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RESEARCH PAPER

Over-expression of mitogen-activated protein kinase phosphatase-2 enhances adhesion molecule expression and protects against apoptosis in human endothelial cells

Mashael Al-Mutairi*, Sameer Al-Harthi, Laurence Cadalbert and Robin Plevin

Division of Physiology and Pharmacology, University of Strathclyde, Strathclyde Institute for Biomedical Sciences, Glasgow, UK

Correspondence
Robin Plevin, Division of Physiology and Pharmacology, University of Strathclyde, Strathclyde Institute for Biomedical Sciences, 27 Taylor Street, Glasgow G4 0NR, UK.
E-mail: r.plevin@strath.ac.uk

*Current address: Department of Medical Laboratory Sciences, Faculty of Allied Health Sciences, Health Sciences Center, Kuwait University, Safat 13110, Kuwait

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BACKGROUND AND PURPOSE
We assessed the effects of over-expressing the dual-specific phosphatase, mitogen-activated protein (MAP) kinase phosphatase-2 (MKP-2), in human umbilical vein endothelial cells (HUVECs) on inflammatory protein expression and apoptosis, two key features of endothelial dysfunction in disease.

EXPERIMENTAL APPROACHES
We infected HUVECs for 40 h with an adenoviral version of MKP-2 (Adv.MKP-2). Tumour necrosis factor (TNF)-α-stimulated phosphorylation of MAP kinase and protein expression was measured by Western blotting. Cellular apoptosis was assayed by FACS.

KEY RESULTS
Infection with Adv.MKP-2 selectively abolished TNF-α-mediated c-Jun-N-terminal kinase (JNK) activation and had little effect upon extracellular signal-regulated kinase or p38 MAP kinase. Adv.MKP-2 abolished COX-2 expression, while induction of the endothelial cell adhesion molecules, intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM), two NFκB-dependent proteins, was not affected. However, when ICAM and VCAM expression was partly reduced by blockade of the NFκB pathway, Adv.MKP-2 was able to reverse this inhibition. This correlated with enhanced TNF-α-induced loss of the inhibitor of κB (IκB)α loss, a marker of NFκB activation. TNF-α in combination with NFκB blockade also increased HUVEC apoptosis; this was significantly reversed by Adv.MKP-2. Protein markers of cellular damage and apoptosis, H2AX phosphorylation and caspase-3 cleavage, were also reversed by MKP-2 over-expression.

CONCLUSIONS AND IMPLICATIONS
Over-expression of MKP-2 had different effects upon the expression of inflammatory proteins due to a reciprocal effect upon JNK and NFκB signalling, and also prevented TNF-α-mediated endothelial cell death. These properties may make Adv.MKP-2 a potentially useful future therapy in cardiovascular diseases where endothelial dysfunction is a feature.

Abbreviations
Adv., adenovirus; HUVECs, human umbilical vein endothelial cells; ICAM, intercellular adhesion molecule; IKK, inhibitory kappa B kinase; JNK, c-Jun-N-terminal kinase; MKP-2, MAP kinase phosphatase-2; TNF-α, tumour necrosis factor-α; VCAM, vascular cell adhesion molecule

Introduction

It is now accepted that perturbation in normal endothelial cell function is a key initiator of cardiovascular disease including atherosclerosis, renal failure and diabetes (Cines *et al.*, 1998; Esper *et al.*, 2006). Endothelial cells activated by factors such as cytokines, free radicals and hypoxia increase the expression of a number of pro-inflammatory proteins such as interleukin-6 and the chemokine CCL2 (MCP-1); COX-2, which produces prostaglandins; and adhesion molecules such as E-selectin, intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM). This results in a functional shift towards reduced vasodilatation and a pro-inflammatory state (Esper *et al.*, 2006). In addition, endothelial cell apoptosis is also implicated in a number of cardiovascular conditions. This includes atherosclerosis (Tricot *et al.*, 2000; Rossig *et al.*, 2001), myocardial ischaemia (Scarabelli *et al.*, 2001; 2002; Zhao *et al.*, 2009), heart failure (Rossig *et al.*, 2000) and lower limb ischaemia (Xie *et al.*, 2006). Pharmacological approaches to prevent either of these events may therefore be of benefit in certain disease conditions.

The mitogen-activated protein (MAP) kinases play key roles in the regulation of endothelial cell function through phosphorylation of a number of cytosol and nuclear targets (Yu *et al.*, 2007). There are three main families of MAP kinases: the extracellular signal-regulated kinases (ERKs), the p38 MAP kinases and the c-Jun N-terminal kinases (JNKs). While ERK is associated with endothelial cell survival (Yu and Sato, 1999; Pintus *et al.*, 2003; Mavria *et al.*, 2006), both p38 MAP kinase and JNK are strongly associated with inflammation and endothelial cell apoptosis (Nakagami *et al.*, 2001; Wadgaonkar *et al.*, 2004). While JNK is linked to expression of COX-2 and E-selectin (Min and Pober, 1997; Nakagami *et al.*, 2001; Naderer and McConville, 2008), a larger body of evidence implicates JNK in the regulation of cell death (Sabapathy *et al.*, 1999; Tournier *et al.*, 2000). In endothelial cells, JNK is associated with apoptosis mediated by agents such as cytokines, hydrogen peroxide (Wang *et al.*, 1999) and high glucose concentration (Ho *et al.*, 2000). Recently, the JNK inhibitor SP600125 (Bennett *et al.*, 2001) has been shown to reverse apoptosis in endothelial cells (Fu *et al.*, 2006; Karahashi *et al.*, 2009); however, SP600125 may not be as selective as first indicated (Cameron *et al.*, 2003). Thus, novel approaches to selectively inhibit JNK activation in endothelial cells could be useful.

In an attempt to depress JNK activity and prevent endothelial cell inflammation and death, we developed an adenoviral version of the MAP kinase phosphatase-2 (Adv.MKP-2) (Misra-Press *et al.*, 1995). MKP-2 is a member of the dual-specific phosphatase family (Keyse, 2008), which functions to dephosphorylate the MAP kinases. It is nuclear targeted due to the presence of two nuclear location sequences (Sloss *et al.*, 2005), and although specific for ERK and JNK *in vitro* (Chu *et al.*, 1996), in a number of cell systems, MKP-2 showed selectivity for JNK (Robinson *et al.*, 2001; Cadalbert *et al.*, 2005). We used this adenovirus to over-express MKP-2 in endothelial cells, and determined the profile of kinase inhibition and the effect upon a number of end points associated with endothelial cell activation.

In this study, we show that over-expression of MKP-2 abolished JNK activity in human umbilical vein endothelial cells (HUVECs) and abolished COX-2 expression in response to tumour necrosis factor (TNF)-α. In contrast, ICAM and VCAM expression was not affected by Adv.MKP-2. However, when ICAM and VCAM expression was reduced by blocking the NFκB pathway, MKP-2 partly reversed inhibition of ICAM and VCAM expression. This effect correlated with enhanced loss of the inhibitor of κB (IκB)α, indicative of cross-talk regulation of the NFκB pathway by MKP-2. Apoptosis, induced by TNF-α in combination with NFκB blockade, was also inhibited following Adv.MKP-2 expression. This effect correlated with a reduction in the phosphorylation of the nuclear protein H2AX and cleavage of caspase-3, a cytosolic effector caspase of the apoptosis pathway. These data suggested that MKP-2 may be a useful mode of therapy in conditions where endothelial cell apoptosis is a feature, not only by inhibiting JNK signalling, but by enhancing NFκB activation. We also show for the first time that MKP-2 can regulate cytosolic-initiated, apoptotic signalling events, despite being a nuclear enzyme.

Methods

Cell culture

Cryopreserved primary HUVECs were purchased from Lonza (Slough, UK), and were grown in endothelial basal media (EBM-2), supplemented with endothelial growth media (EGM-2, Lonza) containing single aliquots of defined supplements [2% fetal bovine serum, 0.2 mL hydrocortisone, 2 mL rh fibroblast growth factor-B, 0.5 mL vascular endothelial growth factor, 0.5 mL R^3-insulin-like growth factor-1, 0.5 mL ascorbic acid, 0.5 mL rh epidermal growth factor, 0.5 mL GA-1000 and 0.5 mL heparin (concentrations not disclosed by the company)].

Cells were incubated at 37°C in humidified air with 5% CO_2. All experiments were performed between passages 2 and 5.

