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RESEARCH PAPER

Okadaic acid induces matrix metalloproteinase-9 expression in fibroblasts: crosstalk between protein phosphatase inhibition and β-adrenoceptor signalling

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BACKGROUND AND PURPOSE
Interactions between protein phosphatase inhibition and matrix metalloproteinase (MMP)-9 expression have implications for tissue remodelling after injury. Stimulation of β-adrenoceptors could affect such interactions as isoprenaline increases protein phosphatase 2A (PP2A) activity and MMP-9 abundance. We investigated the effect of okadaic acid (OA) on MMP-9 expression to assess interactions between phosphatase inhibition and β-adrenoceptor signalling in fibroblasts.

EXPERIMENTAL APPROACH
Fibroblasts were exposed to OA alone and in combination with isoprenaline. Effects on MMP-9 expression and intracellular signalling were studied using promoter assays, Western blot analysis and siRNA methodologies.

KEY RESULTS
Okadaic acid increased MMP-9 abundance in human cardiac ventricular fibroblasts, NIH3T3 fibroblasts and hepatic stellate cells. This effect was unaffected by PP2A knockdown in NIH3T3 cells. OA increased phosphorylation of NF-κB, but not NF-κB promoter activity, IκBα degradation, or nuclear translocation of p65-NF-κB. Exposure to SB202190 (p38 MAPK), U0126 (ERK1/2) and NF-κB III inhibitor revealed that OA induced MMP-9 activity through p38 MAPK. Isoprenaline inhibited OA-mediated MMP-9 expression in NIH3T3, in a β-arrestin 2- and PP2A-dependent manner. Mutation of the activator protein-1 (AP-1) and NF-κB binding sites demonstrated that OA-induced MMP-9 activity was mediated through the AP-1 but not NF-κB sites. The latter mediated the inhibitory effect of isoprenaline on OA-induced MMP-9 promoter activity.

CONCLUSION AND IMPLICATIONS
Okadaic acid induced MMP-9 activity through p38 MAPK and was inhibited by isoprenaline via a pathway involving β-arrestin 2, PP2A and an NF-κB binding motif. These findings elucidate how phosphoprotein phosphatases and adrenoceptors may modulate tissue remodelling by affecting fibroblast function.

Abbreviations
AP-1, activator protein-1; HCVF, human cardiac ventricular fibroblast; I-1, inhibitor-1; ISO, isoprenaline; OA, okadaic acid; PP, protein phosphatase; MMP, matrix metalloproteinase

Introduction

Reversible phosphorylation of proteins by kinases and phosphatases is an essential regulatory mechanism controlling cell function. However, relatively few serine/threonine protein phosphatases (PPs) control the de-phosphorylation of a multitude of phosphoproteins within the cell. In the mammalian heart, approximately 90% of PP activity is attributed to PP1

and PP2A (El-Armouche and Eschenhagen, 2009). These in turn are attenuated by inhibitor-1 (I-1), inhibitor-2 and DARP-32, and by inhibitor 1 of PP2A and inhibitor 2 of PP2A respectively (Shi, 2009).

Although PP1 activity and I-1 expression is altered in patients with end-stage heart failure (Neumann *et al.*, 1997; El-Armouche *et al.*, 2004), their contribution to disease progression has not been established in man. Nevertheless, in mice over-expressing PP1 there is evidence of cardiac remodelling manifest as fibrosis and ventricular dilation (Carr *et al.*, 2002). Similarly, PP2A over-expression is associated with impaired cardiac function, cardiac hypertrophy and fibrosis (Gergs *et al.*, 2004). Furthermore, pharmacological inhibition of PP2A reduces myocardial infarct size in rabbits (Weinbrenner *et al.*, 1998). Hence, PP1 and PP2A are likely to have a key regulatory role in degradation of the extracellular matrix as the PP inhibitor okadaic acid (OA) increases collagenase and stromelysin-1 mRNA expression in rat chondrocytes (Grumbles *et al.*, 1996) and human fibrosarcoma cells (Westermarck *et al.*, 1994).

We have previously shown that mixed α_1/β-adrenoceptor antagonism attenuates matrix metalloproteinase (MMP) activity and tips the degradative balance to a less degradative phenotype in heart failure patients, and that adrenoceptor stimulation increases MMP-9 promoter activity (Song *et al.*, 2006). These observations support earlier studies implicating adrenoceptors in the modulation of MMP abundance. For example, isoprenaline increased MMP-2 in myocytes and conditioned media of myocytes (Coker *et al.*, 2001; Menon *et al.*, 2005), while noradrenaline enhanced LPS-induced MMP-9 expression in human monocytes; the latter effect is prevented by β_1- but not β_2- or α-adrenoceptor antagonists (Speidl *et al.*, 2004). Thus, there is interplay between adrenoceptors, PPs and PP inhibitors. For example, β-adrenoceptor stimulation increases PP1 and PP2A activity in rat heart (Boknik *et al.*, 2000), but inhibits PP1 activity independently of effects on PP2A in guinea pig ventricular myocardium (Gupta *et al.*, 2002). Isoprenaline also de-phosphorylates ERK1/2 MAPK through activation of PP2A in keratinocytes (Pullar *et al.*, 2003) and increases phosphorylation of I-1 in guinea pig ventricular myocardium, but decreases its expression in rat heart (El-Armouche *et al.*, 2007).

There is emerging evidence that PP1 and PP2A are likely to have a key regulatory role in tissue remodelling in human disease states such as heart failure, but there is no consensus on the mechanisms that might underpin their involvement, and specifically whether interplay with adrenoceptors is a contributing factor. This study aimed to elucidate the influence of phosphatase inhibition on MMP-9 expression and the associated signalling pathway in fibroblasts. Our findings indicate that OA induced MMP-9 activity through p38 MAPK and was inhibited by isoprenaline via a signalling pathway involving β-arrestin 2 and PP2A.

Methods

Cell culture

Mouse fibroblasts (NIH3T3; European Collection of Cell Cultures, Salisbury, UK), human adult cardiac ventricular fibroblasts (HCVFs; TCS Cellworks, Buckingham, UK) and human hepatic stellate (LX-2) cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with either 10% newborn calf serum (NIH3T3) or 10% fetal bovine serum (HCVF, LX-2) with antibiotics (100 U·mL^{-1} penicillin and 100 mg·mL^{-1} streptomycin). For all experiments cells were allowed to attach for 24 h, and medium was changed to phenol red-free DMEM containing 5% charcoal-stripped fetal calf serum 18 h prior to the start of the experiment.

Cell viability (MTT assay)

NIH3T3 fibroblasts seeded in 48-well plates (2×10^4 cells per well) were treated with OA (1–100 nmol·L^{-1}; Calbiochem, Notthingham, UK), isoprenaline (1 μmol·L^{-1}), doxorubicin hydrochloride (2.5 μmol·L^{-1}), medium or dimethyl sulphoxide (DMSO) (0.2% v/v) for 24 h. One hour before the end of the incubation time, MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide; 5 mg·mL^{-1}] was added. At the end of the incubation period, the culture medium was removed and the purple formazan deposits dissolved in DMSO. Formazan production was quantified by spectrophotometry at a wavelength of 540 nm with a reference wavelength of 650 nm (BioTek, EL 808, Bedfordshire, UK).

MMP-9 ELISA and gelatin zymography

Cells were seeded in 24-well plates (4×10^4 cells per well) and exposed to OA (1–100 nmol·L^{-1}), or isoprenaline (1 μmol·L^{-1}), OA (40 nmol·L^{-1}) alone and in combination; DMSO and medium were included as vehicle controls. For the inhibitor studies, cells were pre-incubated (1 h) with NF-κB inhibitor III (1 μmol·L^{-1}), SB202190 (5 μmol·L^{-1}) or U0126 (5 μmol·L^{-1}; Calbiochem) prior to addition of OA (40 nmol·L^{-1}). All stock solutions were prepared in water, with the exception of SB202190, U0126 and NF-κB inhibitor III, which were prepared in DMSO (final concentration ≤0.01% v/v). Conditioned medium was collected after 24 h, and cell debris was removed by centrifugation (5000× *g*, 5 min). Total MMP-9 abundance was measured by ELISA according to the manufacturer's protocol (Total MMP-9, Quantikine ELISA Kit, R&D Systems, Abingdon, UK).

MMP-9 activity was measured by gelatin zymography as previously described (Song *et al.*, 2006). In brief, conditioned medium denatured in a non-reducing sample buffer (50 mmol·L^{-1} Tris-HCl, pH 6.8; 0.1% bromophenol blue, 10% glycerol and 2% SDS; final concentrations) was subjected to electrophoresis on an 8% SDS-polyacrylamide gel co-polymerized with gelatin (0.1% w/v). Recombinant murine latent MMP-9 (Prospec, AnaSpec, Cambridge, UK) was used to verify MMP species and for assessment of gel-to-gel variability. Following electrophoresis, gels were washed in 2.5% Triton X-100 and incubated overnight at 37°C in a pH 7.6 buffer (composition 50 mmol·L^{-1} Tris-HCl, 10 mmol·L^{-1} $CaCl_2$, 50 mmol·L^{-1} NaCl). Gels were stained with 0.25% (w/v) Coomassie blue R-250 in a glacial acetic acid : methanol : distilled H_2O mixture (1:3:6). Following destaining, gels were photographed and lytic activity (clear bands on blue background) quantified using a GeneGenius Gel documentation system with Gene Tools analysis software (Syngene, Cambridge, UK). To ensure the results were not confounded by non-specific protease activity, identical gels were incubated in either the standard incubation buffer or one supplemented with EDTA (20 mmol·L^{-1}) to inhibit gelatinase activity.

Real-time quantitative PCR

Total RNA was isolated using TRI REAGENT™, and reverse transcribed using anchored oligo-dT_{23} primers and M-MLV Reverse Transcriptase following treatment with DNase I (Invitrogen, Paisley, UK). The expression of murine MMP-9 (NM_013559), murine β-actin (NM_007393), human MMP-9 (NM_004994) or human GAPDH (NM_002046) were analysed by quantitative PCR using QuantiTec Primer Assays (Qiagen, Crawley, UK), and a QuantiTect SYBR Green RT-PCR Kit on a 7900HT Real-Time PCR System (Applied Biosystems, Paisley, UK). For each primer pair, a no template control was included, for which the signal was negligible. Gel electrophoresis was used to check the size of the specific amplicons of interest and the absence of other PCR products. The specificity of each reaction was assessed by melting curve analysis. mRNA expression was analysed according to Pfaffl (Pfaffl, 2001) by determination of amplification efficiency for each primer set ($R^2 \geq 0.98$).

