dyn cm^{-2} on its downstream surface. This variability could contribute to the heterogeneity in the response of cells to flow.

Numerous studies have implicated the cytoskeleton in the mechanism(s) by which cells are able to sense and then respond to altered shear stresses. The decentralised model of Davies [1] postulates that shear forces acting on the lumenal cell surface are rapidly disseminated to other sites within the cell (e.g. cell–cell contacts, focal adhesions, nuclear envelope) by elements of the cytoskeleton. The possible involvement of the intermediate filament (IF) system as an intracellular force-transmitting pathway has received comparatively little attention to date when compared to either actin- or tubulin-based components. However, direct evidence for mechanical coupling between surface integrins and the nucleus via cytoplasmic IF was obtained using microbeads coated with fibronectin or RGD-containing peptide [54] and recent studies of cells expressing GFP-tagged vimentin show that IF undergo rapid displacement shortly after commencing flow [55,56]. Moreover, immunofluorescence studies have shown that fine bundles of IF make direct contact with the cytoplasmic face of focal adhesions [57], most likely via plectin, a well-documented cytoskeletal 'linker' protein [58]. Contact with the extracellular matrix is established through interaction of the $\alpha_v\beta_3$ integrin with the G domain of the α4 subunit of laminin [59]. The ability to prevent flow-mediated induction of NF-κB transcriptional activity by treating cells with LM609 blocking antibody to the $\alpha_v\beta_3$ integrin [10] suggests that vimentin-associated cell–matrix adhesions, and plectin-based connections with the cytoplasmic IF network, could play a critical role in the mechanotransduction process.

In summary, our results show that laminar flow induces NF-κB-dependent gene regulation in human endothelial cells via a mechanism that involves NIK-IKK1/2 signaling and degradation of NF-κB inhibitor proteins by the 26S proteasome pathway. The process does not involve TPL-2 kinase or PKC. Enhanced expression of the luciferase reporter seen in our experiments confirms that NF-κB induced by flow is transcriptionally active.

Acknowledgements

We are grateful to colleagues mentioned in the text who generously donated antibodies and/or constructs for use in this work. We thank David Roche, Sean Earnshaw and Jim Allen (Photographic Unit, School of Biology) for their expert photographic assistance. The study was assisted by grants (to FWF and RTH) from the British Heart Foundation (PG/1998162) and The Wellcome Trust (FWF).

References

[1] P.F. Davies, Flow-mediated endothelial mechanotransduction, Physiol. Rev. 75 (1995) 519–560.
[2] P.F. Davies, K.A Barbee, M.V. Volin, A. Robotewskyj, J. Chen, J.L. Griem, M.N. Wernick, E. Jacobs, D.C. Polacek, N. DePaola, A.I. Barakat, Spatial relationships in early signaling events of flow-mediated endothelial mechanotransduction, Annu. Rev. Physiol. 59 (1997) 527–549.
[3] S. Jalali, Y.-S. Li, M. Sotoudeh, S. Yuan, S. Li, S. Chien, J.Y.-J. Shyy, Shear stress activates p60src-Ras-MAPKs signaling pathways in vascular endothelial cells, Arterioscler. Thromb. Vasc. Biol. 189 (1998) 227–234.
[4] H. Tseng, T.E. Peterson, B.C. Berk, Fluid shear stress stimulates mitogen-activated protein kinase in endothelial cells, Circ. Res. 77 (1995) 869–878.
[5] T. Ishida, T.E. Peterson, N.L. Kovach, B.C. Berk, MAP kinase acti-