Adenoviral infections

Adenoviral vectors encoding dominant-negative IκB kinase (IKK)β (Adv.DN-IKKβ) and MKP-2 (Adv.MKP-2) were created in-house using the Adeno-X virus purification kit from Clontech Laboratories, Inc. (Mountain View, CA, USA). The DN-IKKβ plasmid was originally a gift from Dr D. Goeddel (Tularik Inc., San Francisco, CA, USA). Large-scale production of high-titre recombinant adenovirus was performed by routine methods (Nicklin *et al.*, 2004). HUVECs, when approximately 50–60% confluent, were incubated with adenovirus up to 300 pfu per cell for 40 h in endothelial growth media. Cells were stimulated with agonist for the indicated times in complete medium.

Western blotting

Proteins (15 μg) were separated by either 8.5 or 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto nitrocellulose. The membranes were blocked for non-specific binding for 2 h in 2% BSA (w/v) diluted in NATT buffer [20 mM Tris, 150 mM NaCl, 0.2% (v/v) Tween-20, pH 7.4]. Membranes were then incubated overnight with primary antibody diluted in 0.2% BSA (w/v) in NATT buffer at room temperature. The membranes were then washed with NATT buffer for 90 min and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h. After a further 90 min wash, the membranes were subjected to ECL reagent and exposed to Kodak X-ray film for appropriate time (Kodak, Amsterdam, the Netherlands).

JNK activity

Cells were stimulated as described, and the reaction was terminated by rapid aspiration and the addition of ice-cold phosphate-buffered saline (PBS). The cells were solubilized in 20 mM HEPES buffer, pH 7.7, containing 50 mM NaCl, 0.1 mM EDTA, 0.1 mM Na_3VO_4, 0.1 mM PMSF, 10 $\mu g \cdot mL^{-1}$ aprotinin, 10 $\mu g \cdot mL^{-1}$ leupeptin and 1% (w/v) Triton X-100. Lysates were clarified by centrifugation for 5 min at 24 232× *g*, and equal amounts of protein were incubated with 20 μg of GST-c-Jun-(5–89) immobilized on glutathione–Sepharose at 4°C for 3 h. Beads were then washed three times in solubilization buffer and twice in 25 mM HEPES buffer, pH 7.6, containing 20 mM β-glycerophosphate, 0.1 mM NaV_3O_4 and 2 mM dithiothreitol. Precipitates were then incubated with the same buffer containing 25 μM/0.5 μCi of ATP/[γ-^{32}P] ATP in a final volume of 30 μL at 30°C for 30 min. The reactions were terminated by the addition of 4× SDS sample buffer and aliquots of each sample subjected to electrophoresis on 11% SDS–PAGE. Phosphorylation of GST-c-Jun was then determined by autoradiography.

Immunofluorescence

HUVECs were grown to 50–60% confluency on no. 0 glass coverslips (Merck Biosciences, Nottingham, England). After adenovirus infection (40 h), the cells were washed three times with ice-cold PBS prior to fixation with 1 mL of 3% paraformaldehyde solution. The coverslips were then incubated with 0.1% Triton X-100 in PBS for 5 min at room temperature. The coverslips were washed three times in PBS and three times with 1% BSA diluted in PBS (w/v) for 5 min followed by incubation with primary monoclonal MKP-2 antibody (1:200 in 1% BSA) for 1 h. Again, the coverslips were washed with ice-cold PBS and incubated with secondary monoclonal antibody (1:400 in 1% BSA) mixed with 100 $ng \cdot mL^{-1}$ of DAPI for 45 min. Following three washes with ice-cold PBS, Mowiol was used to mount coverslips onto slides for visualization by EP-1 fluorescence light microscopy (Sigma, Poole, UK).

Flow cytometry assay of apoptosis

Cells were infected for 40 h then stimulated for a further 24 h prior to analysis. The cells were trypsinized and then centrifuged at 192× *g* for 2 min. The pellet was then resuspended in 500 μL of 1× annexin binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$). Phycoerythyrin–Annexin V and 7-AAD were added to the cells according to the manufacturer's instructions, and the samples were read in the FACS scan flow cytometer using FACS Diva software (FACS scan, Becton Dickinson, Oxford, UK). The data were analysed using FACS Diva (Becton Dickinson) and RCS Express (De Novo Software, Thornhill, Canada) software. A total of 10 000 events were measured per sample. Gating was determined using PE–Annexin V FL-2 and 7–AAD FL-3 standards attached to beads (Becton Dickinson), and preliminary experiments were conducted using paraformaldehyde and serum deprivation to define apoptotic and necrotic populations as outlined by the manufacturer's instructions.

Data analysis

Each figure represents one of at least four separate experiments. Western blots were scanned on an Epson perfection 1640SU scanner using Adobe photoshop 5.0.2 software (Adobe Systems Incorporated, Dublin, Ireland). For gels, densitometry

measurement was performed using the Scion Image program. Data were expressed as mean ± SEM. Statistical analysis was performed by one-way ANOVA with Dunnett's post-test.

Materials

All reagents were from Sigma (Poole, UK), unless otherwise stated. TNF-α was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies were purchased as follows: p65 NFκB, IκBα, phospho and total ERK, MKP-2 and IKKβ from Santa Cruz Biotechnology; phospho-p38 and -JNK from Invitrogen, Paisley, UK; COX-2 from Cayman Chemicals (Ann Arbor, MI, USA); anti-human VCAM-1 and ICAM-1 from R&D Systems (Abingdon, UK); H2AX (Ser-139) from Upstate Biotechnology Inc. (Lake Placid, NY, USA); cleaved caspase-3 from Cell Signaling Biotechnology (Beverly, MA, USA); FITC anti-rabbit and HRP-conjugated secondary antibody from Jackson Immuno Research Laboratories Inc. (West Grove, PA, USA).

Results

The effect of adenoviral infection with MKP-2 (Adv.MKP-2) on TNF-α-stimulated JNK activation was assessed in HUVECs (Figure 1). Imunofluorescent staining (Figure 1A) showed no endogenous MKP-2 expression in LacZ-infected control cells, however, following Adv.MKP-2 infection at a maximum concentration of 300 m.o.i., greater than 95% of the cells stained for nuclear MKP-2. Under these conditions, Adv.MKP-2 caused a significant inhibition of JNK phosphorylation, stimulated by TNF-α (20 ng·mL^{-1}), the response being virtually abolished at 200–300 pfu per cell (Figure 1B, $P < 0.01$). Both p46 and p54 isoforms were similarly affected. Adv.MKP-2 also reduced TNF-α-stimulated JNK kinase activity *in vitro* (Figure 1C, $P < 0.01$ compared to TNF-α stimulation) and phosphorylation of c-Jun, an immediate downstream target of JNK (Figure 1D, $P < 0.01$).

We also examined ERK and p38 MAP kinase phosphorylation to confirm that the effect of MKP-2 was specific to JNK signalling (Figure 2). TNF-α-stimulated ERK phosphorylation was maximal at 15–30 min, two- to threefold of basal values. Neither this response, or basal levels, were affected by MKP-2 infection up to 300 pfu per cell (Figure 2A). TNF-α stimulated a substantial increase in p38 MAP kinase phosphorylation; however, this response was not significantly affected by Adv.MKP-2 infection (Figure 2B).

We next sought to determine if by a selective effect upon JNK, MKP-2 was able to regulate the expression of ICAM, VCAM and COX-2 (Figure 3). For both ICAM and VCAM, infection with 300 pfu per cell of Adv.MKP-2 had no inhibitory effect upon TNF-α-stimulated induction, and in fact, a minor non-significant enhancement of both ICAM and VCAM expression could be observed (Figure 3A,B). In contrast, TNF-α-induced COX-2 expression was essentially abolished following infection with Adv.MKP-2 (Figure 3C, $P < 0.001$). A similar finding was also observed for HUVECs stimulated with LPS (not shown), suggesting that the effect of MKP-2 was not agonist dependent.

Studies suggest that in HUVECs, COX-2, ICAM and VCAM are regulated by NFκB signalling, so we sought to assess possible synergy effects of MKP-2 over-expression and NFκB inhibition (Figures 4–6). Initially, we utilized an adenoviral dominant-negative form of IκB kinase β (DN-IKKβ), a kinase which plays a key role in regulating NFκB (Gomez *et al.*, 2005; MacKenzie *et al.*, 2007). Infection of HUVECs with 300 pfu per cell of DN-IKKβ resulted in a substantial, almost complete, inhibition of the expression of all three proteins induced in response to TNF-α (Figure 4). Conditions were then established to allow simultaneous infection with both viruses, and we then assessed the effects of the two interventions in combination (Figures 5 and 6). We found that for COX-2 (Figure 5), Adv.DN-IKKβ and Adv.MKP-2 infection alone substantially reduced protein expression (% inhibition; Adv.MKP-2 = 88 ± 0.1, Adv.DN-IKKβ = 65 ± 9). In combination, the inhibition was not significantly greater (% inhibition 98.0 ± 3%) than for either agent alone. For ICAM and VCAM, however, the situation was markedly different (Figure 6). While DN-IKKβ substantially reduced ICAM and VCAM, co-expression of MKP-2 was able to almost completely reverse this inhibition, and expression of both proteins was similar to that in LacZ controls. As shown previously, MKP-2 alone did not significantly affect ICAM or VCAM expression. Control Western blotting also indicated that the reversal was not due to an inhibitory effect of MKP-2 upon DN-IKKβ expression nor *vice versa*, as neither protein was affected by infection with the other adenovirus.

