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-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 p21waf1/cip1 and p27kip1.

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 (Ahlawalia 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, adenosiviral 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 (Ahlawalia 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 37 amino acid intracellular tail that contains a Gi/o-coupling domain (Anand-Srivastava et al., 1996; Murthy and Makhlof, 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-β1 (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 Gi/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/Gi/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 Gi/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 µg·mL⁻¹; Promocell, Heidelberg, Germany) and endothelial cell growth supplement (50 µg·mL⁻¹). Cells were grown in a 0.1% gelatin-coated flask in a humidified incubator with 5% CO₂ 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 × 10⁶) 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 10⁴ cells·per well (rat) or 10 x 10⁴ 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% CO₂ in air. HUVEC (Promocell; Heidelberg, Germany) were plated into 96-well plates at a density of 1000 cells·per well in triplicate, in EGM™-2 medium supplemented with a Bullet kit for 24 h in a humidified incubator with 5% CO₂ 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™-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**

AoSMC and HUVEC were seeded in 10 cm culture dishes and incubated at 37°C in a humidified incubator containing 5% CO₂ 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 Gᵢₒ 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, phenylmethysulfonyl fluoride (1 mM), Na₃VO₄ (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 [Thr202/Tyr204] (both 1:500; New England Biolabs, Hertfordshire, UK), anti-cyclin D1 (1:1000), anti-p27kip1 (1:1000; both New England Biolabs) and anti-p21waf1/cip1 (1:500; BD Pharmingen, Oxford, UK).

**RNA isolation and RT-PCR**

Total RNA was extracted from RAoSMC and HUVEC (~5 x 10⁶ 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.
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 cANF4–23 (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 cell 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).
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 Gαo 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 p21waf1/cip1 and p27kip1 (Figure 6), but not the cell cycle promoter cyclin D1 (data not shown). In comparison, HUVEC treated with CNP...
(100 pM) exhibited a time-dependent increase in cyclin D1 (Figure 6), without a concomitant increase in either p21waf1/cip1 (data not shown) or p27kip1 (data not shown). Pretreatment with PD98059 (30 μM) reduced the expression of p21waf1/cip1 and p27kip1 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.
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 signaling; 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 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.

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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 anti-mitogenic 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 signaling 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

![Figure 4](image-url)

**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 ≥ 8 from ≥6 separate experiments.
this CNP-triggered activation of the pathway is also NPR3-dependent. This dovetails well with the thesis that G- 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 p21waf1/cip1 and p27kip1, as demonstrated by the ability of PD98059 to block the inhibition of vascular smooth muscle growth and activation of p21waf1/cip1 and p27kip1.

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 p21waf1/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 5**
The NPR3 antagonist M372049 and the G-protein inhibitor Perussis 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 Perussis 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.
1/2-dependent up-regulation of p21\textsuperscript{waf1/cip1} and p27\textsuperscript{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 a critical 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...
atherosclerosis, restenosis and other inflammatory vascular disorders.

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**Conflict of interest**

None.

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