RESEARCH PAPER

Natriuretic peptide receptor-3 underpins the disparate regulation of endothelial and vascular smooth muscle cell proliferation by C-type natriuretic peptide

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BACKGROUND AND PURPOSE

C-type natriuretic peptide (CNP) is an endothelium-derived vasorelaxant, exerting anti-atherogenic actions in the vasculature and salvaging the myocardium from ischaemic injury. The cytoprotective effects of CNP are mediated in part via the G_i-coupled natriuretic peptide receptor (NPR)3. As GPCRs are well-known to control cell proliferation, we investigated if NPR3 activation underlies effects of CNP on endothelial and vascular smooth muscle cell mitogenesis.

EXPERIMENTAL APPROACH

Proliferation of human umbilical vein endothelial cells (HUVEC), rat aortic smooth muscle cells (RAoSMC) and endothelial and vascular smooth muscle cells from NPR3 knockout (KO) mice was investigated *in vitro*.

KEY RESULTS

CNP (1 pM–1 μM) facilitated HUVEC proliferation and inhibited RAoSMC growth concentration-dependently. The pro- and anti-mitogenic effects of CNP were blocked by the NPR3 antagonist M372049 (10 μM) and the extracellular signal-regulated kinase (ERK) 1/2 inhibitor PD98059 (30 μM) and were absent in cells from NPR3 KO mice. Activation of ERK 1/2 by CNP was inhibited by *Pertussis* toxin (100 ng·mL^{-1}) and M372049 (10 μM). In HUVEC, ERK 1/2 activation enhanced expression of the cell cycle promoter, cyclin D1, whereas in RAoSMC, ERK 1/2 activation increased expression of the cell cycle inhibitors p21$^{waf1/cip1}$ and p27^{kip1}.

CONCLUSIONS AND IMPLICATIONS

A facet of the vasoprotective profile of CNP is mediated via NPR3-dependent ERK 1/2 phosphorylation, resulting in augmented endothelial cell proliferation and inhibition of vascular smooth muscle growth. This pathway may offer an innovative approach to reversing the endothelial damage and vascular smooth muscle hyperplasia that characterize many vascular disorders.

Abbreviations

ANP, atrial natriuretic peptide; CNP, C-type natriuretic peptide; ERK 1/2, extracellular signal-regulated kinase 1/2; HUVEC, human umbilical vein endothelial cell; I/R, ischaemia reperfusion; ICAM-2, intercellular adhesion molecule-2; KO, knockout; NPR3, natriuretic peptide receptor-3; PCI, percutaneous coronary intervention; PMEC, pulmonary microvascular endothelial cells; RAoSMC, rat aortic smooth muscle cells; WT, wild type

Introduction

A key role for C-type natriuretic peptide (CNP) in regulating cardiac and vascular function has recently emerged (Ahluwalia and Hobbs, 2005; Potter *et al.* 2009). This cardiovascular homeostatic role is perhaps best exemplified by its ability to control blood flow in the resistance vasculature (Chauhan *et al.*, 2003), to reduce the reactivity of leukocytes and platelets (Scotland *et al.*, 2005), to govern the pacemaker current in the heart (Rose *et al.*, 2004) and to protect against myocardial ischaemia/reperfusion (I/R) injury (Hobbs *et al.*, 2004; Wang *et al.* 2007) and cardiac hypertrophy (Tokudome *et al.*, 2004; Soeki *et al.* 2005; Wang *et al.*, 2007). In contrast to the endocrine actions of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), the vascular actions of CNP are autocrine or paracrine in nature, as this peptide is stored and released by vascular endothelial cells (Suga *et al.*, 1992; Chen and Burnett, Jr, 1998). This local endothelial production, coupled to the vasodilator, anti-platelet and anti-leukocyte effects of CNP, imply it has the potential to exert a multi-pronged anti-atherosclerotic influence on the blood vessel wall. This concept is supported by several previous reports. For example, adenoviral delivery of CNP slows neointimal hyperplasia and promotes re-endothelialization in vein grafts (Ohno *et al.*, 2002) and following balloon angioplasty (Ueno *et al.*, 1997; Morishige *et al.* 2000; Doi *et al.*, 2001; Kuhnl *et al.*, 2005; Pelisek *et al.* 2006), and maintains blood flow and capillary density after hind-limb ischaemia (Yamahara *et al.*, 2003). Furthermore, systemic infusion of CNP prevents development of intimal thickening in damaged carotid arteries (Furuya *et al.*, 1993) and increases the number of proliferating endothelial cells in pulmonary hypertension (Itoh *et al.*, 2004). CNP expression is also highly up-regulated in human coronary atherosclerotic lesions (Naruko *et al.*, 1996) and in the neointima following percutaneous coronary intervention (PCI) (Naruko *et al.*, 2005). CNP's sibling, ANP, has been shown to act in an endocrine manner to facilitate re-vascularization after I/R injury (Kuhn *et al.*, 2009).

Activation of the guanylate cyclase-linked natriuretic peptide receptor NPR2 (receptor nomenclature follows Alexander *et al.*, 2009) underlies many of the biological actions of CNP (Ahluwalia *et al.*, 2004), but recent evidence suggests that several cardioprotective effects (vasodilatation of the resistance vasculature, anti-platelet and anti-leukocyte effects) of the peptide are dependent on a different receptor, NPR3 (Hobbs *et al.*, 2004; Rose *et al.* 2004; Ahluwalia and Hobbs, 2005). Unlike other NPR subtypes, NPR3 does not possess a guanylate cyclase functionality but rather possesses a short 37amino acid intracellular tail that contains a $G_{i/o}$-binding domain (Anand-Srivastava *et al.*, 1996; Murthy and Makhlouf, 1999). As a consequence, G-protein coupling confers a positive signalling role to NPR3 in certain tissues, including inhibition of adenylate cyclase activity (Pagano and Anand-Srivastava, 2001) and activation of phospholipase C-β_3 (Murthy *et al.*, 2000). NPR3 is a homodimer with each subunit possessing a single transmembrane domain (He *et al.*, 2001). This structure is quite distinct from the archetypal heptahelical GPCR family (Gudermann *et al.* 1995; Nurnberg *et al.*, 1995; Gether, 2000) and more like the $G_{i/o}$-coupled growth factor receptors, such as those triggered by sphingosine-1-phosphate and insulin-like growth factor-1 (Zondag *et al.*, 1998; Hallak *et al.* 2000). This raises the possibility that NPR3/$G_{i/o}$ signalling may play a key role in governing cell proliferation.

Indeed, an important facet of CNP bioactivity that contributes to its cardioprotective function is an ability to promote re-endothelialization (Furuya *et al.* 1993; Ueno *et al.*, 1997; Morishige *et al.*, 2000; Doi *et al.* 2001; Ohno *et al.*, 2002; Yamahara *et al.*, 2003; Kuhnl *et al.* 2005; Pelisek *et al.*, 2006) while concomitantly inhibiting vascular smooth muscle cell growth (Furuya *et al.*, 1993; Cahill and Hassid, 1994; Hutchinson *et al.*, 1997). This dichotomous activity may prove important not only in understanding the mechanisms underpinning vascular homeostasis but may also offer a pharmacological means to differentially regulate endothelial and vascular smooth muscle cell proliferation to slow pathogenesis and facilitate tissue repair in diseased blood vessels. If this therapeutic potential of CNP is to be harnessed, it is imperative that mechanisms underlying this unique pharmacodynamic profile are delineated.

Although the guanylate cyclase-linked NPR2 has been documented to reduce vascular smooth muscle cell growth in a cGMP-dependent manner, the potential for the $G_{i/o}$-coupled NPR3 to act as a regulator of cell growth in the vasculature has not been elucidated. Here, we have sought to characterize the role of CNP/NPR3 signalling in endothelial and vascular smooth muscle cell proliferation and define the subcellular signalling cascades that underlie these novel actions.

Methods

Animals

All animal care and experimental procedures complied with the UK Animals (Scientific Procedures) Act 1986 and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996) and were approved by the University College London Ethics Committee.

Aortic smooth muscle cell (AoSMC) isolation and characterization

AoSMC were isolated from adult Sprague–Dawley rats (male; 200–250 g) or wild-type (WT) and NPR3 knockout (KO) mice (male; 25–30 g) by enzymatic digestion, as described previously (Chamley-Campbell *et al.*, 1979). Cells were used between passages 4 and 10.

Biomagnetic purification of mouse pulmonary microvascular endothelial cells (PMEC)

PMEC were isolated from WT and NPR3 KO mice by a combination of methods previously described (Hartwell *et al.*, 1998; Kuhlencordt *et al.* 2004). Animals were killed by cervical dislocation, and the lungs were removed and placed in Dulbecco's modified Eagle's medium (DMEM)/Nutrient Mixture F12 (DMEM/F12; Invitrogen, Paisley, UK) on ice. The lung tissue was then washed three times in DMEM/F12, and the central cartilaginous portion was removed. The remaining tissue was dissected and incubated with 0.1% collagenase, in

DMEM/F12, for 1 h at 37°C. The tissue was then passed through a 19-gauge needle to disperse cells and subsequently, through a 70 μm filter, to remove debris. The cells were centrifuged (200× g, 5 min, 21°C) and then re-suspended in DMEM/F12 containing 20% heat-inactivated foetal calf serum (HI FCS), penicillin (100 U·mL^{-1}), streptomycin (100 μg·mL^{-1}), endothelial cell growth supplement/heparin (1 μL·mL^{-1}; Promocell, Heidelberg, Germany) and endothelial cell growth supplement (50 μg·mL^{-1}). Cells were grown in a 0.1% gelatine-coated flask in a humidified incubator with 5% CO_2 in air at 37°C. The media were changed after 24 h and changed every 2/3 days until cells were 80–90% confluent; Dynabeads (4 × 10^6) coated in sheep anti-rat IgG (Invitrogen) were washed three times in PBS and then incubated overnight with 5 μg anti-intercellular adhesion molecule (ICAM)-2 (CD102, endothelial specific marker; BD Biosciences, Oxford, UK) on a rotator. The Dynabeads were then washed three times in PBS, media were aspirated and the Dynabeads were added to the isolated pulmonary vascular cells for 1 h in a humidified incubator at 37°C. Cells were then trypsinized and magnetically separated. Cells were washed and magnetically separated a second time and then plated on a 0.1% gelatin-coated flask. Cells displayed a typical cobblestone appearance (data not shown) and were used up to passage 4.