The potential for MKP-2 to influence NFκB-dependent gene expression was further examined at the level of NFκB signalling (Figure 7). Pre-incubation with Adv.MKP-2 had a minor effect upon basal IκBα levels in HUVECs; however, infection with Adv.MKP-2 was able to markedly potentiate submaximal TNF-α-induced IκBα loss (Figure 7A). As expected, pre-incubation with DN-IKKβ partially reversed the agonist-stimulated IκBα loss; however, further infection with Adv.MKP-2 restored the loss.

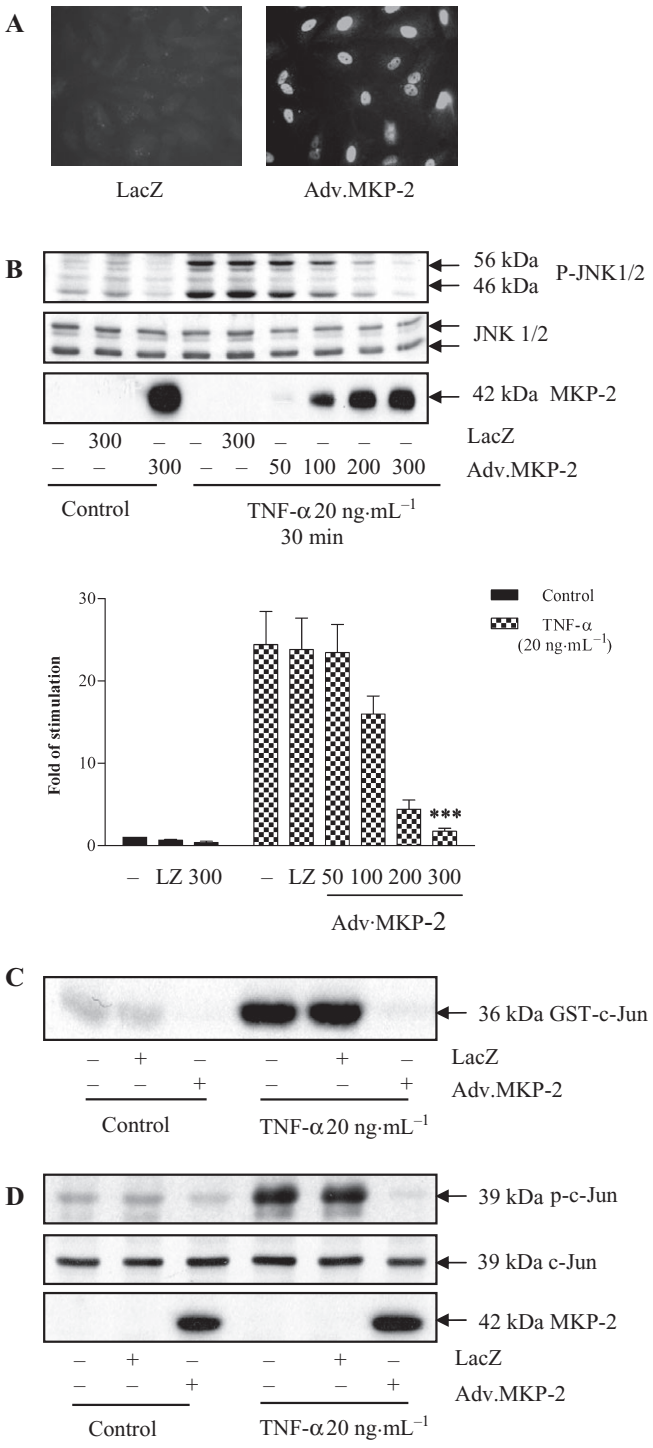

Figure 1

Adv.MKP-2 expression inhibits TNF-α-stimulated JNK phosphorylation and activation in HUVECs. HUVECs were infected with 300 pfu per cell of Adv.MKP-2 or Lac Z (A, C and D), or increasing concentrations as indicated (B) for 40 h prior to stimulation with TNF-α for a further 30 min (B–D). In (A), cells were stained for MKP-2 expression. Samples were also assessed for phospho-JNK (P-JNK 1/2; B), JNK activity (C) and phospho-c-Jun content (p-c-Jun; D) as outlined in the Methods section. Results from gels were quantified using scanning densitometry. Each value represents the mean ± SEM of at least four experiments. ***$P < 0.001$ compared to TNF-α stimulation alone.

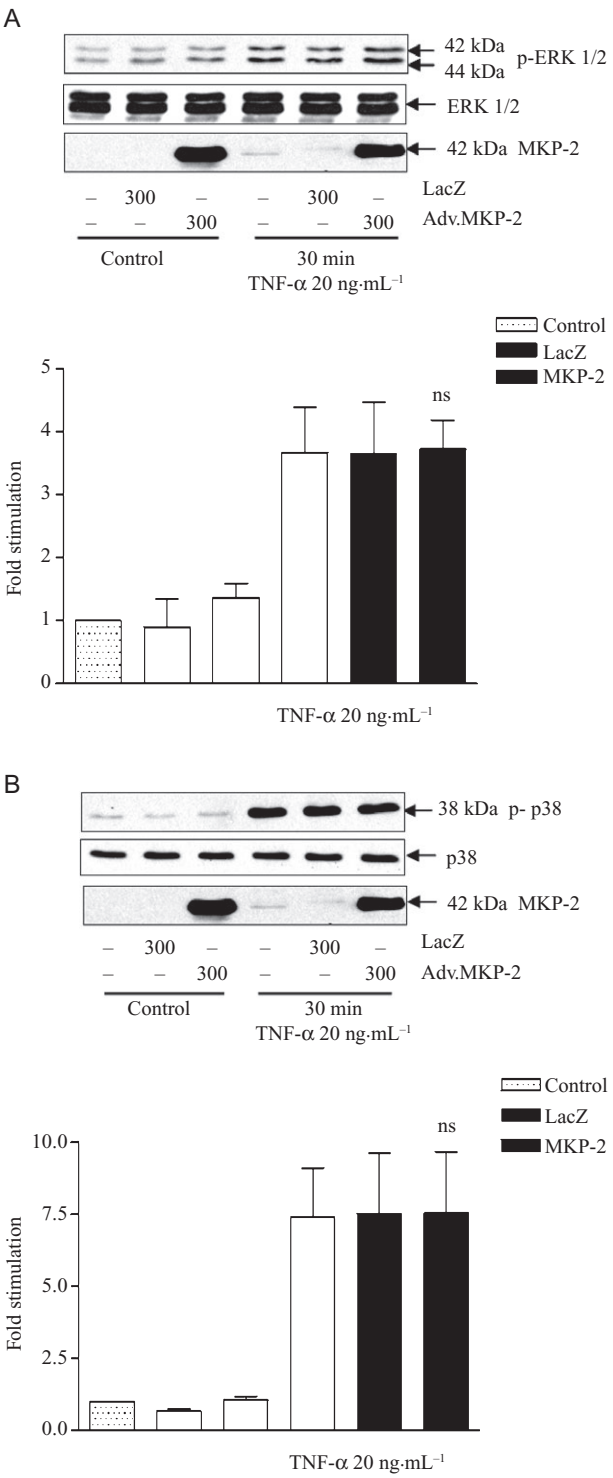

Figure 2

Effect of Adv.MKP-2 expression on TNF-α-stimulated ERK or p38 MAP kinase phosphorylation.HUVECs were infected with 300 pfu per cell of Adv.MKP-2 or LacZ control for 40 h prior to stimulation with TNF-α for a further 30 min. Samples were assessed for phospho-ERK (A) and phospho-p38 MAP kinase (p-p38; B) as outlined in the Methods section. Results from gels were quantified using scanning densitometry. Each value represents the mean ± SEM of at least four experiments. ns, not significant compared to TNF-α stimulation alone.