vation by flow in endothelial cells. Role of β1 integrins and tyrosine kinases, Circ. Res. 79 (1996) 310–316.
[6] C. Yan, M. Takahashi, M. Okuda, J.-D. Lee, B.C. Berk, Fluid shear stress stimulates big mitogen-activated protein kinase 1 (BMK1) activity in endothelial cells, J. Biol. Chem. 274 (1999) 143–150.
[7] H.J. Hsieh, N.Q. Li, J.A. Frangos, Pulsatile and steady flow induces c-fos expression in human endothelial cells, J. Cell. Physiol. 154 (1993) 143–151.
[8] N. Resnick, T. Collins, W. Atkinson, D.T. Bonthron, C.F. Dewey Jr., M.A. Gimbrone Jr., Platelet-derived growth factor B chain promoter contains a *cis*-acting fluid shear-stress-responsive element, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 4591–4595.
[9] S. Chien, S. Li, J.J.-Y. Shyy, Effects of mechanical forces on signal transduction and gene expression in endothelial cells, Hypertension 31 (1998) 162–169.
[10] I.S. Bhullar, Y.S. Li, H. Miao, E. Zandi, M. Kim, J.Y. Shyy, S. Chien, Fluid shear stress activation of IκB kinase is integrin-dependent, J. Biol. Chem. 273 (1998) 30544–30549.
[11] L.M. Khachigian, N. Resnick, M.A. Gimbrone Jr., T. Collins, NF-κB interacts functionally with the platelet-derived growth factor B-chain shear-stress response element in vascular endothelial cells exposed to fluid shear stress, J. Clin. Invest. 96 (1995) 1169–1175.
[12] Q. Lan, K.O. Mercurius, P.F. Davies, Stimulation of transcription factors NF-κB and AP1 in endothelial cells subjected to shear stress, Biochem. Biophys. Res. Commun. 201 (1994) 950–956.
[13] J.Y. Shyy, Y.S. Li, M.C. Lin, W. Chen, S. Yuan, S. Usami, S. Chien, Multiple *cis*-elements mediate shear stress-induced gene expression, J. Biomech. 28 (1995) 1451–1457.
[14] T. Nagel, N. Resnick, C.F. Dewey, M. Gimbrone, Vascular endothelial cells respond to spatial gradients in fluid shear stress by enhanced activation of transcription factors, Arterioscler. Thromb. Vasc. Biol. 19 (1999) 1825–1834.
[15] C.H. Resnick, M.A. Gimbrone, Hemodynamic forces are complex regulators of endothelial gene expression, FASEB J. 9 (1995) 874–882.
[16] I. Alkalay, A. Yaron, A. Hatzubai, A. Orian, A. Ciechanover, Y. Ben-Neriah, Stimulation-dependent IκBα phosphorylation marks the NF-κB inhibitor for degradation via the ubiquitin–proteasome pathway, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 10599–10603.
[17] L. Baldi, K. Brown, G. Franzoso, U. Siebenlist, Critical role for lysine-21 and lysine-22 signal-induced, ubiquitin-mediated proteolysis of IκBα, J. Biol. Chem. 271 (1996) 376–379.
[18] J.A. DiDonato, M. Hayakawa, D.M. Rothwarf, E. Zandi, M. Karin, A cytokine-responsive IκB kinase that activates the transcription factor NF-κB, Nature 388 (1997) 548–554.
[19] F. Mercurio, H. Zhu, B.W. Murray, A. Shevchenko, B.L. Bennett, J. Li, D.B. Young, M. Barbosa, M. Mann, A. Manning, et al., IKK-1 and IKK-2: cytokine-activated IκB kinases essential for NF-κB activation, Science 278 (1997) 860–866.
[20] C.H. Regnier, H.Y. Song, X. Gao, D.V. Goeddel, Z. Cao, M. Rothe, Identification and characterization of an IκB kinase, Cell 90 (1997) 373–383.
[21] J.D. Woronicz, X. Gao, Z. Cao, M. Rothe, D.V. Goeddel, IκB kinase β: NF-κB activation and complex formation with IκB kinase-alpha and NIK, Science 278 (1997) 866–869.
[22] E. Zandi, D.M. Rothwarf, M. Delhase, M. Hayakawa, M. Karin, The IκB kinase complex (IKK) contains two kinase subunits, IKKα and IKKβ, necessary for IκB phosphorylation and NF-κB activation, Cell 91 (1997) 243–252.
[23] D.M. Rothwarf, E. Zandi, G. Natoli, M. Karin, IKKγ is an essential regulatory subunit of the IκB kinase complex, Nature 395 (1998) 297–300.
[24] M. Roff, J. Thomson, M.S. Rodriguez, J.-M. Jacque, F. Baleux, F. Arenzana-Seisdedos, R.T. Hay, Role of IκBα ubiquitination in signal-induced activation of NF-κB in vivo, J. Biol. Chem. 271 (1996) 7844–7850.