Due to the severely reduced fecundity and viability of NPR3 KO animals, the availability of PMEC from these mice was significantly restricted and prevented more extensive studies employing NPR3-deficient cells.

Assessment of cell proliferation by 5-bromo-2′-deoxyuridine (BrdU) incorporation

AoSMC were seeded into 96-well plates at a density of 25 x 10^3 cells·per well (rat) or 10 x 10^3 cells·per well (mouse) in triplicate, in DMEM containing penicillin (100 U·mL^{-1}), streptomycin (100 μg·mL^{-1}), L-glutamine (2 mM) and 10% FCS for 24 h in a humidified incubator with 5% CO_2 in air. HUVEC (Promocell; Heidelberg, Germany) were plated into 96-well plates at a density of 1000 cells·per well in triplicate, in EGM^{TM}-2 medium supplemented with a Bullet kit for 24 h in a humidified incubator with 5% CO_2 in air. PMEC were seeded at 500 cells·per well (96-well plate) for 24 h in growth medium (as above).

Cell proliferation was assessed by determining BrdU incorporation using a commercially available assay (Roche Diagnostics, East Sussex, UK). Proliferation was determined in an essentially identical manner for each cell type. Cells were exposed to media (DMEM for AoSMC and PMEC; EGM^{TM}-2 for HUVEC) containing 0.1% HI FCS (without supplementation for endothelial cells) for 24 h to stop growth. Cells were then returned to corresponding media containing 10% HI FCS (including supplements for endothelial cells) and treated with CNP (ranging from 1 pM–1 μM depending on the cell type) in the absence and presence of inhibitors or vehicle (where appropriate) for 24 h. BrdU was added during the S phase (20 h post treatment), and incorporation was measured at 24 h.

Parallel experiments were also conducted to confirm that BrdU incorporation matched cell number under representative conditions. Here, cells were counted using a haemocytometer following Trypan blue staining at 24 h.

Immunoblotting

RAoSMC and HUVEC were seeded in 10 cm culture dishes and incubated at 37°C in a humidified incubator containing 5% CO_2 in air until confluent. AoSMC were treated with CNP (1 μM) for 0, 5, 10, 15, 30, 60, 90 or 120 min, and HUVEC were treated with CNP (100 pM) for 0, 0.5, 1, 3, 6 or 24 h. In certain experiments, cells were pre-incubated with the $G_{i/o}$ G-protein inhibitor *Pertussis* toxin (100 ng·mL^{-1}; 16 h) or the selective NPR3 antagonist M372049 (Veale *et al.*, 2000; Villar *et al.*, 2007; 10 μM; 30 min). The medium was then removed, and cells were washed with PBS. Phospho-homogenization buffer [Tris (10 mM), pH 7.4, NaCl (50 mM), NaPPi (30 mM), EDTA (2 mM), NaF (50 mM), 1% Triton X-100, phenylmethylsulfonyl fluoride (1 mM), Na_3VO_4 (1 mM) and benzamidine, leupeptin, antipain and aprotinin; all (1 μg·mL^{-1})] was added. Cells were scraped, transferred to 1.5 mL tubes and centrifuged (13 793× g, 5 min, 4°C). Supernatants were retained and analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (as described previously; Connelly *et al.*, 2003) using the following primary antibodies: anti-p44/p42 and anti-phospho-p44/p42 [Thr^{202}/Tyr^{204}] (both 1:500; New England Biolabs, Hertfordshire, UK), anti-cyclin D1 (1:1000), anti-p27^{kip1} (1:1000; both New England Biolabs) and anti-p21$^{waf1/cip1}$ (1:500; BD Pharmingen, Oxford, UK).

RNA isolation and RT-PCR

Total RNA was extracted from RAoSMC and HUVEC (~5 × 10^6 cells) using the RNeasy mini kit (Qiagen, West Sussex, UK), and RT-PCR was carried out using a One-Step RT-PCR kit (Qiagen), as we have described previously (Connelly *et al.*, 2003). Reverse transcription was carried out at 50°C for 30 min followed by thermal cycling conditions as follows: 95°C for 15 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. PCR products were resolved on a 1% agarose gel.

The primer sequences (Sigma Genosys, Cambridgeshire, UK) used for RAoSMC and HUVEC are shown in Tables 1 and 2 respectively.

Measurement of cGMP

Cellular cGMP concentrations were measured using a commercially available assay (R&D Systems, Oxford, UK), as described previously (Baliga *et al.*, 2008).

Data analysis

All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Densitometric analyses were performed using AlphaEaseFC (AlphaInnotech, Cannock, UK). All data are plotted graphically as mean values, with vertical bars representing standard error of the mean (SEM). One-way ANOVA followed by Bonferroni's multiple comparison test was used to assess differences between individual experimental conditions.

Materials

All reagents were purchased from Sigma-Aldrich (Poole, UK), except for SB203580 and SP600125, which were obtained from Axxora, Nottingham, UK.

Table 1

Primer sequences and product sizes used for NPR mRNA determination in RAoSMC

Receptor		Primer sequence	Product size
NPR1	Sense	5′-CATCCTGGACAACCTGC-3′	714
	Antisense	5′-TAGGTCCGAACCTTGCC-3′	
NPR2	Sense	5′-CATGGCAGGACAATCGAACC-3′	720
	Antisense	5′-TGCCTGCACCTTTGTGATATCG-3′	
NPR3	Sense	5′-GGCTCAATGAGGAGGATTACGTG-3′	558
	Antisense	5′-AATCTTCCCGCAGCTCTCGATG-3′	

Table 2

Primer sequences and product sizes for NPR mRNA determination in HUVEC

Receptor		Primer sequence	Product size
NPR1	Sense	5′-CATCCTGGACAACCTGC-3′	714
	Antisense	5′-TAGGTCCGAACCTTGCC-3′	
NPR2	Sense	5′-GGCAGGACAATCGCACCC-3′	717
	Antisense	5′-TGCCTGCACCTTTGTGATAACG-3′	
NPR3	Sense	5′-GGCTCAATATGGAGGATTACGTT-3′	558
	Antisense	5′-AATCTTCCCGTAATTCCCGATG-3′	

Results

NPR-C underpins the pro- and anti-proliferative effects of CNP

CNP (1 nM–1 μM) caused a concentration-dependent inhibition of cell proliferation in RAoSMC, whereas in HUVEC, CNP (1 pM–1 nM) caused a concentration-dependent augmentation of cell growth (of equal magnitude to vascular endothelial growth factor (VEGF) 10 ng·mL⁻¹; data not shown; Figure 1). Representative studies also confirmed that BrdU incorporation was representative of cell number (data not shown). From these data, concentrations of CNP that elicited maximal effects in vascular smooth muscle and endothelial cells (1 μM and 100 pM, respectively) were chosen for further experimentation.

In RAoSMC, CNP (1 μM) significantly inhibited cell proliferation, an effect that was attenuated in the presence of the selective NPR3 antagonist M372049 (Veale *et al.*, 2000; Villar *et al.*, 2007) (10 μM; Figure 1). In WT mouse AoSMC, CNP (1 μM) produced a marked inhibition of growth; however, in AoSMC from NPR3 KO mice, the inhibitory effect of CNP was absent (Figure 2). The basal growth of AoSMC from NPR3 KO mice was significantly increased in comparison to WT mouse AoSMC (Figure 2).

In HUVEC, CNP (100 pM; Figure 1) and the selective NPR3 agonist cANF$^{4-23}$ (100 pM; data not shown) stimulated cell growth; the proliferative effect of both peptides was blocked by M372049 (10 μM). CNP (100 pM) increased proliferation in WT PMEC, whereas in PMEC from NPR3 KO mice, CNP (100 pM) had very little effect (Figure 2). Moreover, the basal growth of PMEC from NPR3 KO mice was lower in comparison with that of WT PMEC (Figure 2).

Identification of NPRs in AoSMC and HUVEC

To confirm the expression of NPR3, and other NPR subtypes, in vascular smooth muscle and endothelial cells, RT-PCR was carried out on total RNA isolated from RAoSMC and HUVEC. mRNA for all three natriuretic peptide receptors (NPR1, NPR2 and NPR3) was detected in both cell types (Figure 3).

Determination of cellular cGMP levels in response to CNP

In order to provide further evidence that activation of NPR2, and consequent increases in cellular cGMP concentrations do not underpin the effects of CNP on RAoSMC and HUVEC, cGMP levels were measured in both cell types.