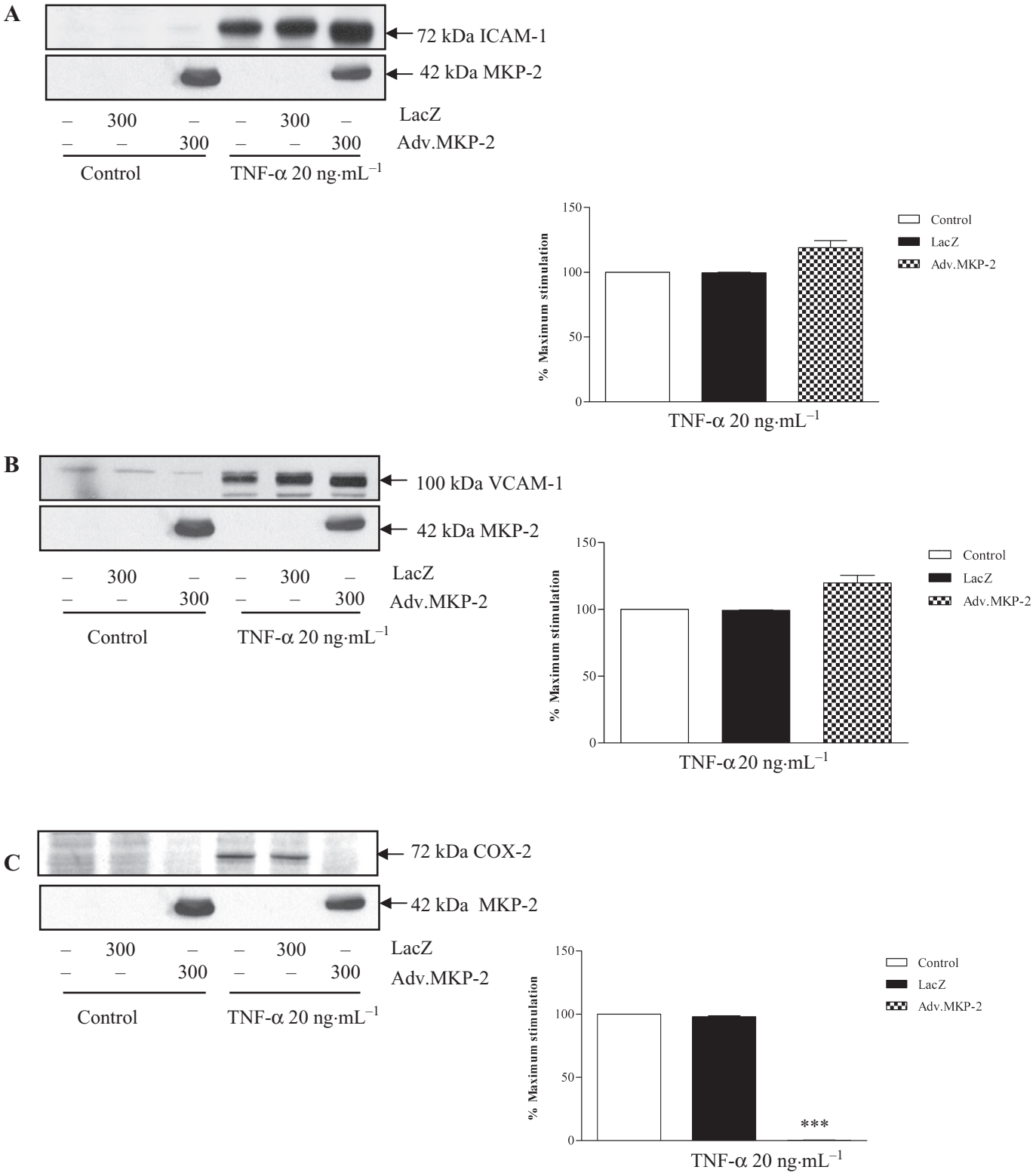

Figure 3

Differential effect of Adv.MKP-2 on TNF-α-stimulated ICAM and VCAM, and COX-2 expression in HUVECs. HUVECs were infected with 300 pfu per cell of Adv.MKP-2 or LacZ control for 40 h prior to stimulation with TNF-α for a further 24 h. Samples were assessed for MKP-2 levels, ICAM (A), VCAM (B) or COX-2 (C) by Western blotting. Results from gels were quantified using scanning densitometry. Each value represents the mean ± SEM of at least four experiments. ***$P < 0.001$ compared to agonist stimulation alone.

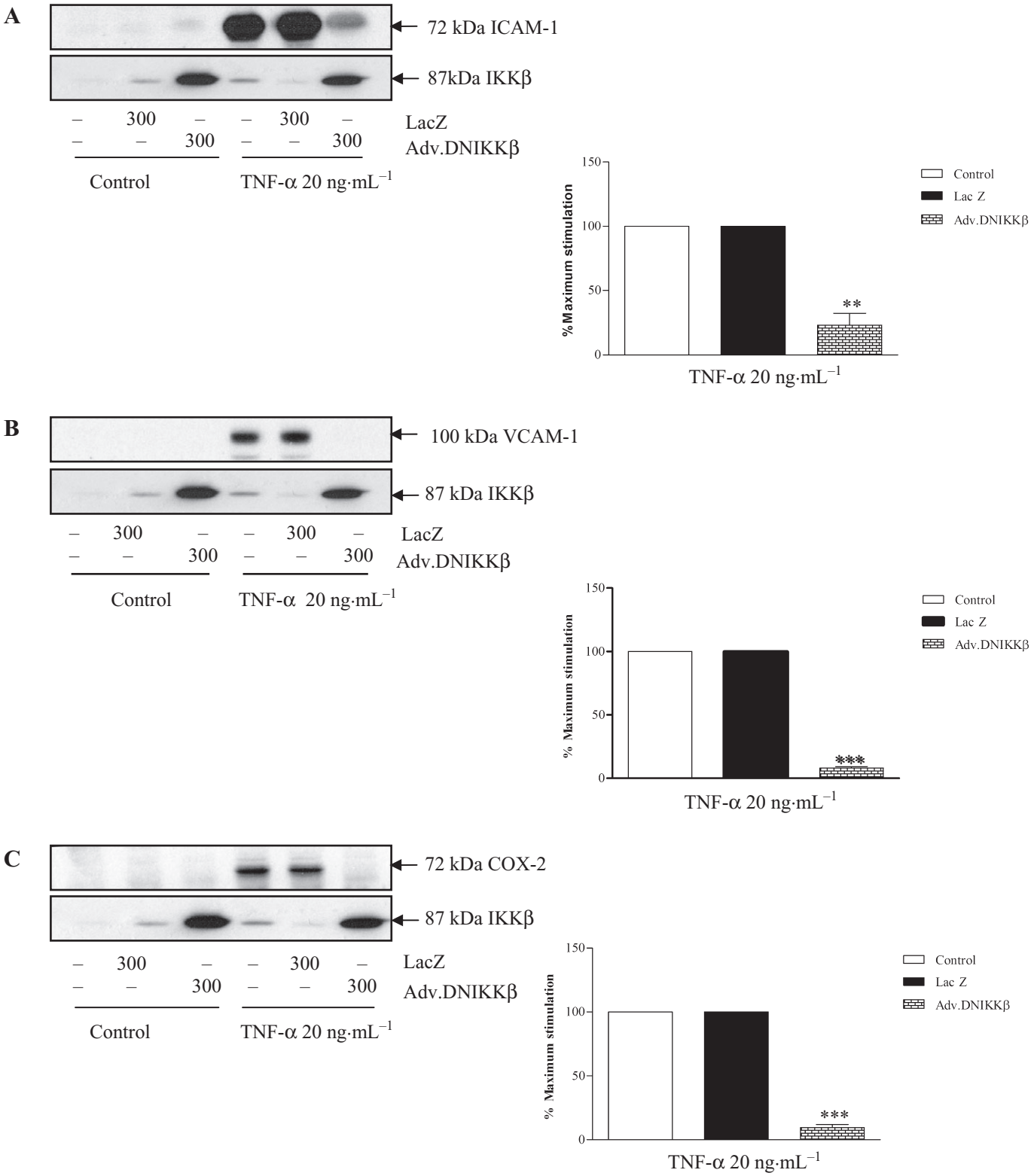

Figure 4

Inhibition of NFκB signalling inhibits TNF-α-stimulated ICAM, VCAM and COX-2 expression in HUVECs. HUVECs were infected with 300 pfu per cell Adv.DN-IKKβ or LacZ control for 40 h prior to stimulation with TNF-α for a further 24 h. Samples were assessed for ICAM (A), VCAM (B) and COX-2 (C). Results from gels quantified using scanning densitometry. Each value represents the mean ± SEM of at least four experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to agonist stimulation alone.

