Biphasic effect of p21<sup>Cip1</sup> on smooth muscle cell proliferation: Role of PI 3-kinase and Skp2-mediated degradation

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Received 3 May 2005; received in revised form 2 August 2005; accepted 26 August 2005
Available online 5 October 2005
Time for primary review 24 days

Abstract

Objective: Proliferation of vascular smooth muscle cells (VSMC) is an important event in atherogenesis, in-stent restenosis and late vein-graft failure. Cell-cycle progression is positively regulated by cyclin:cdk complexes and negatively regulated by cyclin-dependent kinase inhibitors, including p21<sup>Cip1</sup>. Here we investigate the mechanisms regulating p21<sup>Cip1</sup> levels in VSMCs and its role in controlling VSMC proliferation.

Methods and results: We studied the S-phase-associated kinase protein-2 (Skp2), an F-box protein implicated in the ubiquitination of p21<sup>Cip1</sup>. Overexpression of wild-type Skp2 or dominant-negative Skp2 decreased or increased p21<sup>Cip1</sup> levels, respectively. Interestingly, levels of endogenous p21<sup>Cip1</sup> and Skp2 were both increased in a phosphoinositide PI 3-kinase-dependent manner in late G<sub>1</sub> phase. Increased expression of p21<sup>Cip1</sup> occurred despite significantly increased Skp2-mediated proteasomal degradation. To determine the role of p21<sup>Cip1</sup> in regulating VSMC proliferation, we used adenovirus-mediated overexpression and small-interfering RNA to elevate or silence p21<sup>Cip1</sup> expression, respectively. Overexpression of p21<sup>Cip1</sup> significantly inhibited VSCM proliferation. p21<sup>Cip1</sup> silencing also inhibited proliferation and increased apoptotic cell death.

Conclusions: Taken together, this data demonstrates that a balance between PI 3-kinase-driven upregulation and Skp2-mediated degradation controls the level of p21<sup>Cip1</sup>, which regulates VSMC proliferation in a biphasic manner. Low levels of p21<sup>Cip1</sup> are also essential to counter apoptosis during cell-cycle progression.

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Keywords: Smooth muscle cell; Proliferation; p21<sup>Cip1</sup>; Skp2; PI 3-kinase

1. Introduction

Proliferation of vascular smooth muscle cells (VSMC) is an important event during the development atherosclerosis and contributes to the failure of clinical interventions used to treat patients with CHD such as in-stent restenosis and late vein graft failure. During atherogenesis or in response to vessel injury, VSMC proliferation is stimulated by a number of peptide growth factors released from platelets and VSMCs, and by interactions with the vascular ECM [1]. Ultimately, these stimuli regulate SMC proliferation at the level of the cell cycle. Progression through the G1 phase of the cell cycle is regulated by the cyclins (A, D and E), which associate and activate their catalytic partners, the cyclin-dependent kinases (cdk4 and cdk2). Activation of the cdk's in this way results in the hyper-phosphorylation of retinoblastoma protein (Rb), release of the Rb-bound E2F transcription factor and initiation S-phase specific gene expression [2]. This allows the cell to progress through the G1 restriction point, beyond which proliferation becomes mitogen independent. Progression through the G1 restriction point is clearly an important step during proliferation and is subject to multiple levels of regulation. The activity of the cyclin:cdk complexes is negatively regulated by the cyclin-dependent kinase inhibitors (CDKIs) and in particular the Cip/Kip family of CDKIs (p21<sup>Cip1</sup>, p27<sup>Kip1</sup> and p57<sup>Kip2</sup>). Numerous studies have demonstrated that elevated levels of CDKIs are associated with growth arrest at the G1 restriction point [3,4]. CDKI levels are typically downregulated in late G1,
relieving cdk inhibition. For example, p27Kip1 levels are high in quiescent VSMC in culture and in healthy uninjured arteries where proliferation rates are extremely low, but are downregulated in response to mitogen stimulation in vitro or vascular injury in vivo [5,6]. This downregulation is mediated by ubiquitin-dependent proteasomal degradation of p27Kip1 and occurs independently of changes in p27Kip1 gene expression [7]. We recently demonstrated that S-phase kinase associated protein-2 (Skp2), an F-box protein and component of the SCFSkp2 ubiquitin-ligase promotes down-regulation of p27Kip1 and S-phase entry in VSMCs [7]. However, it is not known if Skp2 also plays a role in the regulation of other members of the Cip/Kip family in VSMCs. Here we demonstrate that in contrast to p27Kip1, p21Cip1 levels increase during late G1 and have a growth permissive effect in VSMC at low levels but an inhibitory effect at higher levels. Hence p21Cip1 regulates proliferation in these cells in a biphasic manner. Furthermore, we show that the balance between a PI 3-kinase-dependent increase in p21Cip1 gene expression and the Skp2-mediated degradation of p21Cip1 protein determines p21Cip1 levels during late G1 and the ability of SMCs to proliferate.