Basal cGMP concentrations were 10-fold higher in RAoSMC compared with HUVEC (Figure 3). CNP (1 μM RAoSMC; 100 pM HUVEC) increased cGMP concentrations in RAoSMC and HUVEC approximately sevenfold and twofold respectively. In both cells types, the increase in cGMP produced by CNP was identical in the presence of M372049 (10 μM; Figure 3), a concentration of the NPR3 antagonist that significantly reversed the effects of CNP on cell growth (Figure 1).
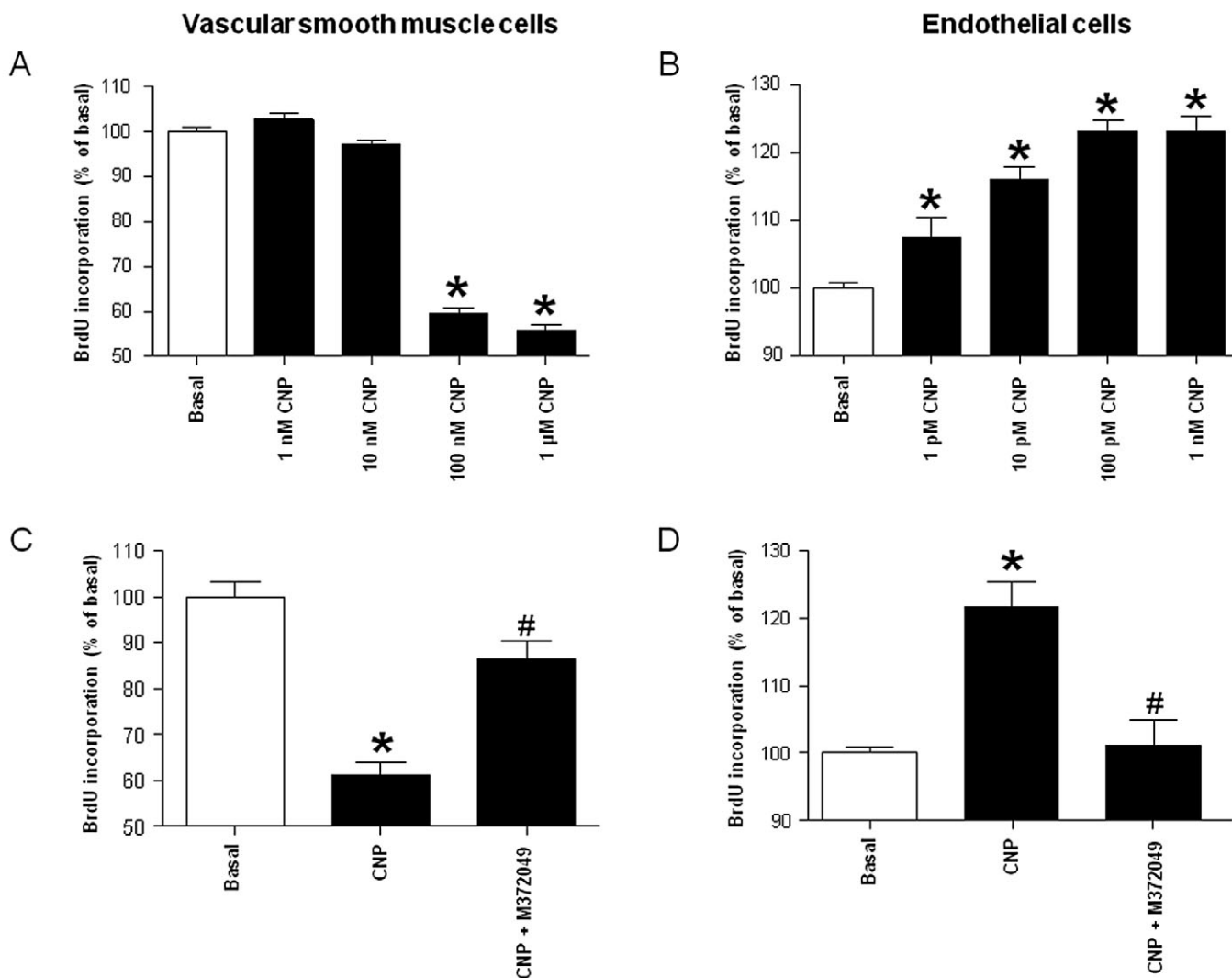
Figure 1

The mitogenic effects of CNP are mediated via NPR3. BrdU incorporation in (A) RAoSMC and (B) HUVEC treated with increasing concentrations of CNP (1 pM–1 µM; 24 h), (C) RAoSMC treated with CNP (1 µM; 24 h) in the absence or presence of M372049 (10 µM) and (D) HUVEC treated with CNP (100 pM; 24 h) in the absence or presence of M372049 (10 µM). Data shown are means $\pm$ SEM expressed as a percentage of basal growth. *$P < 0.05$ versus basal, #$P < 0.05$ versus CNP alone. $n \geq 12$ from ≥ 4 separate experiments.

Extracellular signal regulated kinase (ERK) 1/2 phosphorylation underlies the effects of CNP/NPR-C on cell growth

Neither the p38 inhibitor SB203580 (30 µM) nor the c-Jun N-terminal kinase (JNK) inhibitor SP600125 (3 µM) reversed the effects of CNP on RAoSMC or HUVEC proliferation (data not shown). However, the ERK 1/2 inhibitor PD98059 (30 µM) attenuated the ability of CNP to both increase HUVEC growth and reduce RAoSMC proliferation (Figure 4).

In RAoSMC, ERK 1/2 phosphorylation was transiently and significantly increased in the presence of CNP (1 µM), with a peak effect observed at 10 min after treatment (Figure 4); this effect was blocked in the presence of M372049 (10 µM; Figure 5). In HUVEC, CNP (100 pM) elicited a time-dependent increase in ERK 1/2 phosphorylation (comparable to that produced by VEGF, 10 ng·mL⁻¹; data not shown),

which reached a maximum at 30 min (Figure 4); again, this effect was blocked in the presence of M372049 (10 µM; Figure 5).

In addition, in both cell types, treatment with the $G_{i/o}$ inhibitor *Pertussis* toxin (100 ng·mL⁻¹), at a concentration that blocks the vasorelaxant effects of CNP in isolated resistance arteries (Chauhan *et al.*, 2003), abolished CNP-induced ERK 1/2 phosphorylation (Figure 5).

CNP/NPR-C signalling selectively alters the expression of cell cycle proteins in RAoSMC and HUVEC

RAoSMC treated with CNP (1 µM) exhibited a time-dependent increase in the cell cycle inhibitors p21[waf1/cip1] and p27[kip1] (Figure 6), but not the cell cycle promoter cyclin D1 (data not shown). In comparison, HUVEC treated with CNP
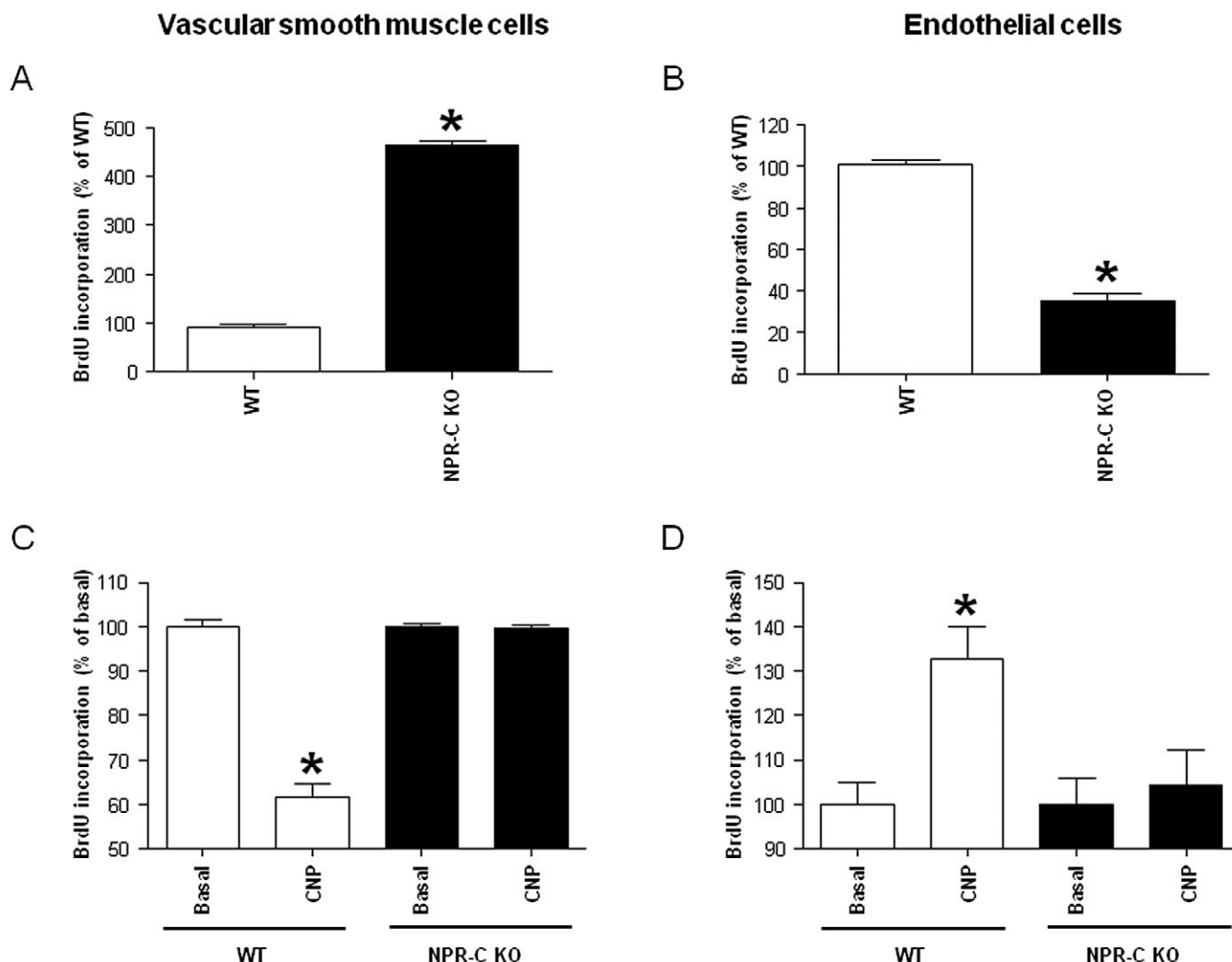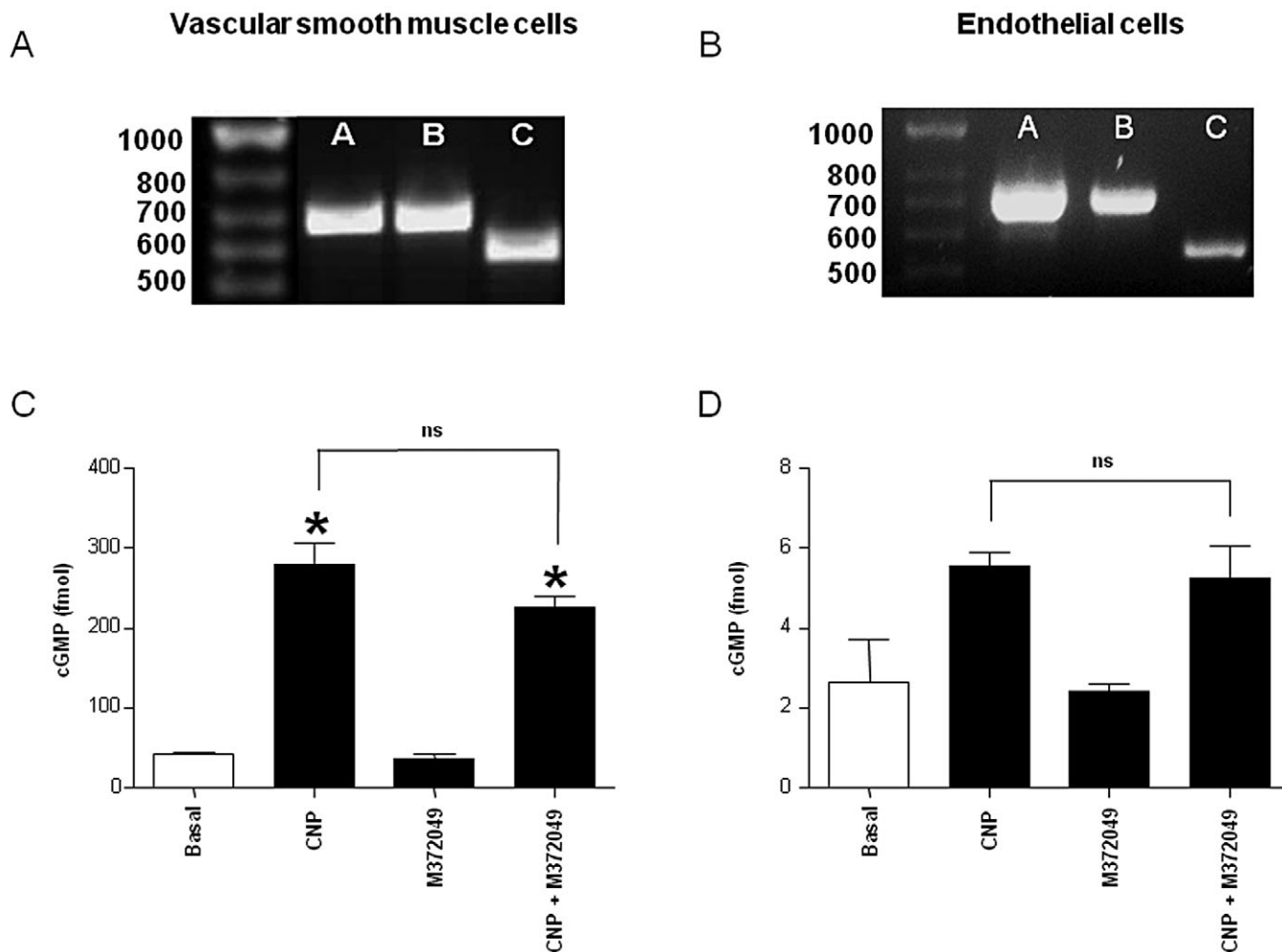
Figure 2