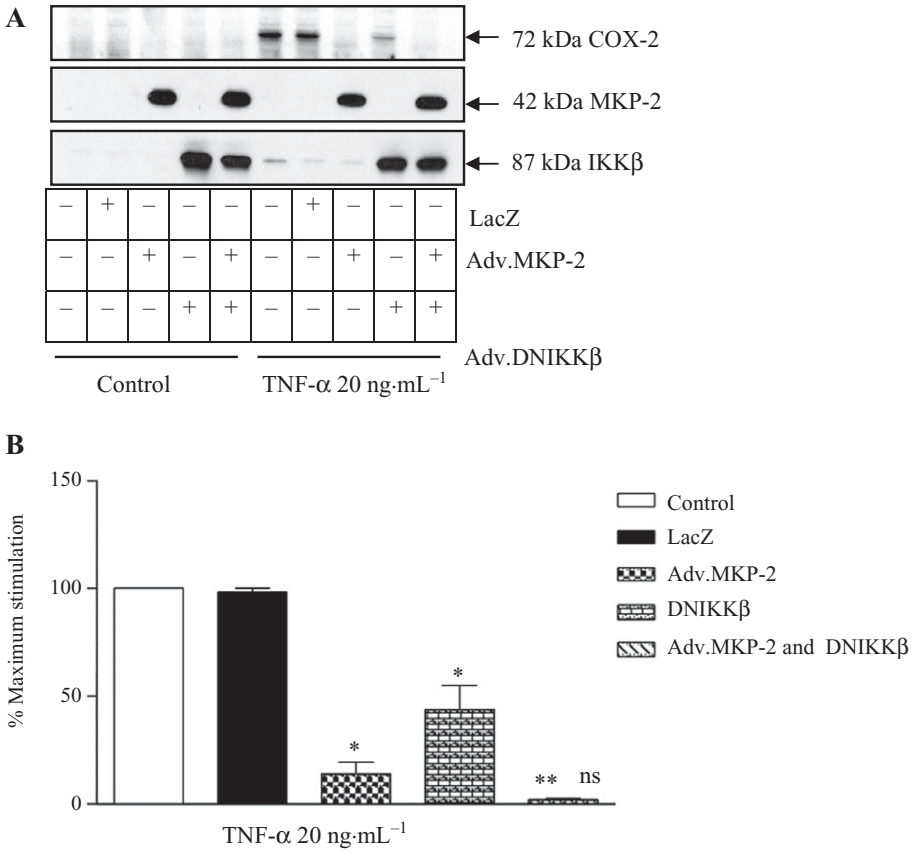

Figure 5

Combined effect of DN-IKKβ and MKP-2 co-expression on TNF-α-stimulated COX-2 induction in HUVECs. HUVECs were infected with 300 pfu per cell Adv.DN-IKKβ or Adv.MKP-2 alone, or in combination for 40 h prior to stimulation with TNF-α for a further 24 h. Samples were assessed for COX-2 expression (A) as outlined in the Methods section. Gels were quantified by densitometry (B), and each value represents the mean ± SEM from four experiments. *$P < 0.05$ and **$P < 0.01$ compared to agonist stimulation alone. ns, not significant compared to Adv.MKP-2 inhibition.

Individual expression of MKP-2 and DN-IKKβ was not influenced by co-infection, and LacZ at higher concentrations (600 pfu per cell) did not mimic the effects of the combination, suggesting a real effect of Adv.MKP-2.

We also examined the potential for MKP-2 to reverse endothelial cell apoptosis (Figure 8). We adopted the strategy of using TNF-α in combination with DN-IKKβ not only to mirror conditions for ICAM and VCAM expression, but because inhibition of NFκB signalling is a prerequisite in most cell types, including endothelial cells, for TNF-α-induced apoptosis. Cells stimulated with TNF-α alone showed a minor, non-significant increase in apoptosis (% apoptosis; control = 1.73 ± 1.23, TNF-α = 5.23 ± 1.04, ns). However, in the presence of DN-IKKβ, there was a clear increase in apoptosis (Figure 8A,B). Co-expression of MKP-2 significantly reversed this increase apoptosis by approximately 50% ($P < 0.05$). Again, control Western blotting demonstrated no difference in relative expression of either DN-IKKβ or MKP-2 alone, or in combination at this later time (Figure 8C).

Finally, we sought to confirm that the reversal by MKP-2 may have an effect upon JNK-regulated nuclear proteins involved in regulating apoptosis and nuclear damage (Figure 9). TNF-α alone gave a minor increase in the phosphorylation of H2AX, a nuclear histone 2A family protein, while in combination with DN-IKKβ, phosphorylation was markedly increased (Figure 9A). We found that Adv.MKP-2 was able to reverse TNF-α/DN-IKKβ-mediated phosphorylation of H2AX ($P < 0.05$). As an additional negative control, we also assessed the formation of the cleaved caspase-3 products, p19 and p17, pro-apoptotic proteins generated in the cytosol (Figure 9B). Unexpectedly, we found that MKP-2 over-expression resulted in a complete reversal of caspase-3 cleavage, stimulated by TNF-α in combination with DN-IKKβ.

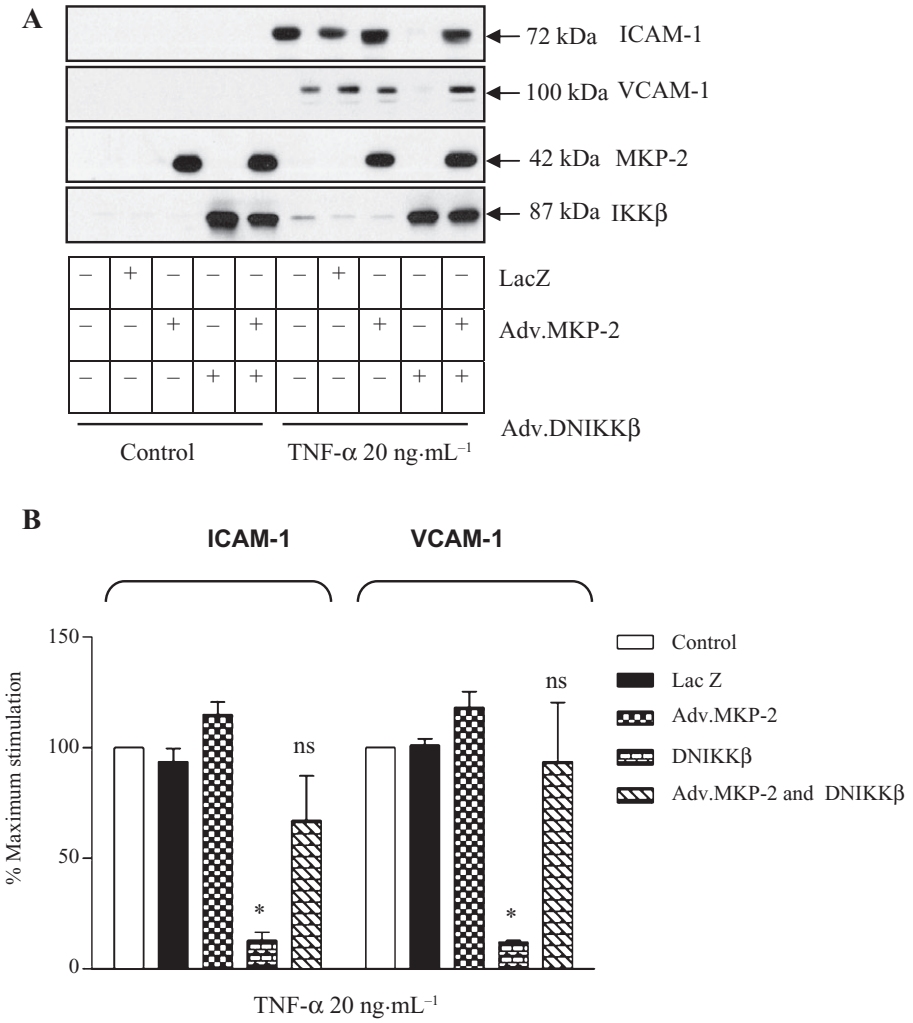

Figure 6

MKP-2 co-expression reverses DN-IKKβ inhibition of ICAM and VCAM expression in TNF-α-stimulated HUVECS. HUVECs were infected with 600 pfu per cell Lac Z, 300 pfu per cell Adv.DN-IKKβ or Adv.MKP-2 alone or in combination for 40 h prior to stimulation with TNF-α for a further 24 h. Samples were assessed for ICAM or VCAM expression (A) as outlined in the Methods section. Data from blots were quantified by densitometry (B), and each value represents the mean ± SEM of at least four experiments. *$P < 0.05$ compared to agonist stimulation alone. ns, not significant compared to either control or Adv.MKP-2 inhibition.