Knockdown of β-arrestin 2 and PP2A by siRNA

NIH3T3 fibroblasts were seeded in 48-well plates (1×10^4 cells per well) or 24-well plates (2×10^4 cells per well) 24 h before transfection with siRNA for β-arrestin 2 (100 nmol·L^{-1}) or the catalytic subunit of PP2A (20 nmol·L^{-1}) using Dharmafect3 transfection reagent according to the manufacturer's instructions. On-Target plus smart pool siRNAs targeting mouse β-arrestin 2 and On-Target plus Non-targeting pool siRNAs (mixture of 4 scrambled siRNAs) were obtained from Dharmacon (Dharmacon, Thermo Fisher Scientific, Inc., Chicago, IL, USA). GeneSolution siRNA 6 (TACGACGAGTGTTTAAGGAA.) targeting the mouse catalytic subunit of PP2A and NON-target siRNA (single scrambled siRNA) were obtained from Qiagen. Cells were transfected for 54 h, before the medium was changed to phenol red-free DMEM containing 5% charcoal stripped fetal calf serum. After a further 72 h, cells were exposed to either OA (40 nmol·L^{-1}), isoprenaline (1 μmol·L^{-1}) alone and in combination, or medium for 24 h. Knockdown of β-arrestin 2 and PP2A protein expression was confirmed in each experiment by Western blot analysis using an anti-β-arrestin 2 antibody (Cell Signaling Technology, Danvers, MA, USA; 1:500 dilution) or anti-PP2A catalytic subunit antibody (Millipore, Tenecula, CA, USA; 1:2000 dilution).

PP2A activity assay

NIH3T3 cells were exposed to OA (40 nmol·L^{-1}), isoprenaline (1 μmol·L^{-1}), alone and in combination, along with medium (vehicle control) for the time indicated. Cells were washed with tris-buffered-saline (pH 7.5), and incubated on ice for 15 min in a lysis buffer of composition: HEPES (50 mmol·L^{-1}, pH 7.5), EDTA (0.1 mmol·L^{-1}), EGTA (0.1 mmol·L^{-1}), NaCl (120 mmol·L^{-1}) and NP-40 (0.5% v/v) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, Arklow, Ireland). Lysates were cleared by centrifugation (5 min, 12 000× *g*, 4°C) and protein content determined by Bradford assay. Equal amounts of protein were analysed for PP2A activity using a DuoSetR IC Active PP2A Assay kit (R&D Systems) according to the manufacturer's instructions. PP2A activity in the samples was derived from the standard curve of absorbance (650 nm) versus concentration of the phosphate standards. All samples and standards were analysed in triplicate and each assay run contained a negative control (no lysates) as a quality control for phosphatase contamination.

Mutational analysis of the NF-κB and activator protein-1 (AP-1) binding sites in the MMP-9 promoter

A 1285 bp sequence of the MMP-9 promoter was produced by PCR from human liver DNA (Biochain, Hayward, CA, USA) using the following primers which contain restriction sites for *Xho*I and *Hind*III: 5′-CCCAAGCTTTGGTGAGGGCAGAG GTGTCT-3′ (analogous to positions +20 up to +1) and 5′-CCGCTCGAGGGGAGGGAGGCTTG GCATAA-3′ (starting at position −1285) (Genersch *et al.*, 2000). The PCR product was amplified using *Phusion*™ high-fidelity DNA polymerase (Finnzymes, Vantaa, Finland). Following 'A-tailing' with Thermo-Start Taq DNA polymerase, the PCR product was inserted into a pGEM-TEasy vector (Promega, Southampton, UK), excised with *Xho*I and *Hind*III (New England Biolabs, Bray, Ireland) and subcloned into a linearized pGL3-Basic vector (Promega). The resulting plasmid is referred to as p1285-luc from here on. Chemically competent TOP10 *Escherichia coli* (transformation efficiency of 2.5×10^8 cfu·μg^{-1} DNA) were transformed with the modified or empty vector by heat shock (42°C for 30 s) and used to inoculate lysogeny broth agar plates pre-incubated with IPTG and X-Gal. Following overnight incubation (37°C), several positive white colonies were grown, and the orientation and sequence correctness of the insert was verified by restriction enzyme digestion and sequencing (DNA Sequencing Facility, University of Cambridge, Cambridge, UK). The p1285-luc construct was used as the backbone for the design of two-base pair mutations in the NF-$\kappa B_{(-600)}$, AP-$1_{(-533)}$ and AP-$1_{(-79)}$ sites. All mutations were designed in accordance with transcription factor binding patterns predicted by AliBaba 2.1. (Grabe, 2000). The NF-κB site was mutated (highlighted in bold) from 5′-GGAATTCCCCA-3′ to 5′-GGAA**C**TC**A**CCA-3′ and both AP-1 sites were mutated from 5′-TGAGTCA-3′ to 5′-TGAGT**TG**-3′. Mutagenesis was performed by GenScript Corporation (Piscataway, NJ, USA).

Cell transfection and reporter assay

NIH3T3 fibroblasts were transfected with 450 ng per well of the wild-type (p1285-luc) and mutated (NF-$\kappa B_{(-600)}$, AP-$1_{(-533)}$ and AP-$1_{(-79)}$) promoter constructs, along with 1 ng pRL-CMV (internal standard) using polyfect (2 μL, Qiagen) in a final volume of 100 μL Opti-Mem (Invitrogen). In complementary experiments, cells were transfected with an NF-κB-luc reporter construct (Stratagene, La Jolla, CA, USA) as above, to assess effects on NF-κB-regulated signal transduction. In all experiments cells were exposed to OA (40 nmol·L^{-1}), isoprenaline (1 μmol·L^{-1}) alone and in combination for either 5 h or 24 h as indicated; TNF-α (100 mg·mL^{-1}; Prospec, Ness-Ziona, Israel) and medium were included as controls. Following washing with PBS, cells were harvested, lysed, and firefly and *Renilla* luciferase activity measured using a Dual-Luciferase Reporter Assay System (Promega).

Western blot analysis

For the analysis of early p38 MAPK and ERK1/2 MAPK phosphorylation events, cells were seeded in six-well plates (3 ×

10^5 cells per well), and cultured in serum-free DMEM for 5 h prior to starting the experiment. In all other experiments, the culture medium was changed to phenol red-free DMEM containing 5% charcoal stripped fetal calf serum 18 h prior to the start of the experiment. Cells were exposed to OA (40 nmol·L^{-1}), isoprenaline (1 μmol·L^{-1}), alone or in combination, along with the appropriate control (5% charcoal stripped fetal calf serum DMEM or DMEM) for the times as indicated. Cells were washed and lysed in boiling Laemmli sample buffer containing 2-β-mercaptoethanol (1% v/v). Samples were subjected to 12% SDS-PAGE and semi-dry transferred to PVDF membrane (Amersham, Buckinghamshire, UK). Membranes were probed overnight at 4°C with the primary antibody [anti-phospho-ERK1/2 MAPK (Thr^{202}/Tyr^{204}, 1:1000), anti-phospho-p38 MAPK (Thr^{180}/Tyr^{182}, 1:1000), anti-phospho-NF-κB p65 (Ser^{536}, 1:1000), anti-phospho-JNK 1/2 MAPK (Thr^{183}/Tyr^{185}, 1:1000), anti-p65 NF-κB (1:1000), anti-lamin A/C (BD Transduction, Oxford, UK; 1:1000), anti-tubulin (Sigma-Aldrich; 1:2000) or anti-IκBα (1:1000)], washed and incubated with an appropriate secondary antibody conjugated to horseradish peroxidase (HRP). Signals were visualized by enhanced chemiluminescence detection (Amersham), captured by digital imaging (LAS-4000, Fujifilm Life Science, Bedfordshire, UK) and quantified using a GeneGenius Gel documentation system. Membranes were stripped and re-probed with a HRP-conjugated β-actin antibody (1:7500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or as stated otherwise. Antibodies were obtained from Cell Signalling Technology or as indicated.

Preparation of nuclear and cytosolic fractions

NIH3T3 cells were exposed to OA (40 nmol·L^{-1}) for 24 h and removed by scraping in PBS supplemented with 6.25 mmol·L^{-1} NaF, 12.5 mmol·L^{-1} β-glycerophosphate, 12.5 mmol·L^{-1} p-nitrophenyl phosphate and 1.25 mmol·L^{-1} $NaVO_3$. Cells were collected (250× *g*, 5 min, 4°C) and incubated for 15 min in ice-cold hypotonic lysis buffer (10 mmol·L^{-1} HEPES pH 7.9, 1.5 mmol·L^{-1} $MgCl_2$, 10 mmol·L^{-1} KCl, 5 mmol·L^{-1} dithiothreitol (DTT), 0.1% v/v Triton X-100) supplemented with a protease inhibitor cocktail. Following centrifugation (250× *g*, 5 min, 4°C) the cell pellet was re-suspended in lysis buffer and homogenized (Pellet Pestle Motor Kontes) for 30 s on ice. The homogenate was centrifuged (8000× *g*, 20 min, 4°C) and the supernatant containing the cytosolic fraction was reserved and stored at –80°C until required. The remaining nuclear pellet was re-suspended in extraction buffer (20 mmol·L^{-1} HEPES pH 7.9, 1.5 mmol·L^{-1} $MgCl_2$, 0.42 mol·L^{-1} NaCl, 0.2 mmol·L^{-1} EDTA, 1.0% v/v IGEPAL-CA-630, 25% v/v glycerol 5 mmol·L^{-1} DTT, protease inhibitor cocktail) and disrupted by homogenization (30 s on ice). The samples were mixed for 30 min at 4°C, cleared by centrifugation (16 000× *g*, 5 min, 4°C), and the supernatants retained. The protein content of the cytosolic and nuclear fractions were determined by Bradford assay, and 10 μg of the cytosolic and 15 μg of the nuclear fraction were analysed by Western blot analysis for expression of p65 NF-κB as described above. Lamin A/C and tubulin protein expression in the samples were assessed as nuclear and cytosolic markers respectively. Blots were re-probed in a crossover fashion to verify integrity of the two fractions; Lamin A/C and tubulin were not detected in the cytosolic and nuclear fractions respectively (data not shown).

Data and statistical analysis

Gelatinolytic activity, MMP-9 expression, cell viability, PP2A activity and Western blot data were normalized to their respective controls, and expressed as a percentage or ratio. MMP-9 and NF-κB reporter data were expressed as the ratio of firefly luciferase to *Renilla* luciferase activity, normalized to control (medium) or p1285-luc promoter activity as appropriate. Data were analysed by one-way ANOVA with *post hoc* analysis (Bonferroni, Dunnett's) as appropriate and expressed as mean ± SEM. A value of $P < 0.05$ was taken to indicate statistical significance.

Materials

OA was supplied by Calbiochem, Merck KGaA, Darmstadt, Germany; isoprenaline, doxorubicin and MTT were from Sigma-Aldrich (Arklow, County Wicklow, Ireland). All other chemicals were obtained from Sigma-Aldrich (Ireland).

Results

Effect of OA on cell viability and MMP-9 activity

Okadaic acid (1–100 nmol·L^{-1}) did not alter cell viability in NIH3T3 fibroblasts at any concentration studied, whilst doxorubicin (2.5 μmol·L^{-1}) decreased cell viability by ~50% compared with untreated cells ($P < 0.05$; Figure 1A).