The mitogenic effects of CNP are absent in NPR3 KO cells. BrdU incorporation in WT and NPR3 KO mouse AoSMC and PMEC under basal conditions (A and B) or treated with CNP (1 µM AoSMC; 100 pM PMEC; 24 h; C and D). Data shown are means ± SEM expressed as a percentage of basal growth. *$P < 0.05$ versus basal or WT. $n \geq 12$ from ≥ 4 separate experiments.

(100 pM) exhibited a time-dependent increase in cyclin D1 (Figure 6), without a concomitant increase in either p21$^{waf1/cip1}$ (data not shown) or p27^{kip1} (data not shown). Pretreatment with PD98059 (30 µM) reduced the expression of p21$^{waf1/cip1}$ and p27^{kip1} in RAoSMC at 24 h and cyclin D1 in HUVEC at 6 h (Figure 7).

Discussion

Endothelium-derived CNP is thought to play a pivotal role in maintaining the patency and integrity of the blood vessel wall and opposing pro-atherogenic stimuli (Ahluwalia and Hobbs, 2005; Potter et al., 2009). A fundamental component of this function may be a differential effect on endothelial and vascular smooth muscle growth and such a dichotomous activity might be exploited pharmacologically to treat several

cardiovascular disorders. For example, in patients undergoing balloon-angioplasty/percutaneous coronary intervention (PCI), re-occlusion of the artery often occurs within 6 months (Fischman et al., 1994; Savage et al., 1998). Such patients are fitted with a bare metal or drug-eluting stent, which acts as a platform for new tissue growth. Current drug-eluting stents release anti-proliferative agents (e.g. sirolimus) that inhibit vascular smooth muscle proliferation, the predominant cause of restenosis. However, these drugs also inhibit endothelial cell proliferation (Matter et al., 2006), which exacerbates the thrombogenicity of the stent surface. The mitogenic effects of endothelium-derived CNP on vascular cells along with its anti-platelet activity (Scotland et al., 2005) therefore provide a promising target for the treatment of restenosis. The beneficial effects of CNP signalling could be equally applied to coronary artery bypass graft (CABG) surgery. In this setting, 13% of saphenous vein grafts occlude within the first month
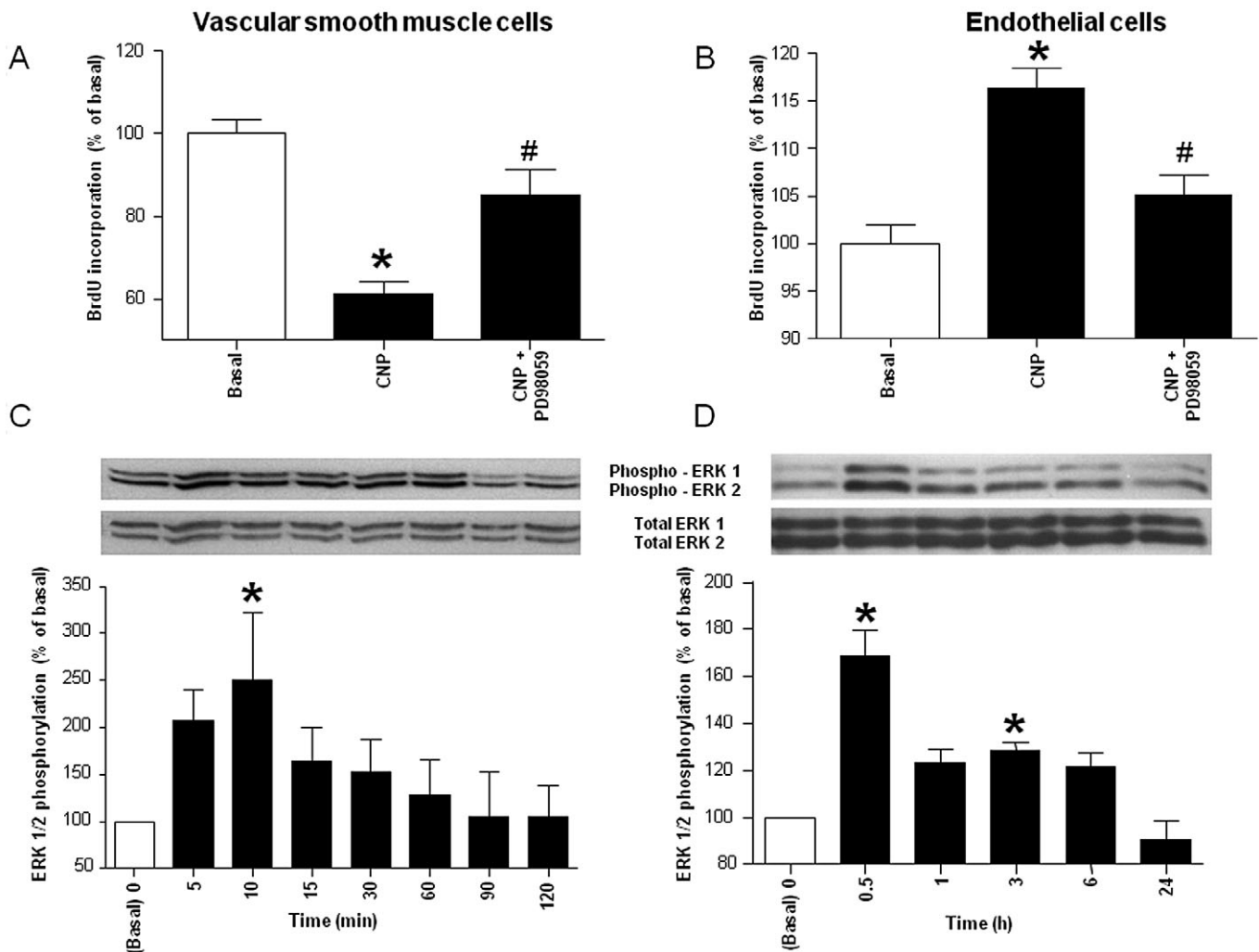
Figure 3

NPR expression and cGMP formation in RAoSMC and HUVEC. RT-PCR analysis of NPR1 (labelled A), NPR2 (B) and NPR3 (C) mRNA expression in (A) RAoSMC and (B) HUVEC (gels representative of three separate experiments). Formation of cGMP in response to CNP in RAoSMC (1 µM; C) and HUVEC (100 pM; D) in the absence and presence of M372049 (10 µM). *$P < 0.05$ versus basal. $n = 9$ observations from three separate experiments.

following surgery, principally due to thrombosis (Bourassa, 1994; Fitzgibbon *et al.*, 1996), and intimal hyperplasia severely affects a further 10% of grafts within one year (Bourassa, 1994; Fitzgibbon *et al.*, 1996). Here too, pharmacological manipulation to mimic CNP signalling may be of therapeutic benefit. Indeed, *in vivo* studies have shown CNP to be beneficial following balloon angioplasty and vein grafting (Doi *et al.*, 2001; Ohno *et al.*, 2002; Qian *et al.*, 2002; Pelisek *et al.*, 2006). Nonetheless, the pathways underpinning the opposing effects of CNP on endothelial and vascular smooth muscle cells remain unclear.