Discussion

In this study, we utilized a dual-specific nuclear phosphatase, MKP-2, as an experimental pharmacological tool, to reverse JNK-mediated responses in HUVECs and thus determine its potential use in clinical conditions involving endothelial cell dysfunction. At the cellular level, our studies revealed a novel point of cross-talk between MKP-2 and the NFκB signalling pathway. We also found that MKP-2 over-expression inhibited apoptosis, and that this effect correlated not only with the dephosphorylation of the pro-apoptotic histone protein H2AX, but with caspase-3 degradation, an event occurring in the cytosol, despite MKP-2 being a nuclear phosphatase. This unique combination of effects suggests that Adv.MKP-2 could represent a novel experimental approach for future treatment of cardiovascular disease in which endothelial cell apoptosis is a feature.

Initially, we demonstrated that HUVECs infected with Adv MKP-2 resulted in the expression of a 42 kDa protein, and as assessed by immunofluorescence, a protein strictly targeted to the nucleus (Sloss *et al.*, 2005). Previous studies have shown that HUVECS express MKP-2 protein endogenously (Wadgaonkar *et al.*, 2004), but no function was assigned for MKP-2 in endothelium. Under our conditions of culture, we found no basal, or agonist-induced, expression of MKP-2. The lack of MKP-2 expression in HUVECs, in contrast to rodent systems, is a common feature of human cellular

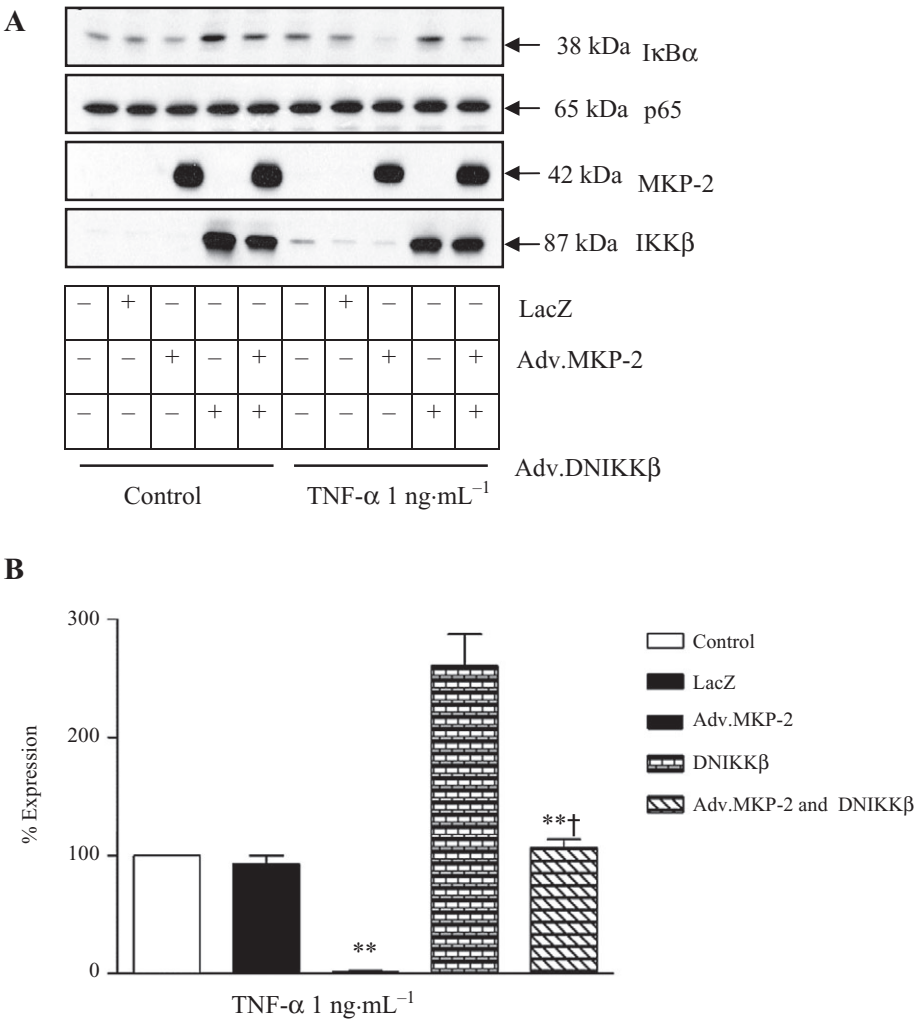

Figure 7

MKP-2 expression reverses DN-IKKβ inhibition of TNF-α-stimulated IκBα loss in HUVECs. HUVECs were infected with 600 pfu per cell Lac Z or 300 pfu per cell Adv.DN-IKKβ or Adv.MKP-2 alone, or in combination for 40 h prior to stimulation with TNF-α (1 ng·mL^{-1}) for a further 30 min. Samples were assessed for IκBα or total p65 NFκB expression as outlined in the Methods section. Each blot is representative of three others. Data from blots were quantified by densitometry (B), and each value represents the mean ± SEM of four experiments. *P < 0.01 compared to TNF-α/DN-IKKβ alone. +P < 0.05 compared to MKP-2 inhibition.

studies and it is at present unclear as to what manipulations are required to promote protein expression. Nevertheless, it allowed the use of Adv.MKP-2 without the potential of contaminating endogenous protein. Our results clearly demonstrated a selective effect of MKP-2 over-expression, the abolition of JNK phosphorylation (Figure 1). This finding is consistent with previous studies in our laboratory examining TNF-α stimulation (Robinson *et al.*, 2001) and genotoxic stress (Cadalbert *et al.*, 2005). Studies *in vitro* show MKP-2 to be selective for JNK and ERK (Chu *et al.*, 1996), and the lack of effectiveness against ERK following stimulation of whole cells may reflect the poor ERK stimulation in response to TNF-α.

In our hands, we found Adv.MKP-2 to be effective against the expression of COX-2, consistent with previous studies showing expression of this protein to be regulated by the JNK pathway (Nieminen *et al.*, 2005; 2006; Wu *et al.*, 2006). By contrast, we found no inhibition of ICAM and VCAM, a finding consistent with some, but not all, studies (De Cesaris *et al.*, 1999; Kobuchi *et al.*, 1999), suggesting cell type-specific differences in the role of JNK in the regulation of adhesion molecule expression. The predominant pathways regulating ICAM and VCAM in most cell types are p38 MAP kinase, which is not a substrate for MKP-2 (Yan *et al.*, 2002; Rahman *et al.*, 2004), and also NFκB (Rahman *et al.*, 1999). However, using MKP-2, we have identified,