[25] A. Yaron, A. Hatzubai, M. Davis, I. Lavon, S. Amit, A.M. Manning, J.S. Anderson, M. Man, F. Mercurio, Y. Ben-Neriah, Identification of the receptor component of the IκBα-ubiquitin ligase, Nature 396 (1998) 590–594.
[26] E. Spencer, J. Jiang, Z.J. Chen, Signal-induced ubiquitination of IκBα by the F-box protein Slimb/β-TrCP, Genes Dev. 13 (1999) 284–294.
[27] P. Tan, S. Fuchs, A. Chen, K. Wu, C. Gomez, Z. Ronai, Z.Q. Pan, Recruitment of a ROC1-CUL1 ubiquitin ligase by Skp1 and HOS to catalyze the ubiquitination of IκBα, Mol. Cell 3 (1999) 527–533.
[28] L. Vuillard, J. Nicholson, R.T. Hay, A complex containing β-TrCP recruits Cdc34 to catalyse ubiquitination of IκBα, FEBS Lett. 455 (1999) 311–314.
[29] J.T. Winston, P. Strack, P. Beer-Romero, C.Y. Chu, S.J. Elledge, J.W. Harper, The SCF b–TRCP–ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IκBα and β-catenin and stimulates IκBα ubiquitination in vitro, Genes Dev. 13 (1999) 270–283.
[30] H. Nakano, M. Shindo, S. Sakon, S. Nishinaka, M. Mihara, H. Yagita, K. Okumura, Differential regulation of IκB kinase alpha and beta by two upstream kinases, NF-κB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 3537–3542.
[31] R. Hofer-Warbinek, J.A. Schmid, C. Stehlik, B.R. Binder, J. Lipp, R. de Martin, Activation of NF-κB by XIAP. The X chromosome-linked inhibitor of apoptosis, in Ecs involves TAK1, J. Biol. Chem. 275 (2000) 22064–22068.
[32] O.N. Ozes, L.D. Mayo, J.A. Gustin, L.M. Pfeffer, D.B. Donner, NF-κB activation by tumour necrosis factor requires the Akt serine–threonine kinase, Nature 401 (1999) 82–85.
[33] I. Dominguez, L. Sanz, F. Arenzana-Seisdedos, M.T. Diaz-Meco, J.L. Virelizier, J. Moscat, Inhibition of protein kinase C-ζ sub-species blocks the activation of an NF-κB-like activity in *Xenopus laevis* oocytes, Mol. Cell Biol. 13 (1993) 1290–1295.
[34] R. de Martin, M. Hoeth, R. Hofer-Warbinek, J.A. Schmid, The transcription factor NF-κB and the regulation of vascular cell function, Arterioscler. Thromb. Vasc. Biol. 20 (2000) E83–E88.
[35] K. Brand, S. Page, G. Rogler, R. Bartsch, R. Kneuchel, M. Page, C. Kaltschmidt, P.A. Baeuerle, D. Neumeier, Activated transcription factor nuclear factor-kappa B is present in the atherosclerotic lesion, J. Clin. Invest. 97 (1996) 1715–1722.
[36] T. Collins, Endothelial nuclear factor κB and the initiation of the atherosclerotic lesion, Lab. Invest. 68 (1993) 499–508.
[37] L. Hajra, A.I. Evans, M. Chen, S.J. Hyduck, T. Collins, M.I. Cybulsky, The NF-κB signal transduction pathway in aortic endothelial cells is primed for activation in regions predisposed to atherosclerotic lesion formation, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 9052–9057.
[38] R.F. Viggers, A.R. Wechezak, L.R. Sauvage, An apparatus to study the response of cultured endothelium to shear stress, J. Biomech. Eng. 108 (1986) 332–337.
[39] Y.J. Shyy, H.J. Hsieh, S. Usami, S. Chien, Fluid shear stress induces a biphasic response of human monocyte chemotactic protein 1 gene expression in vascular endothelium, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 4678–4682.
[40] M.S. Rodriguez, J. Thompson, R.T. Hay, C. Dargemont, Nuclear retention of IκBα protects it from signal-induced degradation and inhibits NF-κB transcriptional activation, J. Biol. Chem. 274 (1999) 9108–9115.
[41] F. Arenzana-Seisdedos, J. Thompson, M.S. Rodriguez, F. Bachelerie, D. Thomas, R.T. Hay, Inducible nuclear expression of newly synthesized IκBα negatively regulates DNA-binding and transcriptional activities of NF-κB, Mol. Cell. Biol. 15 (1995) 2689–2696.
[42] A.S. Baldwin, The NF-κB and IκB proteins: new discoveries and insights, Annu. Rev. Immunol. 14 (1996) 649–683.
[43] M. Fornerod, M. Ohno, M. Yoshida, I.W. Mattaj, CRM1 is an export receptor for leucine-rich nuclear export signals, Cell 90 (1997) 1051–1060.