Here, we have uncovered a pivotal role for NPR3 in executing these disparate actions of CNP on proliferation of the endothelium and vascular smooth muscle. The pro-mitogenic effect of CNP in HUVEC was blocked by selective NPR3 antagonism with M372049, inhibition of NPR3-linked $G_{i/o}$ protein activity by *Pertussis* toxin and mimicked by the selective NPR3 agonist cANF[4–23], each confirming that NPR3 is the primary receptor involved. In contrast, CNP caused a

marked inhibition of RAoSMC growth that was blocked in the presence of the NPR3 antagonist and *Pertussis* toxin, again confirming the reliance on G_i-coupled NPR3 for these (anti-mitogenic) effects. This reliance on NPR3 was substantiated by measurement of cGMP formation in RAoSMC and HUVEC in response to CNP. In both cell types, CNP caused an identical increase in cGMP concentration in the absence and presence of M372049. As this NPR3 antagonist reversed the effects of CNP on cell growth, but did not alter cGMP generation, the clear deduction is that the underpinning mechanism is cGMP-independent. Finally, and perhaps most convincingly, the pro- and anti-proliferative effects of CNP were absent in endothelial cells and vascular smooth muscle cells isolated from NPR3 KO mice. Indeed, the basal growth in cells lacking NPR3 reflected loss of CNP-dependent signalling; that is, NPR3 KO vascular smooth muscle cells proliferated more quickly, whereas NPR3 KO endothelial cells exhibited a reduced mitogenesis. Therefore, whilst NPR2 and cyclic GMP-dependent inhibition of vascular smooth muscle

Figure 4

ERK 1/2 phosphorylation underlies the effects of CNP on cell growth. BrdU incorporation (A and B) and levels of total and phospho-ERK 1/2 (C and D) in RAoSMC (A and C) and HUVEC (B and D) treated with CNP (RAoSMC 1 μM; HUVEC 100 pM; 24 h) in the absence and presence of PD98059 (30 μM). Total ERK 1/2 and phospho-ERK 1/2 were analysed by Western blot, and bands were quantified by densitometry. Data shown are means ± SEM expressed as a percentage of basal growth or ERK 1/2 phosphorylation. *$P < 0.05$ versus basal, #$P < 0.05$ versus CNP alone. $n \geq 8$ from ≥ 6 separate experiments.

cell proliferation undoubtedly underlie some of the vasoprotective actions of CNP (Furuya *et al.*, 1991; 1993; Hutchinson *et al.*, 1997), and despite the fact that our data confirm that HUVEC and RAoSMC express both NPR2 and NPR3 (at least at the mRNA level), our present study suggests that it is NPR3, primarily, that underlies the dichotomous activity of CNP on endothelial and vascular smooth muscle cell mitogenesis.

It is intriguing to note that the disparate effects of CNP on cell growth occur at distinct concentrations. The ability of CNP to promote endothelial proliferation is maximal at <1 nM, whereas the inhibitory action of CNP on vascular smooth muscle mitogenesis is produced by concentrations of CNP > 10 nM. This concentration-dependent profile suggests that, under physiological conditions in which plasma concentrations of CNP are ~1 pg·mL^{-1}(~0.5 pM; Potter *et al.*, 2009), a positive feedback loop facilitating endothelial

renewal may be the predominant mitogenic effect of (endothelium-derived) CNP, whereas in cardiovascular disease, in which CNP levels are raised, i.e. in the presence of atherosclerotic lesions (Naruko *et al.*, 1996), the antimitogenic activity on vascular smooth muscle may become more overt. Clearly, further study in this regard is warranted.

We have also delineated the intracellular pathways that mediate the disparate proliferative effects of CNP/NPR-C signalling on endothelial and vascular smooth muscle cells. Exposure of vascular smooth muscle cells to CNP causes a rapid, transient increase in the phosphorylation of ERK 1/2, a key arm of the mitogen activated protein kinase cascade that is well established to regulate cell growth (Zondag *et al.*, 1998; Hallak *et al.*, 2000; Pearson *et al.*, 2001). Moreover, both NPR3 antagonism and *Pertussis* toxin attenuate CNP-induced enhancement in ERK 1/2 phosphorylation, suggesting that

## Vascular smooth muscle cells

Endothelial cells
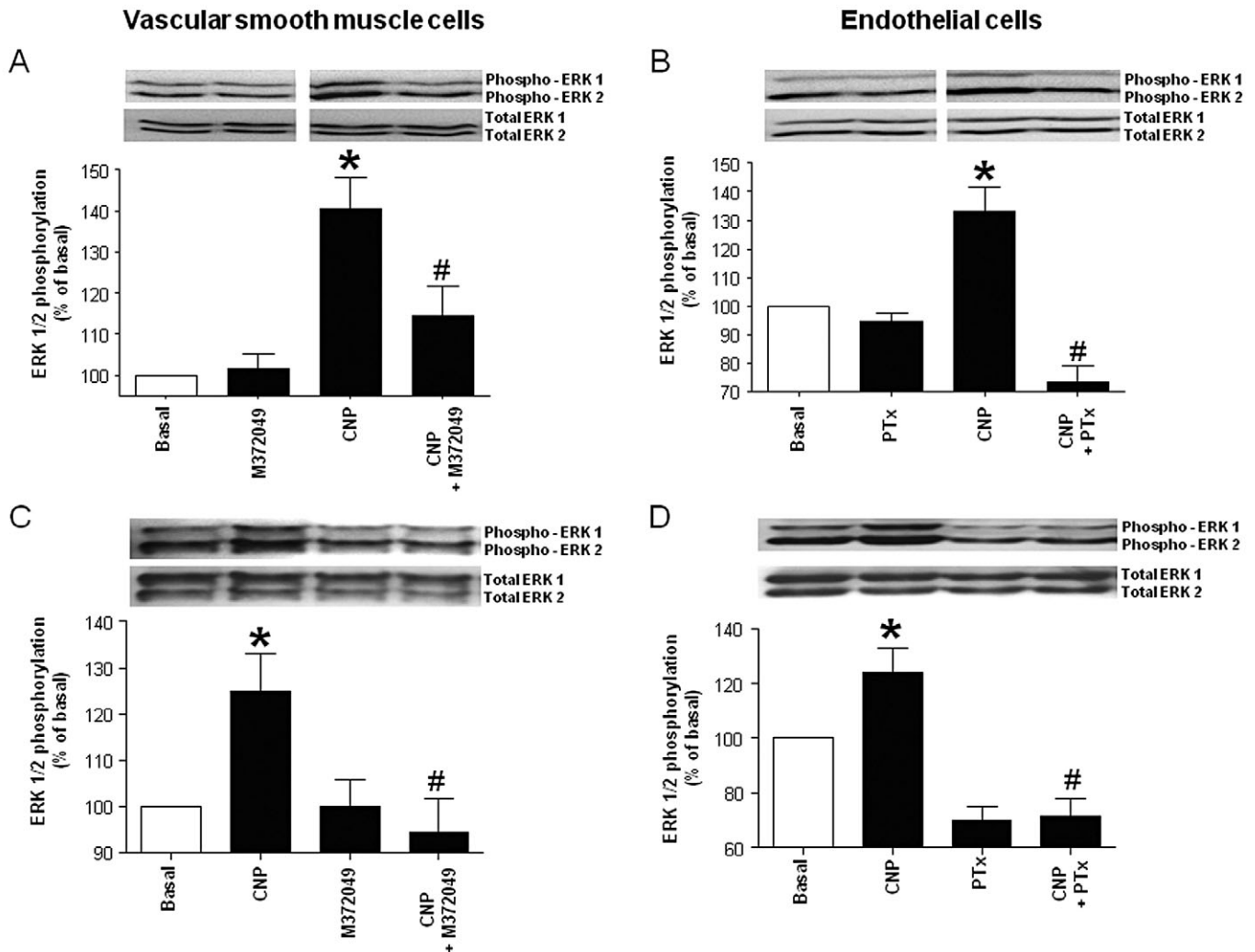
Figure 5

The NPR3 antagonist M372049 and the G_i-protein inhibitor *Pertussis* toxin attenuate CNP-induced ERK 1/2 phosphorylation. Levels of total ERK 1/2 and phospho-ERK 1/2 in (A and C) RAoSMC and (B and D) HUVEC treated with CNP (RAoSMC 1 μM, 10 min; HUVEC 100 pM, 30 min) in the absence and presence of M372049 (10 μM) or *Pertussis* toxin (PTx; 100 ng·mL^{-1}). Total ERK 1/2 and phospho-ERK 1/2 were analysed by Western blot, and bands were quantified by densitometry. Data shown are means ± SEM expressed as a percentage of basal ERK 1/2 phosphorylation. *$P < 0.05$ versus basal; #$P < 0.05$ versus CNP alone. $n = 3–4$.

this CNP-triggered activation of the pathway is also NPR3-dependent. This dovetails well with the thesis that $G_{i/o}$-coupled NPR3 acts in a similar fashion to analogous, single transmembrane domain, G-protein-linked, growth factor receptors (Zondag *et al.*, 1998; Hallak *et al.*, 2000).