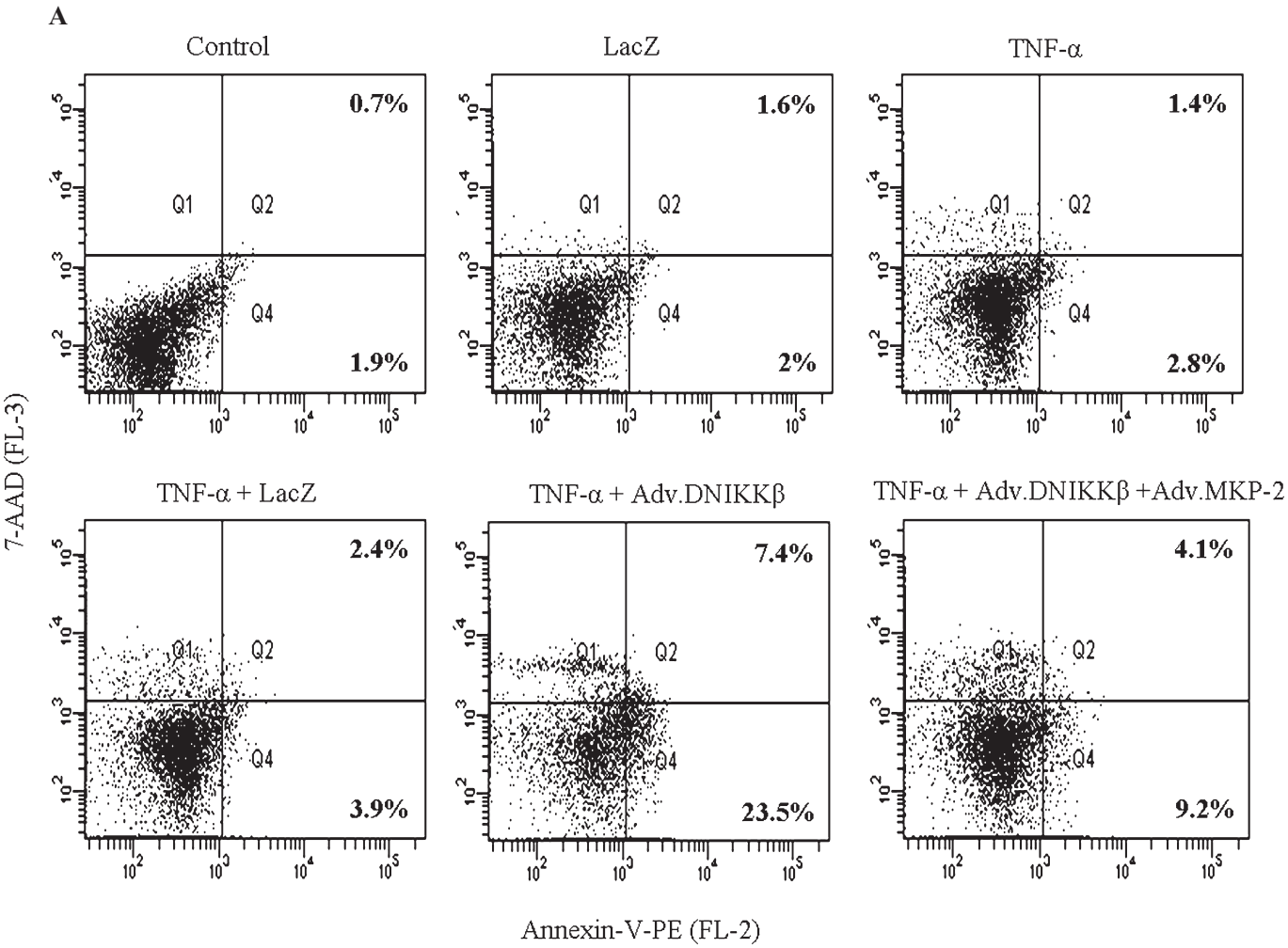

Figure 8

Adv.MKP-2 infection reverses TNF-α-mediated apoptosis in HUVECS. HUVECs were infected with 600 pfu per cell Lac Z or 300 pfu per cell Adv.DN-IKKβ or Adv.MKP-2 alone, or in combination for 40 h prior to stimulation with TNF-α for a further 24 h. Samples were assessed for Annexin V and 7-aminoactinomycin D (7-AAD) staining as outlined in the Methods section (A and B), while in parallel control experiments cell samples were assessed by Western blotting for IKKβ and MKP-2 expression (C). In (B), each value represents the mean ± SEM of at least four experiments. *$P < 0.05$ compared to TNF-α/DN-IKKβ stimulation alone.

for the first time, the potential of JNK inhibition to enhance the expression of ICAM and VCAM, revealed only in conditions of partial NFκB inhibition. This effect was specific for ICAM and VCAM, as COX-2 expression, despite also being NFκB dependent, was also strongly reduced by Adv.MKP-2 alone, and in combination with DN-IKKβ did not show any reversal of inhibition. This suggests a potential for JNK to play a negative role in the expression of ICAM and VCAM. This phenomenon was implied from a single study by Hosokawa *et al.*, (2006), whereby pretreatment with the JNK inhibitor SP600125 resulted in slightly enhanced VCAM expression. Indeed, preliminary results in our laboratory showed a similar phenomenon using SP600125 (not shown), indicating a JNK-mediated effect. However, in this study, we have extended the work, and shown this phenomenon to be correlated with an effect upon NFκB activation, MKP-2 was able to enhance TNF-α-induced IκBα loss or reverse the inhibitory effect of DN-IKKβ. This present study is one of the few demonstrating that long-term JNK down-regulation can enhance NFκB activation (Sanchez-Perez *et al.*, 2002). Overwhelmingly, studies indicate the reverse phenomenon, that NFκB limits the kinetics of JNK signalling (Papa *et al.*, 2006).

We then determined if MKP-2 had an effect upon HUVEC apoptosis, a process also influenced by the interplay between NFκB and JNK signalling. TNF-α mediates apoptosis in HUVECs, but only in certain conditions, such as following cycloheximide

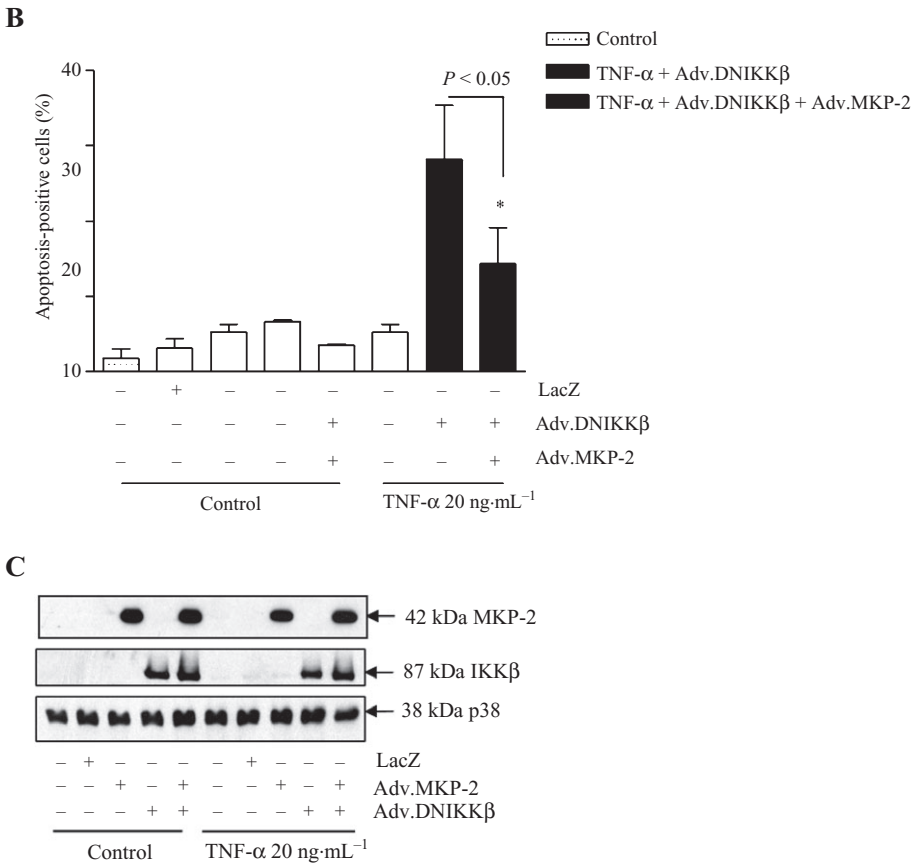

Figure 8
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pretreatment or NFκB inhibition (Wadgaonkar *et al.*, 2004). A number of studies have shown that in endothelial and other cell types, NFκB is able to mediate cellular protection through the enhanced expression of a number of protective proteins including Gadd45b (De Smaele *et al.*, 2001) and XIAP (Tang *et al.*, 2001), which function to suppress JNK activation. Recent studies have also shown that several antioxidant enzymes are under NFκB regulation, and may also contribute to limiting JNK activity (Papa *et al.*, 2006) by inhibiting generation of reactive oxygen species. Under conditions of NFκB inhibition, TNF-α was indeed found to sustain JNK activation in HUVECs (not shown) and cause a significant increase in apoptosis; results consistent with previous findings in endothelial cells (Stehlik *et al.*, 1998). MKP-2 significantly reversed apoptosis; however, the reversal was not total, suggesting the potential of both JNK-dependent and -independent pathways controlling apoptosis. A role for JNK in endothelial cell apoptosis has been previously established using pharmacological inhibitors (Fu *et al.*, 2006; Karahashi *et al.*, 2009); however, only one previous study has demonstrated that MKP-2 can mediate the same inhibition of JNK, and thus regulate cell death (Cadalbert *et al.*, 2005).