2. Experimental procedures

2.1. Materials

This investigation conforms with 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). Male Wistar rats were obtained from Charles River Culture media and additives were obtained from Gibco Life Technologies, Inc. (Paisley, Scotland). Monoclonal antibody to Skp2 was obtained from Zymed (Cambridge, U.K.). Monoclonal antibody to p21Cip1 was obtained from Transduction Laboratories (San Diego, U.S.A.). Rabbit antibody to hyperphosphorylated-Rb, phosphorylated and total AKT/PKB were from Cell Signalling Technology (MA, U.S.A). Adenovirus expressing p21 Cip1 was provided by Elizabeth Nabel (Bethesda, U.S.A.). Adenoviruses expressing wild-type and dominant-negative Skp2 have been described previously [7]. Adenovirus expressing p21Cip1 was provided by Elizabeth Nabel (Bethesda, U.S.A.). Viral stocks were plaque purified, amplified in 293HEK cells, CsCl banded and titrated as previously described [7]. VSMCs were infected with adenovirus at 200 pfu/cell for 3 h. This typically achieves infection efficiencies of 90–100% in rat VSMC.

2.2. Methods

2.2.1. SMC culture and bromo-deoxyuridine labelling

Rat thoracic aorta was excised, cut into 4 mm sections and cultured in DMEM (100 U/ml Streptomycin and 100 mg/ml penicillin, 2 mM glutamine) containing 10% FCS. Isolated VSMCs were prepared using a modification of the explant technique described previously [8]. Isolated VSMCs were rendered quiescent by serum-deprivation for 72 h. VSMC proliferation was quantified by labelling cells with 10 μM BrdU for 18 h. Cells were then fixed in ice cold 70% ethanol and analysed for BrdU incorporation by immuno-histochemistry using a monoclonal anti-BrdU antibody (ICN Biochemicals). BrdU positive cells were visualised with diaminobenzidine staining.

2.3. Western blotting

Isolated VSMCs were plated at a density of 4 × 10^4/cm². Where indicated, cells were synchronised in G0, by serum deprivation for 72 h. Total cell lysates were prepared using SDS-lysis buffer (50 mM Tris–HCl, pH 6.8; 10% glycerol, 1% SDS). Protein content was determined (Micro BCA assay kit, Pierce) and equal amounts of reduced protein (50–100 μg) were separated by polyacrylamide gel electrophoresis and transferred to PVDF membrane (Bio Rad). Membranes were blocked with TBS-T (20 mM Tris–HCl, pH 7.6; 137 mM NaCl, 25 mM KCl, 0.25% Tween-20) containing 6% milk powder before incubation in primary antibody. Specific proteins were detected using HRP-conjugated secondary antibodies (Dako, Ely, U.K.). Peroxidase activity was detected using enhanced chemoluminescence (Amsersham, U.K.).

2.4. Recombinant adenoviruses

Control adenovirus (Ad:control) containing an empty expression cassette has been described previously and was a gift from Dr G.W.G. Wilkinson (University of Wales College of Medicine, Heath Park, Cardiff, U.K.) [7]. Adenoviruses expressing wild-type and dominant-negative Skp2 have been described previously [7]. Adenovirus expressing p21Cip1 was provided by Elizabeth Nabel (Bethesda, U.S.A.). Viral stocks were plaque purified, amplified in 293HEK cells, CsCl banded and titrated as previously described [7]. VSMCs were infected with adenovirus at 200 pfu/cell for 3 h. This typically achieves infection efficiencies of 90–100% in rat VSMC.