To gain mechanistic insight into the cell cycle proteins modified by CNP/NPR3 in RAoSMC and HUVEC to bring about anti- and pro- proliferative effects, respectively, the relative activity of cell cycle proteins was evaluated in each cell. These data revealed that ultimately, ERK 1/2 activation increases expression of the cell cycle inhibitors p21$^{waf1/cip1}$ and p27^{kip1}, as demonstrated by the ability of PD98059 to block the inhibition of vascular smooth muscle growth and activation of p21$^{waf1/cip1}$ and p27^{kip1}.

Classically, phosphorylation and activation of ERK 1/2 is associated with a positive effect on cellular proliferation, as is

observed in vascular smooth muscle cells in response to several mitogens including endothelin-1 and angiotensin II (Watanabe *et al.*, 1996; Yoshizumi *et al.*, 1998). However, there are well-documented exceptions to this model. For example, small GTPases, such as *ras* and *raf*, regulate ERK 1/2 activity to integrate extracellular signals with cell growth, such that activation of ERK1/2 can result in both cell cycle activation and cell cycle arrest (Olson *et al.*, 1998). In accord with this finding, activation of ERK 1/2 leads to stimulation of p21$^{waf1/cip1}$ and cessation of the cell cycle in a number of cell types, including vascular smooth muscle (Pumiglia and Decker, 1997; Olson *et al.*, 1998; Ray *et al.*, 1999). This mechanism appears particularly pertinent to the anti-mitogenic effects of NO (Ishida *et al.*, 1999; Kibbe *et al.*, 2000; Sato *et al.*, 2000; Bauer *et al.*, 2001), sodium salicylate (Marra *et al.*, 2000) and relaxin (Mookerjee *et al.*, 2009), all of which elicit ERK
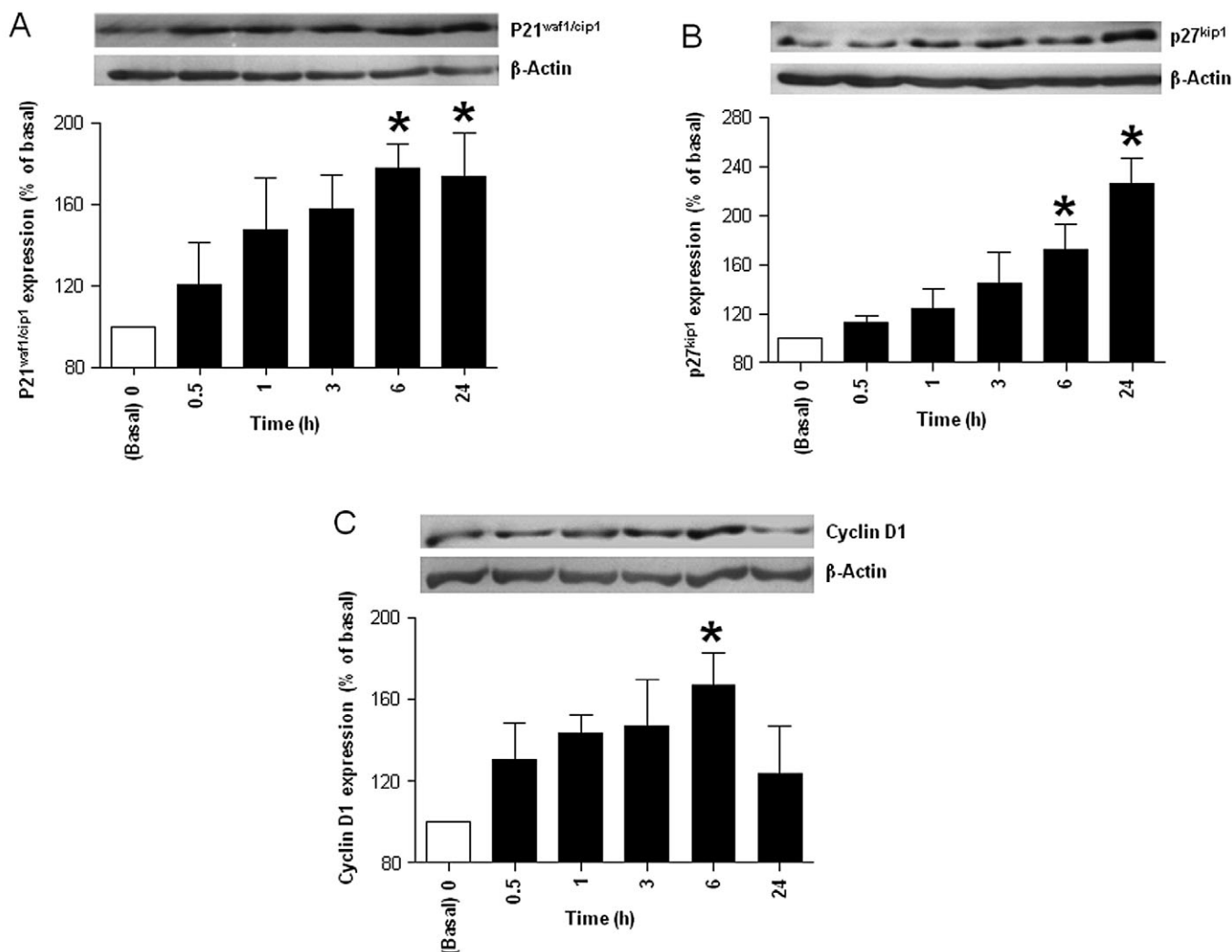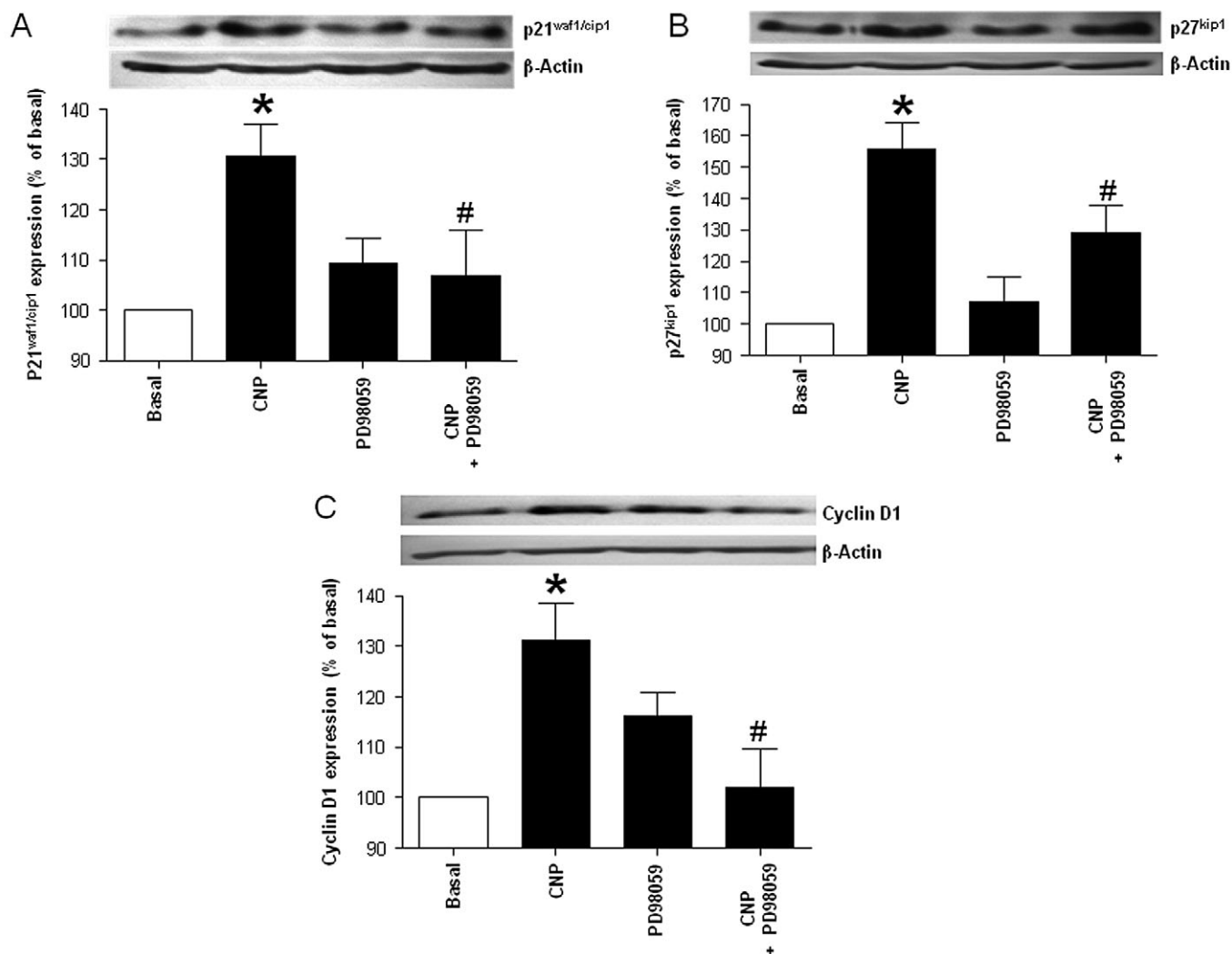
Figure 6

CNP disparately regulates cell cycle protein expression in RAoSMC and HUVEC. Expression of (A) p21[waf1/cip1] and (B) p27[kip1] in RAoSMC treated with CNP (1 μM; 24 h) and (C) cyclin D1 in HUVEC treated with CNP (100 pM; 24 h). p21[waf1/cip1], p27[kip1] and cyclin D1 were analysed by Western blot, and bands were quantified by densitometry. Data shown are means ± SEM expressed as a percentage of basal cell cycle protein expression. *$P < 0.05$ versus basal. $n = 3–4$.