A representative zymogram shows clear bands of gelatinolytic activity at approximately 105 kDa, 72 kDa and 62 kDa corresponding to proMMP-9, proMMP-2 and MMP-2 in conditioned medium from NIH3T3 cells (Figure 1B). No lytic activity corresponding to MMP-9 was detected. OA caused a concentration-dependent increase in proMMP-9 activity in NIH3T3 fibroblasts ($P < 0.05$; Figure 1B,C), and in MMP-9 activity in hepatic stellate cells compared with baseline ($P < 0.05$; Figure 1D). These data indicate that the effect of OA on MMP-9 expression is a generalized phenomenon across a number of cells involved in extracellular matrix remodelling. ProMMP-2 and MMP-2 activity was not altered by OA at the concentrations studied in either cell line (data not presented).

Effect of isoprenaline on OA-induced MMP-9 abundance

Okadaic acid increased (40 nmol·L^{-1}; $P < 0.05$) proMMP-9 gelatinolytic activity by 40% in NIH3T3 cells compared with control. However, when combined with isoprenaline (1 μmol·L^{-1}), proMMP-9 activity was not increased ($P < 0.05$; Figure 2A,B). To complement this, effects on total MMP-9 protein expression were assessed by ELISA. OA (40 nmol·L^{-1}) increased MMP-9 protein and on combination with isoprenaline, this effect was inhibited ($P < 0.05$; Figure 2C). Although MMP-9 or proMMP-9 gelatinolytic activity was not detectable in culture medium from the HCVF cells under the conditions tested, OA increased MMP-9 mRNA expression by ~2.5-fold and, as observed in NIH3T3 cells,

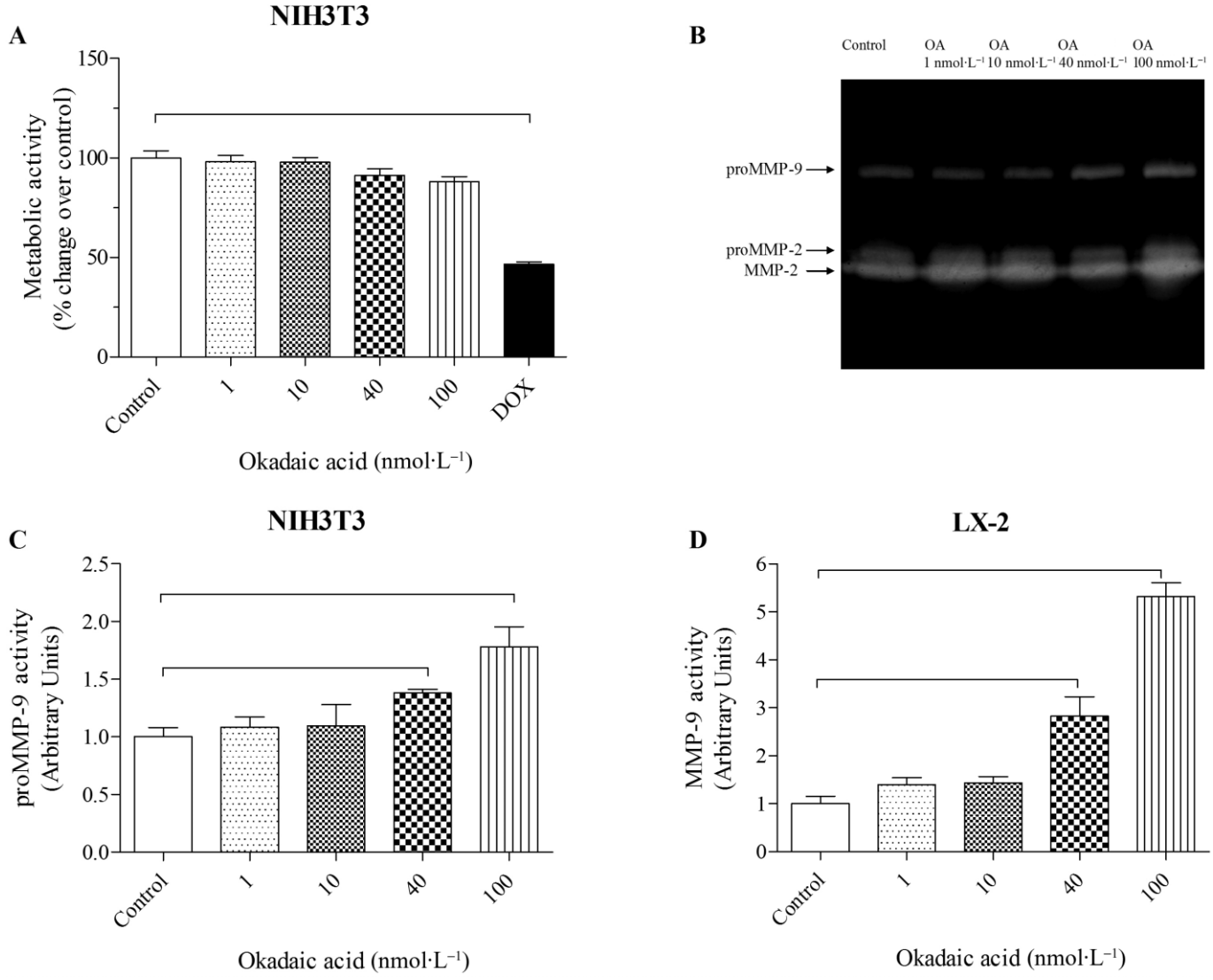

Figure 1

Effects of OA on cell viability and MMP-9 activity in NIH3T3 fibroblasts and LX-2 cells. (A) Cell viability was assessed in NIH3T3 cells following exposure to OA (1–100 nmol·L^{-1}), doxorubicin (DOX; 2.5 μmol·L^{-1}) or medium (control) for 24 h. (B) A representative zymogram showing the effects of OA (1–100 nmol·L^{-1}) on gelatinolytic activity in NIH3T3 cells. (C) Concentration-dependent effects of OA on proMMP-9 activity in conditioned medium from NIH3T3 cells. (D) Concentration-dependent effects of OA on MMP-9 activity in conditioned medium from LX-2 hepatic stellate cells. Data are expressed as a percentage (A; mean ± SEM; $n = 4$) or fold increase of control (C, D; mean ± SEM; $n = 4$). Data were analysed by one-way ANOVA with *post hoc* analysis (Dunnett's). Horizontal bars indicate a statistical significance of $P < 0.05$.

OA in combination with isoprenaline prevented this increase in MMP-9 mRNA expression ($P < 0.05$; Figure 2D). Incubation with isoprenaline alone did not alter MMP-9 activity or abundance in NIH3T3 cells or mRNA expression in HCVFs (Figure 2B–D), indicating a common response across a number of cell lines.

The effect of β-arrestin 2 knockdown on MMP-9 activity

Transfection of NIH3T3 cells with β-arrestin 2 siRNA decreased basal expression of β-arrestin 2 by approximately 70% (Figure 3A), while transfection with NON-Target siRNA had no effect. In β-arrestin 2 knockdown cells, OA (40 nmol·L^{-1}) increased proMMP-9 activity compared with control ($P < 0.05$; Figure 3B). Interestingly, the inhibitory effect of isoprenaline (1 μmol·L^{-1}) on OA-mediated proMMP-9 activity was prevented Figure 3B). Isoprenaline did not alter proMMP-9 activity in conditioned medium from β-arrestin 2 siRNA-transfected cells. siRNA transfection (non-target and β-arrestin 2) did not affect basal proMMP-9 activity compared to mock transfected cells (Figure 3C).

Effect of OA and isoprenaline on PP2A activity

Okadaic acid (40 nmol·L^{-1}) inhibited PP2A activity by 75% at 24 h in NIH3T3 cells (OA vs. control: 26.8 ± 3.36 vs. 100

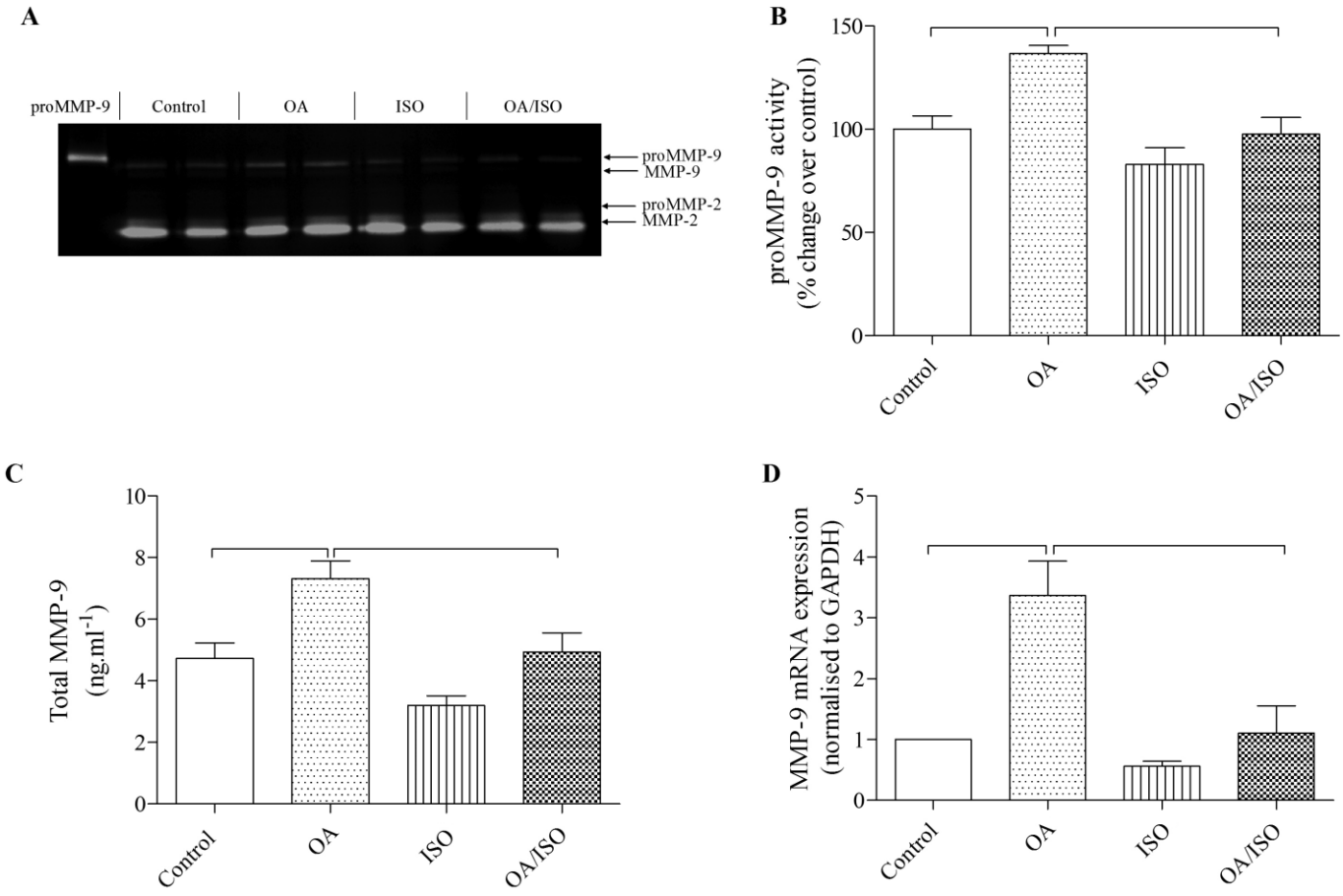

Figure 2

Effects of OA (40 nmol·L^{-1}) and isoprenaline (ISO; 1.0 μmol·L^{-1}), alone and in combination on MMP-9 abundance. (A) A representative zymogram, (B) MMP-9 activity and (C) total MMP-9 protein expression in conditioned medium from NIH3T3 fibroblasts, and (D) on relative mRNA expression in HCVFs. Cells exposed to medium were included as control. Data are expressed as fold increase of control or in ng·mL^{-1} (mean ± SEM; $n = 4$). Data were analysed by one-way ANOVA with *post hoc* analysis (Bonferroni). Horizontal bars indicate a statistical significance of $P < 0.05$.