In investigating the correlation between reversal of cell death and effects upon pro-apoptotic proteins, we made a series of novel observations. Initially, we found that TNF-α in combination with DN-IKKβ resulted in increased phosphorylation of H2AX, a histone protein involved in regulating cellular responses to DNA damage (Kinner *et al.*, 2008). Initial studies indicated that this protein was regulated by phosphoinositide-3-kinase-related protein kinases (Fernandez-Capetillo *et al.*, 2004); however, our work suggests phosphorylation by JNK, in agreement with a recent study in HeLa cells (Lu *et al.*, 2006). Interestingly, we also found that MKP-2 over-expression was also able to abolish the formation of cleaved caspase-3 proteins, p19 and p17. Caspases are implicated in endothelial cell apoptosis mediated in response to TNF-α (Daniel *et al.*, 2004) and a number of stressful agents such as high glucose and hydrogen peroxide (Ho *et al.*, 2000; Ramachandran *et al.*, 2002). In some of these models, caspase-3

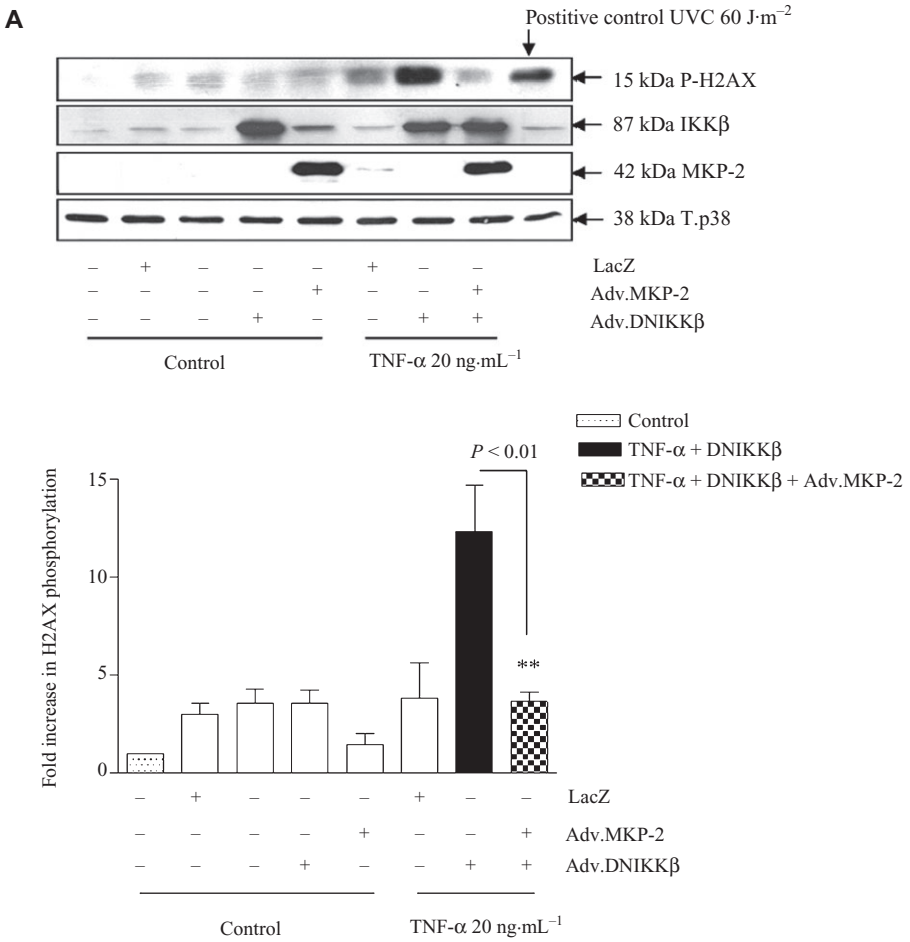

Figure 9

MKP-2 over-expression reverses TNF-α-induced H2AX phosphorylation and caspase-3 cleavage in HUVECs. HUVECs were infected with either 600 pfu per cell Lac Z or 300 pfu per cell Adv.DN-IKKβ or Adv.MKP-2 alone, or in combination for 40 h prior to stimulation for a further 24 h. Samples were assessed for phospho-H2AX (A), caspase 3 cleavage products (19, 17 kDa; B), IKKβ, or MKP-2 expression as outlined in the Methods section. Data from blots were quantified by densitometry. Each value represents the mean ± SEM of at least four experiments. $^{**}P < 0.01$ compared to TNF-α/DN-IKKβ alone. T-p38 represents a loading control.

cleavage is regulated by JNK (Ho *et al.*, 2000; Ramachandran *et al.*, 2002). Translocation of caspase fragments is dependent upon prior cleavage of procaspase 3 in the cytosol and association with recognized substrates (Kamada *et al.*, 2005). Thus, as MKP-2 is strictly localized in the nucleus, even at high viral concentrations, this suggests that to regulate caspase-3 cleavage in endothelial cells, JNK must be firstly phosphorylated within the nucleus, before its translocation to the cytosol. Current dogma suggests roles for JNK both within the nucleus and mitochondria (Dhanasekaran and Reddy, 2008), but a functional link between each compartment has not been established. Alternatively, it may be that a pool of MKP-2 is located in the mitochondria, as is the case with MKP-1 (Rosini *et al.*, 2004), and functions to directly regulate JNK activity within this compartment. In addition is the possibility that long-term down-regulation of JNK has a rebound effect upon the expression of anti-apoptotic proteins such as cFLIP (Suzuki *et al.*, 2003; Albrecht *et al.*, 2009), XIAP (Tang *et al.*, 2001) or A20 (Daniel *et al.*, 2004).

Overall, our studies have identified the potential for over-expression of MKP-2 to down-regulate JNK-dependent COX-2 expression, and up-regulate NFκB-dependent expression of ICAM and VCAM. We also showed that MKP-2 was able to negate TNF-α-stimulated apoptosis by regulating not only nuclear events such as phosphorylation of H2AX, but also cytosolic events such as caspase-3 cleavage. Thus, Adv.MKP-2 has a potential therapeutic use in clinical conditions where JNK-mediated effects upon endothelial apoptosis is a feature. A number of

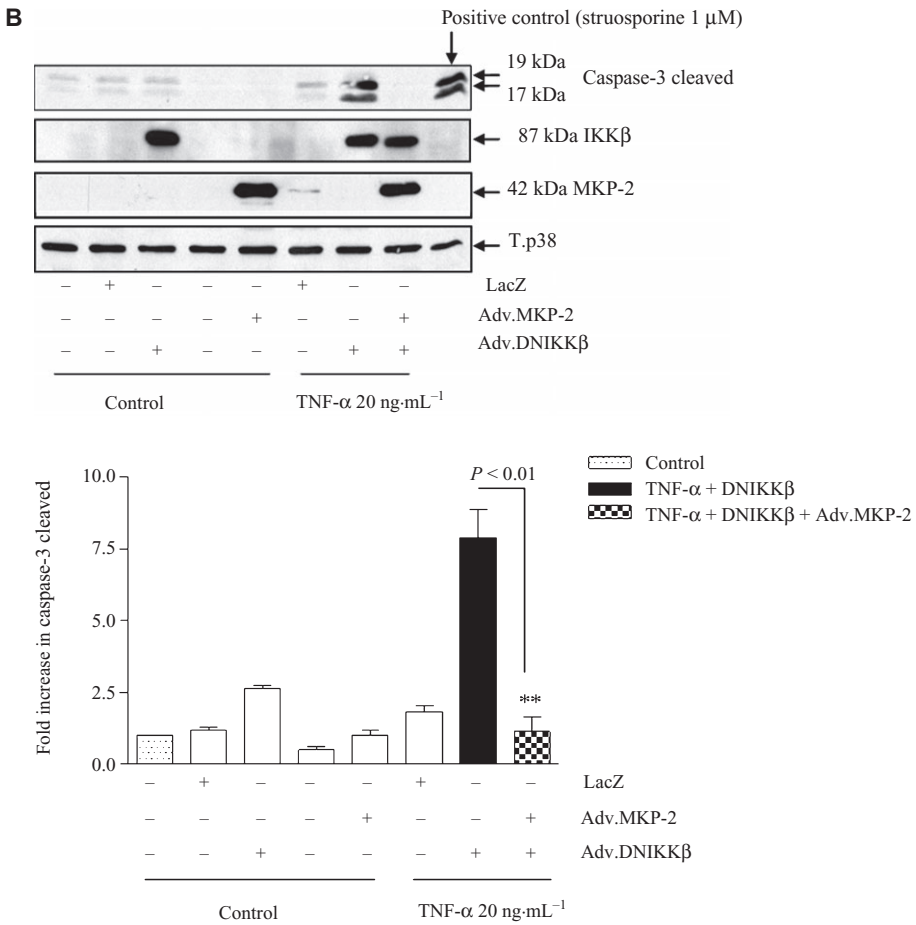

Figure 9
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previous studies have already utilized adenovirus infection to express proteins such TIMP-3 and p53 to inhibit vascular remodelling *in vitro* (George *et al.*, 1998a,b; 2001) and *in vivo* (George *et al.*, 2000). While these approaches are associated primarily with inhibiting smooth muscle cell migration and mediating apoptosis, other studies have delivered adenoviral eNOS to enhance re-endothelialization and inhibit restenosis (Tanner *et al.*, 2004; Sharif *et al.*, 2008). Thus, the anti-apoptotic effects of Adv.MKP-2 over-expression in endothelial cells may be a useful outcome which can be exploited *in vivo*. It will be complicated, however, by effects upon adhesion molecule expression which may enhance migration of macrophages to the sub-endothelial space. This may limit its clinical use.

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Conflict of interest

The authors have no conflict of interest.

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