1/2 -dependent up-regulation of p21[waf1/cip1] and p27[kip1] to inhibit mitogenesis. It is likely that a similar mechanism is triggered by CNP.

In HUVEC, however, NPR3-dependent ERK 1/2 phosphorylation resulted in an increase in the expression of cyclin D1, a protein which complexes with cyclin dependent kinase 4/6 to promote progression through the G1 phase of the cell cycle (Serrano *et al.*, 1993). This observation is compatible with previous studies in endothelial cells, which show that the archetypal, endothelial pro-mitogenic mediators VEGF, fibroblast growth factor-2 and angiopoietin-1 all trigger up-regulation of cyclin D1 to promote proliferation (Pedram *et al.*, 1998; Kanda *et al.* 2005; Fu *et al.*, 2007). Moreover, VEGF has been shown to increase cyclin D1 expression, inhibited by PD98059, thereby implicating ERK 1/2 as a regulator of cyclin D1 expression, as we observed here with CNP (Pedram *et al.*, 1998; Fu *et al.*, 2007). Again, it is clear that ERK

1/2 phosphorylation is acrtical component of the change in cell cycle proteins brought about by CNP/NPR-C, as such changes were absent in the presence of the selective ERK 1/2 inhibitor PD98059.

In summary, we have shown that CNP activation of NPR3 enhanced ERK 1/2 phosphorylation and results in endothelial cell proliferation and, conversely, vascular smooth muscle cell growth inhibition. These data strengthen the evidence supporting NPR3 signal transduction as a principal mechanism conveying the anti-atherogenic actions of this peptide. Together with previous work (Ohno *et al.*, 2002; Qian *et al.*, 2002; Chauhan *et al.*, 2003; Hobbs *et al.*, 2004; Scotland *et al.*, 2005), it is clear that CNP has an important role to play maintaining vascular homeostasis at a local level. The NPR3-dependent pro- and anti-mitogenic properties highlighted in this study endorse CNP, and more specifically NPR3 agonists, as a feasible therapeutic option for the treatment of

Figure 7

The ERK 1/2 inhibitor PD98059 attenuates the effects of CNP on altered cell cycle protein expression in RAoSMC and HUVEC. Expression of (A) p21$^{waf1/cip1}$ and (B) p27^{kip1} in RAoSMC treated with CNP (1 μM; 24 h) in the absence and presence of PD98059 (30 μM) and (C) cyclin D1 in HUVEC treated with CNP (100 pM; 6 h) in the absence and presence of PD98059 (30 μM). p21$^{waf1/cip1}$, p27^{kip1} and cyclin D1 were analysed by Western blot, and bands were quantified by densitometry. Data shown are means ± SEM expressed as a percentage of basal cell cycle protein expression. *$P < 0.05$ versus basal, #$P < 0.05$ versus CNP alone. $n = 3$–5.

atherosclerosis, restenosis and other inflammatory vascular disorders.

Acknowledgements

Conflict of interest

None.

References

Ahluwalia A, Hobbs AJ (2005). Endothelium-derived C-type natriuretic peptide: more than just a hyperpolarizing factor. Trends Pharmacol Sci 26: 162–167.

Ahluwalia A, MacAllister RJ, Hobbs AJ (2004). Vascular actions of natriuretic peptides. Cyclic GMP-dependent and -independent mechanisms. Basic Res Cardiol 99: 83–89.

Alexander SPH, Mathie A, Peters JA (2009). Guide to Receptors and Channels (GRAC), 4th edn. Br J Pharmacol 158 (Suppl. 1): S1–S254.

Anand-Srivastava MB, Sehl PD, Lowe DG (1996). Cytoplasmic domain of natriuretic peptide receptor-C inhibits adenylyl cyclase. Involvement of a pertussis toxin-sensitive G protein. J Biol Chem 271: 19324–19329.

Baliga RS, Zhao L, Madhani M, Lopez-Torondel B, Visintin C, Selwood D *et al.* (2008). Synergy between natriuretic peptides and phosphodiesterase 5 inhibitors ameliorates pulmonary arterial hypertension. Am J Respir Crit Care Med 178: 861–869.

Bauer PM, Buga GM, Ignarro LJ (2001). Role of p42/p44 mitogen-activated-protein kinase and p21waf1/cip1 in the regulation of vascular smooth muscle cell proliferation by nitric oxide. Proc Natl Acad Sci USA 98: 12802–12807.

Bourassa MG (1994). Long-term vein graft patency. Curr Opin Cardiol 9: 685–691.

Cahill PA, Hassid A (1994). ANF-C-receptor-mediated inhibition of aortic smooth muscle cell proliferation and thymidine kinase activity. Am J Physiol 266: R194–R203.

Chamley-Campbell J, Campbell GR, Ross R (1979). The smooth muscle cell in culture. Physiol Rev 59: 1–61.

Chauhan SD, Nilsson H, Ahluwalia A, Hobbs AJ (2003). Release of C-type natriuretic peptide accounts for the biological activity of endothelium-derived hyperpolarizing factor. Proc Natl Acad Sci USA 100: 1426–1431.

Chen HH, Burnett JC, Jr (1998). C-type natriuretic peptide: the endothelial component of the natriuretic peptide system. J Cardiovasc Pharmacol 32 (Suppl. 3): S22–S28.

Connelly L, Jacobs AT, Palacios-Callender M, Moncada S, Hobbs AJ (2003). Macrophage endothelial nitric-oxide synthase autoregulates cellular activation and pro-inflammatory protein expression. J Biol Chem 278: 26480–26487.

Doi K, Ikeda T, Itoh H, Ueyama K, Hosoda K, Ogawa Y *et al.* (2001). C-type natriuretic peptide induces redifferentiation of vascular smooth muscle cells with accelerated reendothelialization. Arterioscler Thromb Vasc Biol 21: 930–936.

Fischman DL, Leon MB, Baim DS, Schatz RA, Savage MP, Penn I *et al.* (1994). A randomized comparison of coronary-stent placement and balloon angioplasty in the treatment of coronary artery disease. Stent Restenosis Study Investigators. N Engl J Med 331: 496–501.

Fitzgibbon GM, Kafka HP, Leach AJ, Keon WJ, Hooper GD, Burton JR (1996). Coronary bypass graft fate and patient outcome: angiographic follow-up of 5,065 grafts related to survival and reoperation in 1,388 patients during 25 years. J Am Coll Cardiol 28: 616–626.

Fu XD, Cui YH, Lin GP, Wang TH (2007). Non-genomic effects of 17beta-estradiol in activation of the ERK1/ERK2 pathway induces cell proliferation through upregulation of cyclin D1 expression in bovine artery endothelial cells. Gynecol Endocrinol 23: 131–137.

Furuya M, Yoshida M, Hayashi Y, Ohnuma N, Minamino N, Kangawa K *et al.* (1991). C-type natriuretic peptide is a growth inhibitor of rat vascular smooth muscle cells. Biochem Biophys Res Commun 177: 927–931.

Furuya M, Aisaka K, Miyazaki T, Honbou N, Kawashima K, Ohno T *et al.* (1993). C-type natriuretic peptide inhibits intimal thickening after vascular injury. Biochem Biophys Res Commun 193: 248–253.

Gether U (2000). Uncovering Molecular Mechanisms Involved in Activation of G Protein-Coupled Receptors. Endocr Rev 21: 90–113.

Gudermann T, Nurnberg B, Schultz G (1995). Receptors and G proteins as primary components of transmembrane signal transduction. Part 1. G-protein-coupled receptors: structure and function. J Mol Med 73: 51–63.

Hallak H, Seiler AE, Green JS, Ross BN, Rubin R (2000). Association of heterotrimeric G(i) with the insulin-like growth factor-I receptor. Release of G(betagamma) subunits upon receptor activation. J Biol Chem 275: 2255–2258.

Hartwell DW, Mayadas TN, Berger G, Frenette PS, Rayburn H, Hynes RO *et al.* (1998). Role of P-selectin cytoplasmic domain in granular targeting in vivo and in early inflammatory responses. J Cell Biol 143: 1129–1141.

He X, Chow D, Martick MM, Garcia KC (2001). Allosteric activation of a spring-loaded natriuretic peptide receptor dimer by hormone. Science 293: 1657–1662.

Hobbs A, Foster P, Prescott C, Scotland R, Ahluwalia A (2004). Natriuretic peptide receptor-C regulates coronary blood flow and prevents myocardial ischemia/reperfusion injury: novel cardioprotective role for endothelium-derived C-type natriuretic peptide. Circulation 110: 1231–1235.

Hutchinson HG, Trindade PT, Cunanan DB, Wu CF, Pratt RE (1997). Mechanisms of natriuretic-peptide-induced growth inhibition of vascular smooth muscle cells. Cardiovasc Res 35: 158–167.

Ishida A, Sasaguri T, Miwa Y, Kosaka C, Taba Y, Abumiya T (1999). Tumor suppressor p53 but not cGMP mediates NO-induced expression of p21(Waf1/Cip1/Sdi1) in vascular smooth muscle cells. Mol Pharmacol 56: 938–946.

Itoh T, Nagaya N, Murakami S, Fujii T, Iwase T, Ishibashi-Ueda H *et al.* (2004). C-type natriuretic peptide ameliorates monocrotaline-induced pulmonary hypertension in rats. Am J Respir Crit Care Med 170: 1204–1211.

Kanda S, Miyata Y, Mochizuki Y, Matsuyama T, Kanetake H (2005). Angiopoietin 1 is mitogenic for cultured endothelial cells. Cancer Res 65: 6820–6827.

Kibbe MR, Li J, Nie S, Watkins SC, Lizonova A, Kovesdi I *et al.* (2000). Inducible nitric oxide synthase (iNOS) expression upregulates p21 and inhibits vascular smooth muscle cell proliferation through p42/44 mitogen-activated protein kinase activation and independent of p53 and cyclic guanosine monophosphate. J Vasc Surg 31: 1214–1228.