± 1.42%; $P < 0.05$; Figure 4A). Isoprenaline (1 μmol·L^{-1}) had no effect on PP2A activity compared to untreated cells nor did it alter OA-mediated inhibition of PP2A activity (Figure 4A). Isoprenaline transiently increased PP2A activity at 5 min (5 min vs. control: 153.9 ± 11 vs. 100 ± 3%; $P < 0.05$) and 15 min (15 min vs. control: 136 ± 11 vs. 100 ± 3%; $P < 0.05$), before returning to baseline by 30 min in NIH3T3 cells.

Effect of knockdown of the catalytic subunit of PP2A on MMP-9 activity

Transfection of NIH3T3 cells with siRNA targeting the catalytic subunit of PP2A reduced basal PP2A expression by 80% (Figure 4B), while transfection with NON-Target siRNA had no effect. Following PP2A knockdown, OA (40 nmol·L^{-1}) increased proMMP-9 activity compared with control ($P < 0.05$; Figure 4C). Surprisingly, isoprenaline (1 μmol·L^{-1}) augmented, rather than attenuated OA-mediated proMMP-9 activity following PP2A knockdown ($P < 0.05$; Figure 4C). Incubation with isoprenaline alone did not alter proMMP-9 activity in conditioned medium from PP2A siRNA-transfected cells compared with control. siRNA transfection (non-target and PP2A) did not alter basal proMMP-9 activity compared with mock transfected cells (Figure 4D).

Effect of OA and isoprenaline on p38 and ERK1/2 MAPK signalling

Prolonged exposure (24 h) to SB202190 (5 μmol·L^{-1}), a p38 MAPK inhibitor, prevented OA-induced MMP-9 expression ($P < 0.05$; Figure 5A). However, U0126 (5 μmol·L^{-1}), a MEK1/2 MAPK inhibitor, did not block the effect of OA on MMP-9 abundance (Figure 5B). Of note, SB202190 and U0126 decreased basal MMP-9 expression by approximately 70% and 40% respectively ($P < 0.05$; Figure 5A,B). OA elicited a biphasic increase in phospho-p38 MAPK levels, which peaked at 3 and 24 h ($P < 0.05$; Figure 5C), while phospho-JNK1/2 MAPK expression peaked at 1 and 3 h ($P < 0.05$; Figure 5D).

As isoprenaline transiently increased PP2A at 7.5 min, we also investigated the early effects of OA and isoprenaline on phospho-p38 MAPK and phospho-ERK1/2 MAPK expression. OA (40 nmol·L^{-1}) did not alter phospho-p38 MAPK or

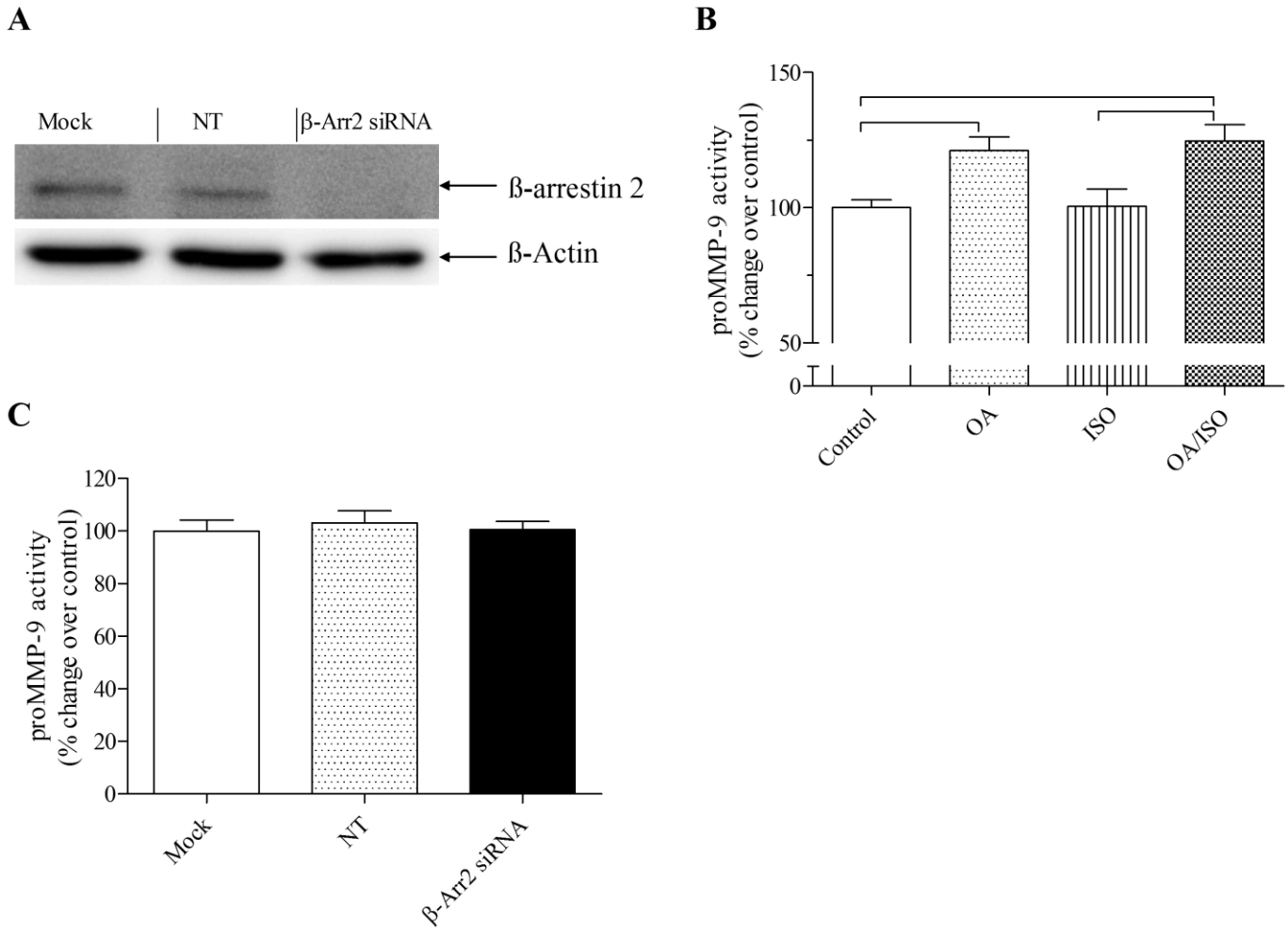

Figure 3

Effect of transfection of NIH3T3 cells with β-arrestin 2 siRNA and NON-Target siRNA (NT) on: (A) β-arrestin 2 and β-actin (internal standard) expression in whole cell lysates, (B) proMMP-9 activity following exposure to OA (40 nmol·L^{-1}) and isoprenaline (ISO; 1 μmol·L^{-1}), alone and in combination, and (C) basal proMMP-9 activity. Data are expressed as a percentage of control (mean ± SEM; n = 6). Data were analysed by one-way ANOVA with *post hoc* analysis (Bonferroni). Horizontal bars indicate a statistical significance of $P < 0.05$.

phospho-ERK1/2 levels at 7.5 min compared with control (Figure 6A,B) in NIH3T3 cells. However, isoprenaline almost doubled expression of phospho-p38 MAPK and phospho-ERK1/2 MAPK ($P < 0.05$; Figure 6A,B); this was not altered by co-exposure to OA (Figure 6A,B).

Assessment of the role of AP-1 and NF-κB transcription factor binding sites on MMP-9 promoter activity

In NIH3T3-transfected cells, mutation of the proximal (AP-$1_{(-79)}$) but not distal (AP-$1_{(-533)}$) AP-1 binding site attenuated basal promoter activity by 50% compared with the p1285-luc (wild type) MMP-9 promoter construct ($P < 0.05$; Figure 7A). Interestingly, mutation of the NF-κB p65 binding motif augmented basal promoter activity by 30% ($P < 0.05$; Figure 7A).

Okadaic acid (40 nmol·L^{-1}) increased p1258-luc MMP-9 promoter activity by 60% compared with the untreated control ($P < 0.05$; Figure 7B), whereas isoprenaline had no effect (Figure 7B). When the AP-1 binding sites were mutated, OA-induced promoter activity was attenuated by ~50% compared with the wild-type MMP-9 promoter construct ($P < 0.05$; Figure 7C). Mutation of the NF-κB$_{(-600)}$ binding site did not affect OA-mediated MMP-9 promoter activity ($P < 0.05$; Figure 7C).

In keeping with the zymography data, isoprenaline (1 μmol·L^{-1}) did not alter p1285-luc AP-$1_{(-79)}$, p1285-luc AP-$1_{(-533)}$ or p1285-luc NF-κB$_{(-600)}$ promoter activity relative to the wild-type construct (Figure 7D). However, it prevented OA-mediated activation of the wild-type MMP-9 promoter ($P < 0.05$; Figure 7B). Isoprenaline in combination with OA increased promoter activity of the NF-κB$_{(-600)}$ mutant construct relative to the wild-type construct ($P < 0.05$; Figure 7E). In contrast, mutation of the AP-1 binding sites (–79 and –533 bp) did not alter MMP-9 promoter activity following co-exposure to isoprenaline and OA, compared with wild type (Figure 7E).