[44] S. Pinol-Roma, G. Dreyfuss, Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm, Nature 355 (1992) 730–732.
[45] J.S. Seeler, A. Dejean, The PML nuclear bodies: actors or extras? Curr. Opin. Genet. Dev. 9 (1999) 362–367.
[46] S. Zhong, P. Salomoni, P.P. Pandolfi, The transcriptional role of PML and the nuclear body, Nat. Cell Biol. 2 (2000) E85–E90.
[47] N. Auphan, J.A. DiDonato, C. Rosette, A. Helmberg, M. Karin, Immunosuppression by glucocorticoids: inhibition of NF-κB activity through induction of IκB synthesis, Science 270 (1995) 286–290.
[48] R. de Martin, B. Vanhove, Q. Cheng, E. Hofer, V. Csizmadia, H. Winkler, F.H. Bach, Cytokine-inducible expression in endothelial cells of an IκBα-like gene is regulated by NF-κB, EMBO J. 12 (1993) 2773–2779.
[49] R.I. Scheinman, P.C. Cogswell, A.K. Lofquist , A.S. Baldwin Jr., Role of transcriptional activation of IκBα in mediation of immunosuppression by glucocorticoids, Science 270 (1995) 283–286.
[50] M.P. Belich, A. Salmeron, L.H. Johnston, S.C. Ley, TPL-2 kinase regulates the proteolysis of the NF-κB inhibitory protein NF-kB1 p105, Nature 397 (1999) 363–368.
[51] V. Lang, J. Janzen, G.Z. Fischer, Y. Someji, S. Beinke, A. Slameron, H. Allen, R.T. Hay, Y. Ben-Neriah, S.C. Ley, βTrCP-mediated proteolysis of NF-κB1 p105 requires phosphorylation of p105 on serines 927 and 932, Mol. Cell. Biol. 23 (2003) 402–413.
[52] K.A. Barbee, P.F. Davies, R. Lal, Shear stress-induced reorganisation of the surface topography of living endothelial cell imaged by atomic force microscopy, Circ. Res. 74 (1994) 163–171.
[53] K.A. Barbee, T. Mundell, R. Lal, P.F. Davies, Sub-cellular distribution of shear stress at the surface of flow-aligned and non-aligned endothelial monolayers, Am. J. Physiol. 268 (1995) H1765–H1772.
[54] A.J. Maniotis, C.S. Chen, D.E. Ingber, Demonstration of mechanical connections between integrins, cytoskeletal filaments and nucleoplasm that stabilise nuclear structure, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 849–854.
[55] B.P. Helmke, R.D. Goldman, P.F. Davies, Rapid displacement of vimentin intermediate filaments in living endothelial cells exposed to flow, Circ. Res. 86 (2000) 745–752.
[56] B.P. Helmke, D.B. Thakker, R.D. Goldman, P.F. Davies, Spatiotemporal analysis of flow-induced intermediate filament displacement in living endothelial cells, Biophys. J. 80 (2001) 184–194.
[57] F.W. Flitney, R.D. Goldman, O. Skalli, K.O. Mercurius, P.F. Davies, Dynamic properties of intermediate filaments in cultured endothelial cells: the effects of controlled fluid shear stress, in: S. Moncada, S. Gross, A. Higgs (Eds.), The Biology of Nitric Oxide, vol. 5. Portland Press, London, 1996, p. 251.
[58] G. Wiche, Role of plectin in cytoskeletal organisation and dynamics, J. Cell. Sci. 111 (1998) 2477–2486.
[59] M. Gonzales, B. Weksler, D. Tsurata, R.D. Goldman, K.J. Yoon, S.B. Hopkinson, F.W. Flitney, J.C.R. Jones, Structure and function of a vimentin-associated matrix adhesion in endothelial cells, Mol. Biol. Cell 12 (2001) 85–100.