Kuhlencordt PJ, Rosel E, Gerszten RE, Morales-Ruiz M, Dombkowski D, Atkinson WJ *et al.* (2004). Role of endothelial nitric oxide synthase in endothelial activation: insights from eNOS knockout endothelial cells. Am J Physiol Cell Physiol 286: C1195–C1202.

Kuhn M, Volker K, Schwarz K, Carbajo-Lozoya J, Flogel U, Jacoby C *et al.* (2009). The natriuretic peptide/guanylyl cyclase–a system functions as a stress-responsive regulator of angiogenesis in mice. J Clin Invest 119: 2019–2030.

Kuhnl A, Pelisek J, Tian W, Kuhlmann M, Rolland PH, Mekkaoui C *et al.* (2005). C-type natriuretic peptide inhibits constrictive remodeling without compromising re-endothelialization in balloon-dilated renal arteries. J Endovasc Ther 12: 171–182.

Marra DE, Simoncini T, Liao JK (2000). Inhibition of vascular smooth muscle cell proliferation by sodium salicylate mediated by upregulation of p21(Waf1) and p27(Kip1). Circulation 102: 2124–2130.

Matter CM, Rozenberg I, Jaschko A, Greutert H, Kurz DJ, Wnendt S *et al.* (2006). Effects of tacrolimus or sirolimus on proliferation of vascular smooth muscle and endothelial cells. J Cardiovasc Pharmacol 48: 286–292.

Mookerjee I, Hewitson TD, Halls ML, Summers RJ, Mathai ML, Bathgate RA *et al.* (2009). Relaxin inhibits renal myofibroblast differentiation via RXFP1, the nitric oxide pathway, and Smad2. FASEB J 23: 1219–1229.

Morishige K, Shimokawa H, Yamawaki T, Miyata K, Eto Y, Kandabashi T et al. (2000). Local adenovirus-mediated transfer of C-type natriuretic peptide suppresses vascular remodeling in porcine coronary arteries in vivo. J Am Coll Cardiol 35: 1040–1047.

Murthy KS, Makhlouf GM (1999). Identification of the G protein-activating domain of the natriuretic peptide clearance receptor (NPR-C). J Biol Chem 274: 17587–17592.

Murthy KS, Teng BQ, Zhou H, Jin JG, Grider JR, Makhlouf GM (2000). G(i-1)/G(i-2)-dependent signaling by single-transmembrane natriuretic peptide clearance receptor. Am J Physiol Gastrointest Liver Physiol 278: G974–G980.

Naruko T, Ueda M, van der Wal AC, van der Loos CM, Itoh H, Nakao K et al. (1996). C-type natriuretic peptide in human coronary atherosclerotic lesions. Circulation 94: 3103–3108.

Naruko T, Itoh A, Haze K, Ehara S, Fukushima H, Sugama Y et al. (2005). C-Type natriuretic peptide and natriuretic peptide receptors are expressed by smooth muscle cells in the neointima after percutaneous coronary intervention. Atherosclerosis 181: 241–250.

Nurnberg B, Gudermann T, Schultz G (1995). Receptors and G proteins as primary components of transmembrane signal transduction. Part 2. G proteins: structure and function. J Mol Med 73: 123–132.

Ohno N, Itoh H, Ikeda T, Ueyama K, Yamahara K, Doi K et al. (2002). Accelerated reendothelialization with suppressed thrombogenic property and neointimal hyperplasia of rabbit jugular vein grafts by adenovirus-mediated gene transfer of C-type natriuretic peptide. Circulation 105: 1623–1626.

Olson MF, Paterson HF, Marshall CJ (1998). Signals from Ras and Rho GTPases interact to regulate expression of p21Waf1/Cip1. Nature 394: 295–299.

Pagano M, Anand-Srivastava MB (2001). Cytoplasmic domain of natriuretic peptide receptor C constitutes Gi activator sequences that inhibit adenylyl cyclase activity. J Biol Chem 276: 22064–22070.

Pearson G, Robinson F, Beers GT, Xu BE, Karandikar M, Berman K et al. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 22: 153–183.

Pedram A, Razandi M, Levin ER (1998). Extracellular signal-regulated protein kinase/Jun kinase cross-talk underlies vascular endothelial cell growth factor-induced endothelial cell proliferation. J Biol Chem 273: 26722–26728.

Pelisek J, Fuchs AT, Kuehnl A, Tian W, Kuhlmann MT, Rolland PH et al. (2006). C-type natriuretic peptide for reduction of restenosis: gene transfer is superior over single peptide administration. J Gene Med 8: 835–844.

Potter LR, Yoder AR, Flora DR, Antos LK, Dickey DM (2009). Natriuretic peptides: their structures, receptors, physiologic functions and therapeutic applications. Handb Exp Pharmacol 191: 341–366.

Pumiglia KM, Decker SJ (1997). Cell cycle arrest mediated by the MEK/mitogen-activated protein kinase pathway. Proc Natl Acad Sci USA 94: 448–452.

Qian JY, Haruno A, Asada Y, Nishida T, Saito Y, Matsuda T et al. (2002). Local expression of C-type natriuretic peptide suppresses inflammation, eliminates shear stress-induced thrombosis, and prevents neointima formation through enhanced nitric oxide production in rabbit injured carotid arteries. Circ Res 91: 1063–1069.

Ray RM, Zimmerman BJ, McCormack SA, Patel TB, Johnson LR (1999). Polyamine depletion arrests cell cycle and induces inhibitors p21(Waf1/Cip1), p27(Kip1), and p53 in IEC-6 cells. Am J Physiol 276: C684–C691.

Rose RA, Lomax AE, Kondo CS, Anand-Srivastava MB, Giles WR (2004). Effects of C-type natriuretic peptide on ionic currents in mouse sinoatrial node: a role for the NPR-C receptor. Am J Physiol Heart Circ Physiol 286: H1970–H1977.

Sato J, Nair K, Hiddinga J, Eberhardt NL, Fitzpatrick LA, Katusic ZS et al. (2000). eNOS gene transfer to vascular smooth muscle cells inhibits cell proliferation via upregulation of p27 and p21 and not apoptosis. Cardiovasc Res 47: 697–706.

Savage MP, Fischman DL, Rake R, Leon MB, Schatz RA, Penn I et al. (1998). Efficacy of coronary stenting versus balloon angioplasty in small coronary arteries. Stent Restenosis Study (STRESS) Investigators. J Am Coll Cardiol 31: 307–311.

Scotland RS, Cohen M, Foster P, Lovell M, Mathur A, Ahluwalia A et al. (2005). C-type natriuretic peptide inhibits leukocyte recruitment and platelet-leukocyte interactions via suppression of P-selectin expression. Proc Natl Acad Sci USA 102: 14452–14457.

Serrano M, Hannon GJ, Beach D (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature 366: 704–707.

Soeki T, Kishimoto I, Okumura H, Tokudome T, Horio T, Mori K et al. (2005). C-type natriuretic peptide, a novel antifibrotic and antihypertrophic agent, prevents cardiac remodeling after myocardial infarction. J Am Coll Cardiol 45: 608–616.

Suga S, Nakao K, Itoh H, Komatsu Y, Ogawa Y, Hama N et al. (1992). Endothelial production of C-type natriuretic peptide and its marked augmentation by transforming growth factor-beta. Possible existence of 'vascular natriuretic peptide system'. J Clin Invest 90: 1145–1149.

Tokudome T, Horio T, Soeki T, Mori K, Kishimoto I, Suga S et al. (2004). Inhibitory effect of C-type natriuretic peptide (CNP) on cultured cardiac myocyte hypertrophy: interference between CNP and endothelin-1 signaling pathways. Endocrinology 145: 2131–2140.

Ueno H, Haruno A, Morisaki N, Furuya M, Kangawa K, Takeshita A et al. (1997). Local expression of C-type natriuretic peptide markedly suppresses neointimal formation in rat injured arteries through an autocrine/paracrine loop. Circulation 96: 2272–2279.

Veale CA, Alford VC, Aharony D, Banville DL, Bialecki RA, Brown FJ et al. (2000). The discovery of non-basic atrial natriuretic peptide clearance receptor antagonists. Part 1. Bioorg Med Chem Lett 10: 1949–1952.

Villar IC, Panayiotou CM, Sheraz A, Madhani M, Scotland RS, Nobles M et al. (2007). Definitive role for natriuretic peptide receptor-C in mediating the vasorelaxant activity of C-type natriuretic peptide and endothelium-derived hyperpolarising factor. Cardiovasc Res 74: 515–525.

Wang Y, de Waard MC, Sterner-Kock A, Stepan H, Schultheiss HP, Duncker DJ et al. (2007). Cardiomyocyte-restricted over-expression of C-type natriuretic peptide prevents cardiac hypertrophy induced by myocardial infarction in mice. Eur J Heart Fail 9: 548–557.

Watanabe G, Lee RJ, Albanese C, Rainey WE, Batlle D, Pestell RG (1996). Angiotensin II activation of cyclin D1-dependent kinase activity. J Biol Chem 271: 22570–22577.

Yamahara K, Itoh H, Chun TH, Ogawa Y, Yamashita J, Sawada N *et al.* (2003). Significance and therapeutic potential of the natriuretic peptides/cGMP/cGMP-dependent protein kinase pathway in vascular regeneration. Proc Natl Acad Sci USA 100: 3404–3409.

Yoshizumi M, Kim S, Kagami S, Hamaguchi A, Tsuchiya K, Houchi H *et al.* (1998). Effect of endothelin-1 (1–31) on extracellular signal-regulated kinase and proliferation of human coronary artery smooth muscle cells. Br J Pharmacol 125: 1019–1027.

Zondag GC, Postma FR, Etten IV, Verlaan I, Moolenaar WH (1998). Sphingosine 1-phosphate signalling through the G-protein-coupled receptor Edg-1. Biochem J 330 (Pt 2): 605–609.