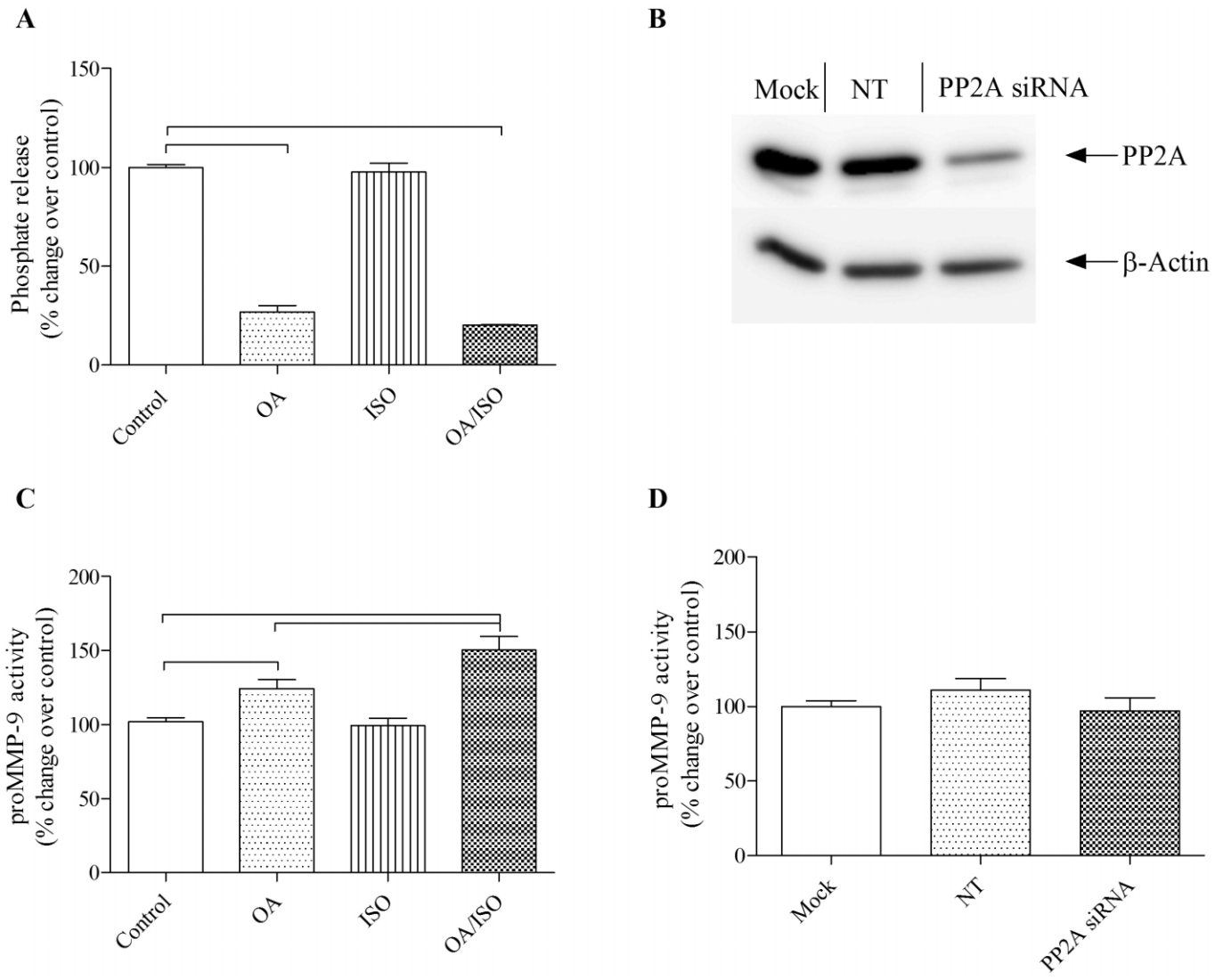

Figure 4

Effect of 24 h exposure to OA (40 nmol·L^{-1}), isoprenaline (ISO;1.0 μmol·L^{-1}) alone and in combination on phosphoprotein phosphatase 2A activity in NIH3T3 cells. Cells exposed to medium were included as control (A). Effect of transfection of NIH3T3 cells with PP2A (catalytic subunit) siRNA and NON-Target siRNA (NT) on: (B) PP2A (catalytic subunit) and β-actin (internal standard) expression in whole cell lysates, (C) proMMP-9 activity following exposure to OA (40 nmol·L^{-1}) and isoprenaline (ISO; 1 μmol·L^{-1}), alone and in combination, and (D) basal proMMP-9 activity. Data are expressed as a percentage of control (mean ± SEM; n = 6). Data were analysed by one-way ANOVA with *post hoc* analysis (Dunnett's and Bonferroni). Horizontal bars indicate a statistical significance of $P < 0.05$.

Effects of OA and isoprenaline on NF-κB and IκBα

In order to extend the data implicating NF-κB in mediating the inhibitory action of isoprenaline on OA-mediated MMP-9 promoter activity, the effects on NF-κB phosphorylation, IκBα degradation and NF-κB reporter activity were studied. Isoprenaline decreased phospho-NF-κB expression at 7.5 min compared with control (isoprenaline vs. control: 0.50 ± 0.04 vs. 1 ± 0.13 AU; $P < 0.05$), whilst OA alone and in combination with isoprenaline had no effect (OA/ isoprenaline vs. isoprenaline: 0.50 ± 0.04 vs. 0.37 ± 0.09 AU). These effects were not accompanied by a change in IκBα expression (isoprenaline vs. control: 1.00 ± 0.26 vs. 1 ± 0.08 AU; OA/isoprenaline vs. isoprenaline alone: 1.03 ± 0.27 vs. 1.00 ± 0.08 AU). However, at 24 h, OA (40 nmol·L^{-1}) caused a doubling in phosphorylation of NF-κB p65 compared with control ($P < 0.05$; Figure 8A). This effect was not accompanied by a reciprocal change in IκBα expression (Figure 8B). At this time point, isoprenaline did not alter phospho-NF-κB or IκBα expression, nor did it alter the OA-induced increase in phospho-NF-κB expression (Figure 8A).

Despite OA increasing NF-κB phosphorylation, it did not alter NF-κB promoter activity at 24 h in NIH3T3 fibroblasts (Figure 8C). Furthermore, isoprenaline alone and in combination with OA did not alter NF-κB promoter activity (Figure 8C), whilst TNF-α (100 ng·mL^{-1}) increased NF-κB pro-

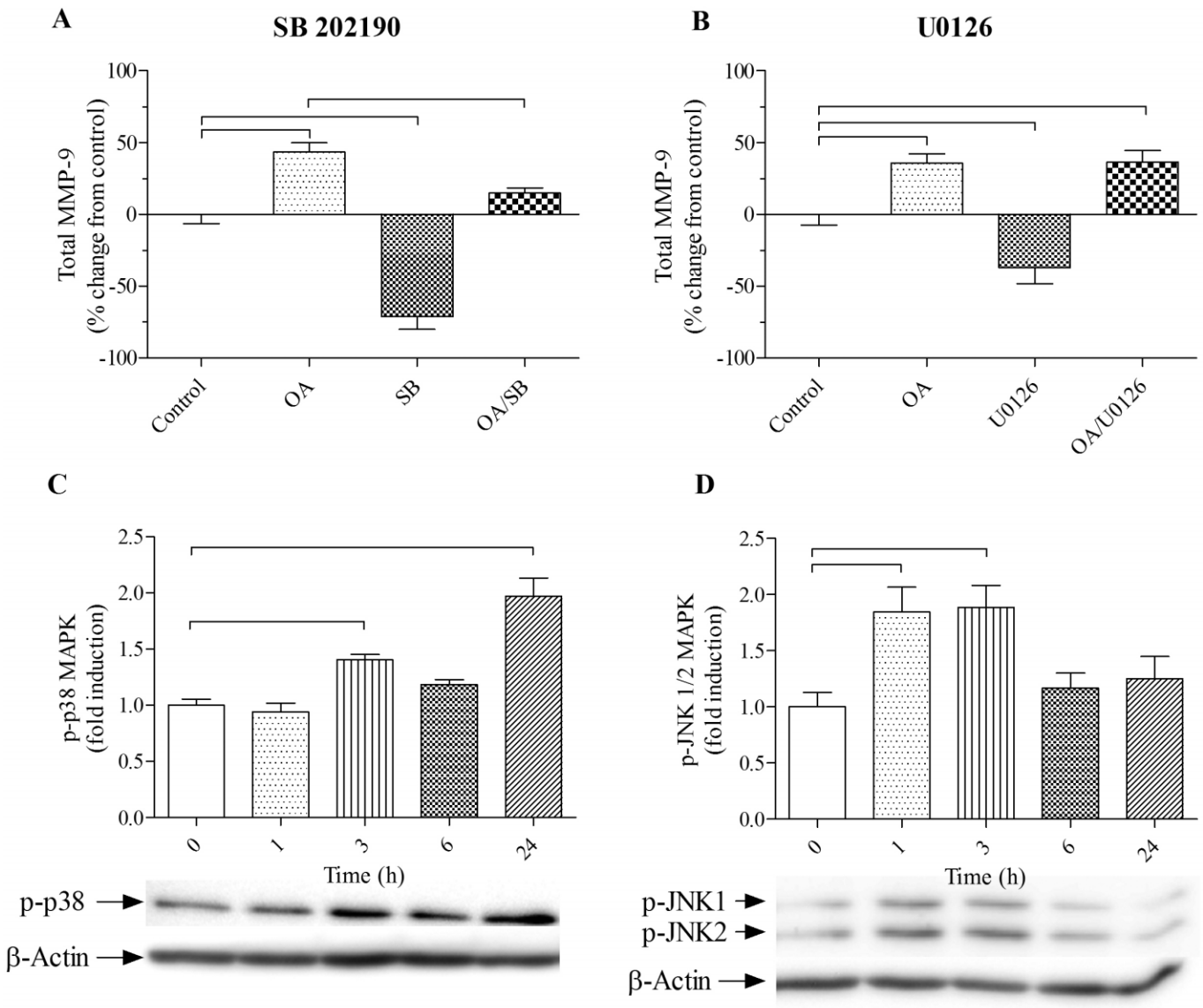

Figure 5

Effect of 24 h exposure to SB202190 (5 μmol·L^{-1}; A), U0126 (5 μmol·L^{-1}; B) or medium (control) on OA-induced MMP-9 expression in conditioned medium from NIH3T3 cells. Temporal effect of OA on phospho-p38 MAPK (C) and phospho-JNK1/2 levels (D) in whole cell lysates of NIH3T3. Data are expressed as percentage over control or fold increase (mean ± SEM; $n = 4$). Data were analysed by one-way ANOVA with *post hoc* analysis (Dunnett's and Bonferroni). Horizontal bars indicate a statistical significance of $P < 0.05$.

moter activity by approximately 30-fold (Figure 8C). Pre-treatment of NIH3T3 cells with an NF-κB inhibitor (NF-κB III inhibitor; 1 μmol·L^{-1}) prior to stimulation with OA confirmed the lack of involvement of NF-κB, as the NF-κB inhibitor did not prevent OA-induced MMP-9 expression (Figure 8D). The NF-κB III inhibitor decreased basal expression of MMP-9 by approximately 40% compared with untreated cells ($P < 0.05$; Figure 8D). To examine the role of NF-κB further, nuclear translocation was assessed. OA did not alter expression levels of the p65 subunit of NF-κB in either nuclear or cytosolic fractions compared with control after 24 h exposure (Figure 8E, F).

Discussion

The present study provides evidence that OA increases MMP-9 abundance in fibroblasts through a p38 MAPK pathway, independent of PP2A inhibition that requires functional AP-1 transcription factor binding sites on the promoter. Furthermore, it demonstrates that isoprenaline inhibits OA-induced MMP-9 expression through a β-arrestin 2- and PP2A-dependent pathway, which requires an NF-κB consensus motif on the promoter. However, this is not associated with altered NF-κB activity or degradation of IκBα.

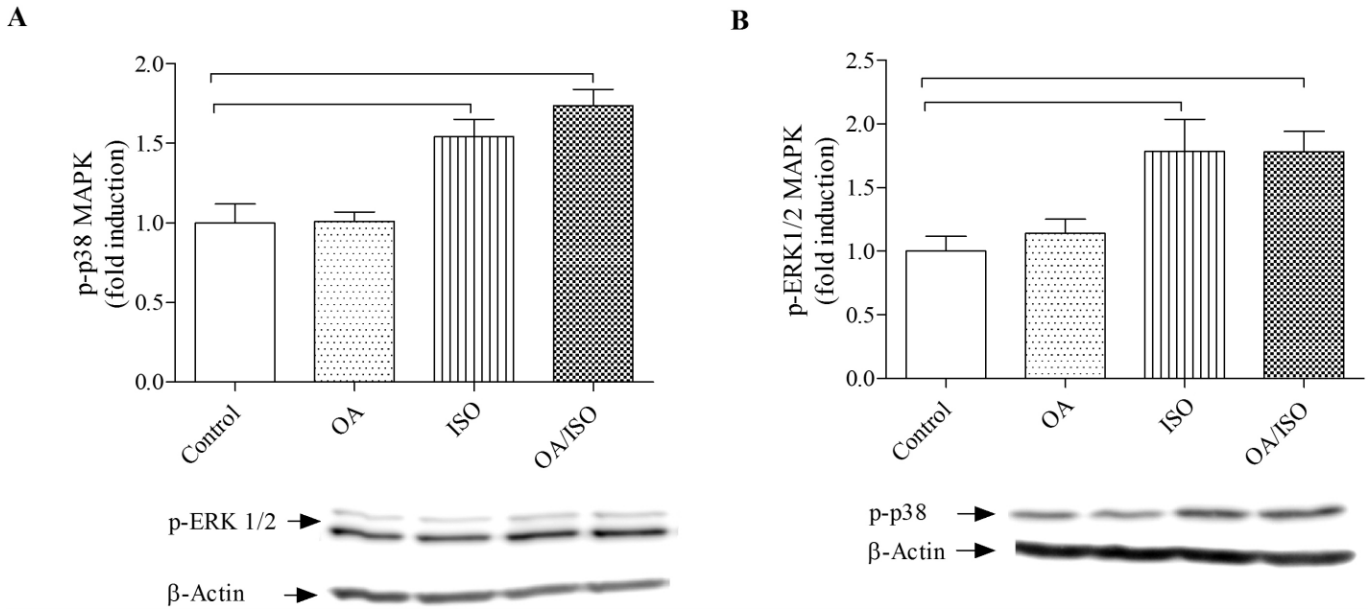

Figure 6

Effect of OA (40 nmol·L^{-1}), isoprenaline (ISO;1 μmol·L^{-1}) alone and in combination on phospho-p38 MAPK (A) and phospho-ERK1/2 MAPK (B) expression in NIH3T3 cells at 7.5 min. Cells exposed to medium were included as control. Expression of β-actin (internal standard) is presented in the lower blots in each image. The positions of phospho-ERK1/2 (Thr^{202}/Tyr^{204}) and p38 MAPK (Thr^{180}/Tyr^{182}) are indicated. Data are expressed as percentage over control or fold increase (mean ± SEM; n = 3). Data were analysed by one-way ANOVA with *post hoc* analysis (Dunnett's and Bonferroni). Horizontal bars indicate a statistical significance of $P < 0.05$.

Okadaic acid is a non-phorbol ester-like tumour promoter, which selectively inhibits PP2A activity at concentrations below 1 × 10^{-6} mol·L^{-1} in cell models (Millward *et al.*, 1999). In the present study OA caused a robust inhibition of phosphoprotein phosphatase 2A activity and increased proMMP-9 activity, mRNA and protein expression and MMP-9 promoter activity in NIH3T3 fibroblasts, hepatic stellate cells (LX-2) and HCVFs. This does not appear to be mediated through PP2A, as knockdown of the catalytic subunit of PP2A failed to induce or prevent OA-induced proMMP-9 activity in NIH3T3 cells. This effect would indicate that OA increased MMP-9 abundance through inhibition of other PP type 2A family members (Shi, 2009) or via an unidentified mechanism.

It is well documented that MMP-9 expression is coupled to ERK1/2, JNK/SAPK and p38 MAPK signalling pathways (Van den Steen *et al.*, 2002). However, no link has been made with regard to OA, which increases phosphorylation of p38 MAPK in T leukaemia cells (Boudreau *et al.*, 2007) and p38, JNK and ERK1/2 MAPK in papilloma-producing mouse keratinocytes (Rosenberger *et al.*, 1999). We found that in NIH3T3 cells, OA elicited a biphasic increase in phospho-p38 that peaked at 3 and 24 h, and at 1 and 3 h for JNK1/2 MAPK. Although only assessed at a single time point (7.5 min), ERK1/2 phosphorylation was not affected. A single time point was chosen as it corresponded to the time point at which isoprenaline transiently increased PP2A activity. While these data clearly demonstrate an effect of OA on p38 and JNK MAPKs, it does not implicate them in the regulation of MMP-9. In this respect, the p38 MAPK inhibitor, SB202190, but not U0126, a MEK1/2 MAPK inhibitor, prevented the OA-mediated increase in MMP-9 abundance. These data are broadly in keeping with early studies showing OA to increase MMP-1 and MMP-13 expression in chondrocytes and fibroblasts (Westermarck *et al.*, 1994; 1998; Grumbles *et al.*, 1996). However, they differ somewhat to our findings in that MMP-1 expression was mediated through ERK1/2, JNK/SAPK and p38 MAPK signalling pathways (Westermarck *et al.*, 1994; 1998).

Surprisingly, isoprenaline prevented the effects of OA on MMP-9 abundance and mRNA expression in NIH3T3 fibroblasts and HCVFs, whilst having no effect itself. Following β-adrenoceptor activation, the receptor is phosphorylated and β-arrestins are recruited. The functional consequences of these events are, receptor desensitization, uncoupling from their associated G-proteins and receptor internalization. More recently, β-arrestins have emerged as scaffolding proteins, which can modify MAPK and other signalling pathways independent of G-protein involvement (Kobayashi *et al.*, 2005; Shenoy *et al.*, 2006). In the present study, knockdown of β-arrestin 2 prevented the inhibitory effect of isoprenaline on OA-increased MMP-9 activity, without affecting OA-augmented MMP-9 activity. When the catalytic subunit of PP2A was knocked out, isoprenaline did not attenuate OA-induced MMP-9 activity. Together, these data show that isoprenaline inhibits OA-induced MMP-9 abundance in a β-arrestin 2- and PP2A-dependent mechanism. Further studies are underway to assess this interaction, as β-arrestin 2 is itself a multifunctional adaptor protein that has the ability to signal through a range of mediators such as MAPKs, Src, NF-κB and phosphoinositide 3-kinase.

The gene for MMP-9 contains multiple transcription factor binding sites including three AP-1 and one NF-κB site.

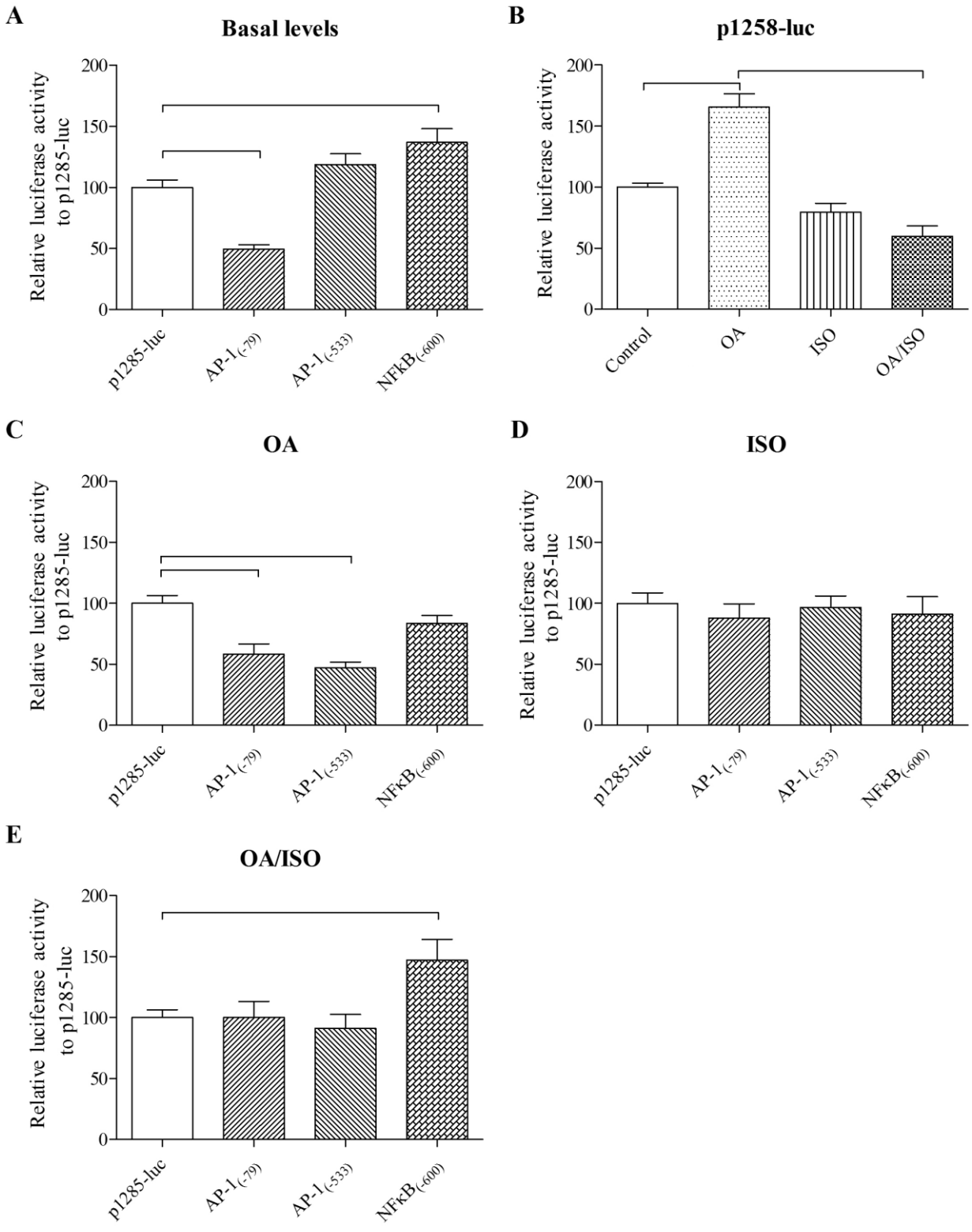

Figure 7

Effect of mutation of the AP-1 and NF-κB binding motifs on MMP-9 promoter activity in response to OA and isoprenaline (ISO, A–E). NIH3T3 fibroblasts were transfected with p1285-luc, p1285-luc AP-$1_{(-79)}$, p1285-luc AP-$1_{(-533)}$ and p1285-luc NF-$\kappa B_{(-600)}$ promoter constructs and exposed to OA (40 nmol·L^{-1}), isoprenaline (1.0 μmol·L^{-1}) alone and in combination for 24 h. Luciferase activity was determined in cell lysates and promoter activity for each mutant was normalized to their individual control and expressed as a ratio to p1285-luc construct (mean ± SEM; $n = 8$). Data were analysed by one-way ANOVA with *post hoc* analysis (Bonferroni). Horizontal bars indicate a statistical significance of $P < 0.05$.

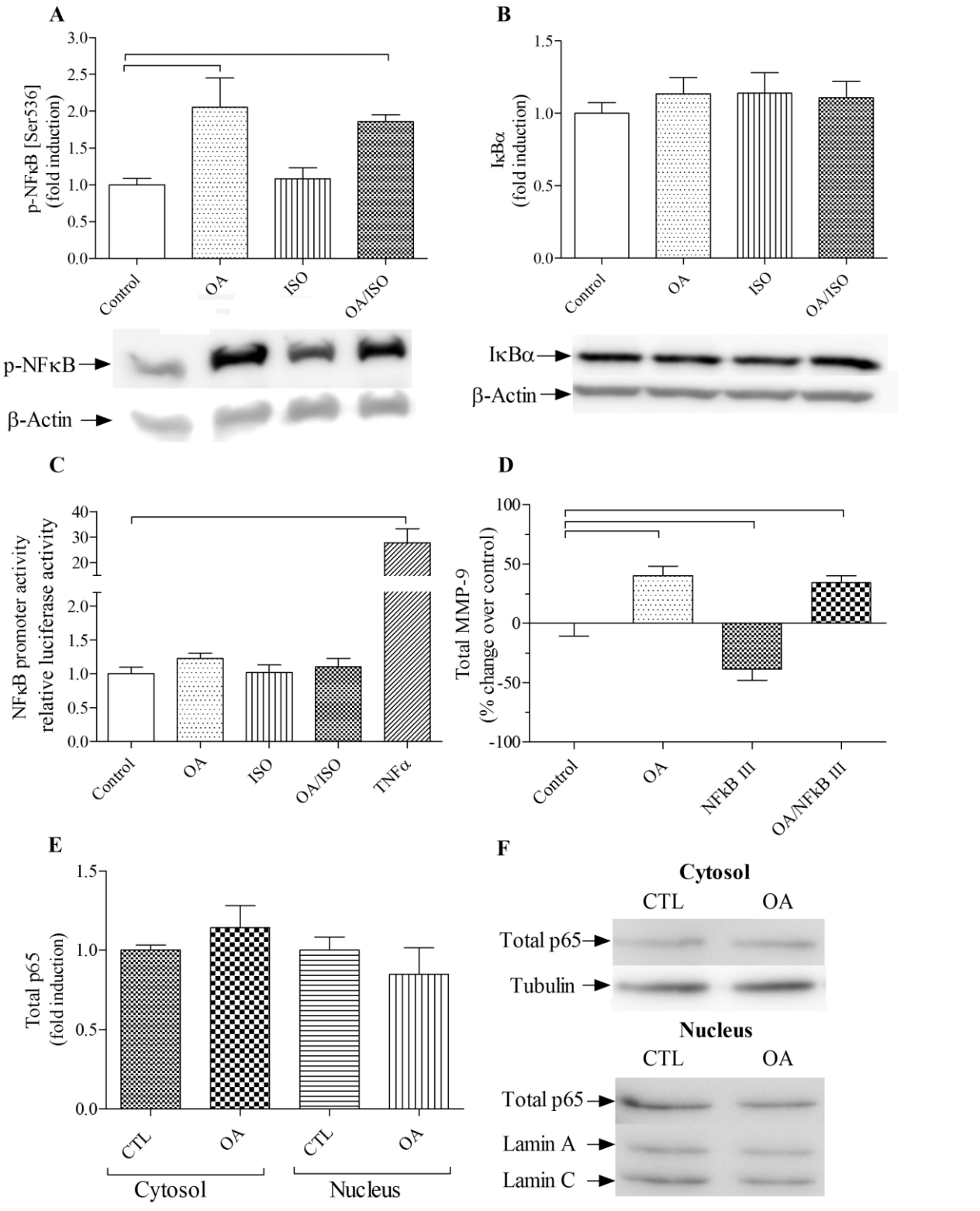

Figure 8

Effect of OA (40 nmol·L^{-1}) and isoprenaline (ISO; 1.0 μmol·L^{-1}) alone and in combination on: (A) phosphorylation of NF-κB p65 (Ser^{536}), (B) IκBα expression and (C) NF-κB promoter activity after 24 h in NIH3T3 fibroblasts. (D) Effects of pre-incubation with NF-κB inhibitor III (1 μmol·L^{-1}) on MMP-9 expression for 24 h in NIH3T3 fibroblasts. Effect of OA on p65 abundance in nuclear and cytosolic fractions of NIH3T3 cells after 24 h (E, F). MMP-9 abundance was analysed using ELISA. Data are expressed as fold or percentage increase over control (mean ± SEM; $n = 3$). Data were analysed by one-way ANOVA with *post hoc* analysis (Dunnett's and Bonferroni). Horizontal bars indicate a statistical significance of $P < 0.05$.

Although MMP-1 and MMP-9 share a TATA box and an AP-1 site in the proximal ~70 bp region of their promoters (Yan and Boyd, 2007), they differ in other transcription factor binding sites, and MMP-1 only has NF-κB-like elements (Borghaei *et al.*, 1997). Using deletion mutants of the MMP-9 promoter, it was noted that both the proximal AP-1 (–79 bp) and distal AP-1 (–533 bp) binding sites were required for full activation of the p1285-luc promoter by OA. Earlier work showing that OA increases expression of JunB, and enhances trans-activation of AP-1 complexes containing c-Jun and JunB (Westermarck *et al.*, 1994) supports this observation. Furthermore, we found that OA increased phosphorylation of p38 MAPK and JNK1/2 MAPK, which lie upstream of c-Jun and JunB. In contrast, the inhibitory effect of isoprenaline on OA-induced MMP-9 promoter activity is mediated by an NF-κB binding motif. This effect, taken in conjunction with the data from the β-arrestin knockout experiments, indicated that isoprenaline inhibited the OA response via a pathway involving β-arrestin 2 and NF-κB. This explanation is strengthened by the observation that over-expression of β-arrestin 1 and β-arrestin 2 decreases NF-κB promoter activity (Witherow *et al.*, 2004), and knockdown of β-arrestin 1 augments TNF-α-induced NF-κB promoter activity. Hence, the NF-κB binding site of the MMP-9 promoter might have a negative regulatory role. Indeed, basal MMP-9 promoter activity was increased when the NF-κB site was mutated in the present study, whilst the proximal but not distal AP-1 is required for basal activation as reported by others (Sato *et al.*, 1993; Ma *et al.*, 2004; Ray *et al.*, 2005). In contrast, the distal AP-1 (–533) is required for full promoter activity in Hela cells (Ma *et al.*, 2004), as is the NF-κB binding motif (Sato *et al.*, 1993; Ma *et al.*, 2004; Ray *et al.*, 2005). Nevertheless, OA-induced MMP-9 promoter activation requires both AP-1 binding sites, but not the NF-κB binding motif in NIH3T3 cells.

Although the NF-κB binding site in the MMP-9 promoter does not influence OA-induced MMP-9 expression, other studies have shown that phosphoprotein phosphatase inhibitors activate NF-κB (Rieckmann *et al.*, 1992; Sun *et al.*, 1995; Traenckner *et al.*, 1995; Miskolci *et al.*, 2003). When we investigated this further, it was found that OA increased phosphorylation of NF-κB (Ser^{536}), which is not unexpected as PP2A de-phosphorylates the p65 subunit of NF-κB *in vitro* (Yang *et al.*, 2001). However, there was no reciprocal change in IκBα degradation, which is at variance with work by Miskolci *et al.*, (2003), showing that OA-mediated activation of NF-κB is accompanied by an increased degradation of IκBα in neutrophils. Nevertheless, OA did not alter NF-κB reporter activity, nor did pharmacological inhibition of NF-κB ($NF\text{-}κB_{III}$ inhibitor) affect OA-mediated MMP-9 expression in the present study. These data, in combination with data from the MMP-9 reporter assays and lack of nuclear translocation of the p65 subunit of NF-κB, strongly indicate that NF-κB is not involved in regulation of MMP-9 by OA. However, the NF-κB binding site is important in mediating the inhibitory effect of isoprenaline on OA-induced MMP-9 promoter activity, which occurred independently of effects on NF-κB promoter activity, IκBα expression or alteration of OA-induced NF-κB phosphorylation. Hsieh *et al.* report that andrographolide inhibits LPS-induced MMP-9 activity by repression of NF-κB transactivation independently of the classical effects of LPS on IκBα, IKKα and IKKβ (Hsieh *et al.*, 2011). Furthermore, it decreased phosphorylation of p65-NF-κB (Ser^{536}) through activation of PP2A, thereby inhibiting nuclear translocation of the p65 unit. While consistent with our view of a non-classical signalling pathway, we did not find OA inducing nuclear translocation of NF-κB, despite increased p65 (Ser^{536}) phosphorylation, which was unaltered by isoprenaline. In the current study, isoprenaline caused a rapid decrease in NF-κB (Ser^{536}) phosphorylation in NIH3T3 fibroblasts. This effect is indicative of inhibition of NF-κB signalling, and supports our suggestion that the NF-κB binding motif in the MMP-9 promoter has a negative regulatory role. Interestingly, the inhibitory effect of isoprenaline on OA-induced MMP-9 abundance was prevented by knockdown of the catalytic unit of PP2A.

There is little consensus regarding the effect of β-adrenoceptor stimulation and cAMP on NF-κB activation, as β-adrenoceptors activate NF-κB in cardiomyocytes (Chandrasekar *et al.*, 2004), whilst in monocytes β-adrenoceptor agonists exert anti-inflammatory effects through repression of IκBα/NF-κB signalling (Farmer and Pugin, 2000). The latter observation is interesting as isoprenaline by itself did not activate NF-κB in NIH3T3 fibroblasts. Nonetheless, Takemoto *et al.* found that chronic stimulation with isoprenaline enhanced NF-κB DNA binding activity in rats (Takemoto *et al.*, 1999). Therefore, it is possible that isoprenaline may alter NF-κB DNA binding activity in the present study, thus regulating MMP-9 promoter activity in a non-classical manner.

In conclusion, OA increased MMP-9 activity via a p38 MAPK signalling cascade and required both AP-1 binding sites. This effect was inhibited by stimulation of β-adrenoceptors and mediated by β-arrestin 2, PP2A and the NF-κB binding site on the MMP-9 promoter. However, this was not associated with altered NF-κB activity or increased degradation of IκBα. These data extend our understanding of how MMP-9 expression is regulated and highlights a possible role of type 2A PPs in tissue remodelling.

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Conflict of interest

The authors state no conflict of interest.

References

Boknik P, Fockenbrock M, Herzig S, Knapp J, Linck B, Luss H *et al.* (2000). Protein phosphatase activity is increased in a rat model of long-term beta-adrenergic stimulation. Naunyn Schmiedebergs Arch Pharmacol 362: 222–231.

Borghaei H, Borghaei RC, Ni X, Pease E, Thornton R, Mochan E (1997). Evidence that suppression of IL-1 induced collagenase mRNA expression by dihomo-gamma-linolenic acid (DGLA) involves inhibition of NF kappa B binding. Inflamm Res 46 (Suppl. 2): S177–S178.

Boudreau RT, Conrad DM, Hoskin DW (2007). Apoptosis induced by protein phosphatase 2A (PP2A) inhibition in T leukemia cells is negatively regulated by PP2A-associated p38 mitogen-activated protein kinase. Cell Signal 19: 139–151.

Carr AN, Schmidt AG, Suzuki Y, del MF, Sato Y, Lanner C *et al.* (2002). Type 1 phosphatase, a negative regulator of cardiac function. Mol Cell Biol 22: 4124–4135.

Chandrasekar B, Marelli-Berg FM, Tone M, Bysani S, Prabhu SD, Murray DR (2004). Beta-adrenergic stimulation induces interleukin-18 expression via beta2-AR, PI3K, Akt, IKK, and NF-kappaB. Biochem Biophys Res Commun 319: 304–311.

Coker ML, Jolly JR, Joffs C, Etoh T, Holder JR, Bond BR *et al.* (2001). Matrix metalloproteinase expression and activity in isolated myocytes after neurohormonal stimulation. Am J Physiol Heart Circ Physiol 281: H543–H551.

El-Armouche A, Eschenhagen T (2009). Beta-adrenergic stimulation and myocardial function in the failing heart. Heart Fail Rev 14: 225–241.

El-Armouche A, Pamminger T, Ditz D, Zolk O, Eschenhagen T (2004). Decreased protein and phosphorylation level of the protein phosphatase inhibitor-1 in failing human hearts. Cardiovasc Res 61: 87–93.

El-Armouche A, Gocht F, Jaeckel E, Wittkopper K, Peeck M, Eschenhagen T (2007). Long-term beta-adrenergic stimulation leads to downregulation of protein phosphatase inhibitor-1 in the heart. Eur J Heart Fail 9: 1077–1080.

Farmer P, Pugin J (2000). Beta-adrenergic agonists exert their 'anti-inflammatory' effects in monocytic cells through the IkappaB/NF-kappaB pathway. Am J Physiol Lung Cell Mol Physiol 279: L675–L682.

Genersch E, Hayess K, Neuenfeld Y, Haller H (2000). Sustained ERK phosphorylation is necessary but not sufficient for MMP-9 regulation in endothelial cells: involvement of Ras-dependent and -independent pathways. J Cell Sci 113 (Pt 23): 4319–4330.

Gergs U, Boknik P, Buchwalow I, Fabritz L, Matus M, Justus I *et al.* (2004). Overexpression of the catalytic subunit of protein phosphatase 2A impairs cardiac function. J Biol Chem 279: 40827–40834.

Grabe N (2000). Alibaba 2.1. http://www.gene-regulation.com/pub/programs.html#alibaba2.

Grumbles RM, Shao L, Jeffrey JJ, Howell DS (1996). Regulation of rat interstitial collagenase gene expression in growth cartilage and chondrocytes by vitamin D3, interleukin-1 beta, and okadaic acid. J Cell Biochem 63: 395–409.

Gupta RC, Neumann J, Watanabe AM, Sabbah HN (2002). Inhibition of type 1 protein phosphatase activity by activation of beta-adrenoceptors in ventricular myocardium. Biochem Pharmacol 63: 1069–1076.

Hsieh CY, Hsu MJ, Hsiao G, Wang YH, Uang CW, Hen SW *et al.* (2011). Andrographolide enhances nuclear factor-κB subunit p65 Ser^{536} dephosphorylation through activation of protein phosphatase 2A in vascular smooth muscle cells. J Biol Chem 286: 5942–5955.

Kobayashi H, Narita Y, Nishida M, Kurose H (2005). Beta-arrestin2 enhances beta2-adrenergic receptor-mediated nuclear translocation of ERK. Cell Signal 17: 1248–1253.

Ma Z, Shah RC, Chang MJ, Benveniste EN (2004). Coordination of cell signaling, chromatin remodeling, histone modifications, and regulator recruitment in human matrix metalloproteinase 9 gene transcription. Mol Cell Biol 24: 5496–5509.

Menon B, Singh M, Singh K (2005). Matrix metalloproteinases mediate beta-adrenergic receptor-stimulated apoptosis in adult rat ventricular myocytes. Am J Physiol Cell Physiol 289: C168–C176.

Millward TA, Zolnierowicz S, Hemmings BA (1999). Regulation of protein kinase cascades by protein phosphatase 2A. Trends Biochem Sci 24: 186–191.

Miskolci V, Castro-Alcaraz S, Nguyen P, Vancura A, Davidson D, Vancurova I (2003). Okadaic acid induces sustained activation of NFkappaB and degradation of the nuclear IkappaBalpha in human neutrophils. Arch Biochem Biophys 417: 44–52.

Neumann J, Eschenhagen T, Jones LR, Linck B, Schmitz W, Scholz H *et al.* (1997). Increased expression of cardiac phosphatases in patients with end-stage heart failure. J Mol Cell Cardiol 29: 265–272.

Pfaffl MW (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45.

Pullar CE, Chen J, Isseroff RR (2003). PP2A activation by beta2-adrenergic receptor agonists: novel regulatory mechanism of keratinocyte migration. J Biol Chem 278: 22555–22562.

Ray A, Bal BS, Ray BK (2005). Transcriptional induction of matrix metalloproteinase-9 in the chondrocyte and synoviocyte cells is regulated via a novel mechanism: evidence for functional cooperation between serum amyloid A-activating factor-1 and AP-1. J Immunol 175: 4039–4048.

Rieckmann P, Thevenin C, Kehrl JH (1992). Okadaic acid is a potent inducer of AP-1, NF-kappa B, and tumor necrosis factor-alpha in human B lymphocytes. Biochem Biophys Res Commun 187: 51–57.

Rosenberger SF, Gupta A, Bowden GT (1999). Inhibition of p38 MAP kinase increases okadaic acid mediated AP-1 expression and DNA binding but has no effect on TRE dependent transcription. Oncogene 18: 3626–3632.

Sato H, Kita M, Seiki M (1993). v-Src activates the expression of 92-kDa type IV collagenase gene through the AP-1 site and the GT box homologous to retinoblastoma control elements. A mechanism regulating gene expression independent of that by inflammatory cytokines. J Biol Chem 268: 23460–23468.

Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S *et al.* (2006). Beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. J Biol Chem 281: 1261–1273.

Shi Y (2009). Serine/threonine phosphatases: mechanism through structure. Cell 139: 468–484.

Song G, Hennessy M, Zhao YL, Li Q, Han WD, Qi Y *et al.* (2006). Adrenoceptor blockade alters plasma gelatinase activity in patients with heart failure and MMP-9 promoter activity in a human cell line (ECV304). Pharmacol Res 54: 57–64.

Speidl WS, Toller WG, Kaun C, Weiss TW, Pfaffenberger S, Kastl SP *et al.* (2004). Catecholamines potentiate LPS-induced expression of MMP-1 and MMP-9 in human monocytes and in the human monocytic cell line U937: possible implications for peri-operative plaque instability. FASEB J 18: 603–605.

Sun SC, Maggirwar SB, Harhaj E (1995). Activation of NF-kappa B by phosphatase inhibitors involves the phosphorylation of I kappa B alpha at phosphatase 2A-sensitive sites. J Biol Chem 270: 18347–18351.

Takemoto Y, Yoshiyama M, Takeuchi K, Omura T, Komatsu R, Izumi Y *et al*. (1999). Increased JNK, AP-1 and NF-kappa B DNA binding activities in isoproterenol-induced cardiac remodeling. J Mol Cell Cardiol 31: 2017–2030.

Traenckner EB, Pahl HL, Henkel T, Schmidt KN, Wilk S, Baeuerle PA (1995). Phosphorylation of human I kappa B-alpha on serines 32 and 36 controls I kappa B-alpha proteolysis and NF-kappa B activation in response to diverse stimuli. EMBO J 14: 2876–2883.

Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA, Opdenakker G (2002). Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). Crit Rev Biochem Mol Biol 37: 375–536.

Weinbrenner C, Baines CP, Liu GS, Armstrong SC, Ganote CE, Walsh AH *et al*. (1998). Fostriecin, an inhibitor of protein phosphatase 2A, limits myocardial infarct size even when administered after onset of ischemia. Circulation 98: 899–905.

Westermarck J, Lohi J, Keski-Oja J, Kahari VM (1994). Okadaic acid-elicited transcriptional activation of collagenase gene expression in HT-1080 fibrosarcoma cells is mediated by JunB. Cell Growth Differ 5: 1205–1213.

Westermarck J, Holmstrom T, Ahonen M, Eriksson JE, Kahari VM (1998). Enhancement of fibroblast collagenase-1 (MMP-1) gene expression by tumor promoter okadaic acid is mediated by stress-activated protein kinases Jun N-terminal kinase and p38. Matrix Biol 17: 547–557.

Witherow DS, Garrison TR, Miller WE, Lefkowitz RJ (2004). Beta-arrestin inhibits NF-kappaB activity by means of its interaction with the NF-kappaB inhibitor IkappaBalpha. Proc Natl Acad Sci U S A 101: 8603–8607.

Yan C, Boyd DD (2007). Regulation of matrix metalloproteinase gene expression. J Cell Physiol 211: 19–26.

Yang J, Fan GH, Wadzinski BE, Sakurai H, Richmond A (2001). Protein phosphatase 2A interacts with and directly dephosphorylates RelA. J Biol Chem 276: 47828–47833.