2.5. Real-time RT-PCR analysis

Total RNA was extracted from isolated SMCs and rat aortic segments using the Qiagen Fibrous tissue RNA isolation protocol. First-strand cDNA was synthesised by random priming using ProStar first-strand synthesis kit (Stratagene, U.S.A.). Quantitative PCR was performed using a Roche Lightcycler with primers for Skp2 (forward 5’-ACCAGCTTCAGCTGGGGATGGG-3’ and reverse 5’-TTCGACAGGTCATGTGCTGAC-3’), 18S ribosomal RNA (forward 5’-CGCGGTTCTATTTGTTGGT-3’ and reverse 5’-CTTCAACCTCAGGACTTTCG-3’), p21Cip1 mRNA (forward primer 5’-TCCTGTTGATGTCCGCCTGTT-3’ and reverse primer 5’-GAAATCTGTAGGGCTGCTGCTGCTG-3’).

2.6. Apoptosis assays

Induction of apoptosis after treatment with small-interfering RNAs was analysed by immuno-fluorescent staining of...
active caspase-3 using a monoclonal active-specific caspase-3 antibody (R&D Systems; Minneapolis, U.S.A.). Briefly, adherent and detached cells were collected and attached to 3-amino propyl tri ethoxysilene (APES)-coated microscope slides using a cyto spin centrifuge. Cells fixed in 3% paraformaldehyde in PBS, permeabilised in PBS/1% Triton-X-100 and immunostained with active-caspase-3 antibody. Cells containing active caspase-3 were visualised using Alexafluor-488 secondary antibody (Molecular Probes).

Increased DNA fragmentation during apoptosis was quantified using a cell death detection ELISA according to the manufacturers instructions (Roche Applied Sciences, Lewes, East Sussex, U.K.).

2.7. Calcium phosphate mediated transfection of small interfering RNAs

Small interfering RNAs were synthesised using the Ambion siRNA Construction Kit (Ambion, Austin, Texas, U.S.A.) according to the manufacturers instructions. To inhibit p21Cip1 expression, equimolar amounts of two siRNAs corresponding to target sequences 5’ AAAG-TATGCCGTCTCTGTTCC 3’ (target 1) and 5’ AACGGTGGAACTTTGACTTCG 3’ (target 2) were used. An siRNA 5’ AAGTGCGTTGC TAGTACCAAC 3’ targeting the luciferase gene was used as a control. Rat SMC were seeded into 6 well plates at 1.5 × 10⁵ cell/well and calcium phosphate mediated transfection performed the next day with siRNA at a final concentration of 100 nM. Cells were washed three times after 6 h and allowed to recover overnight in culture medium containing 10% foetal bovine serum. Cells were analysed for BrdU incorporation or induction of apoptosis 72 h post-transfection.

2.8. Statistical analysis

After calculating means and standard errors of the means, analysis was performed using a two-tailed paired t test. Significant differences were taken when p < 0.05.

3. Results

3.1. Modulation of Skp2 activity by gene transfer affects p21Cip1 levels

We previously demonstrated that Skp2, an F-box protein component of the SCF<sup>Skp2</sup> ubiquitin ligase, regulates proliferation and G1-S transition in SMCs [7]. To investigate the mechanism underlying the regulation of SMC proliferation by Skp2, we determined the role of Skp2 in regulating the levels of the cyclin-dependent kinase inhibitor, p21<sup>Cip1</sup>, using adenovirus vectors capable of expressing wild-type Skp2 (Ad:WT-Skp2) and dominant-negative F-box deleted Skp2, respectively (Fig. 1B). These blots were underexposed so as to clearly visualise the overexpressed exogenous proteins and hence endogenous levels of Skp2 were undetectable. Infection with Ad:WT-Skp2 resulted in a significant decrease (to 39 ± 7.1% compared to Ad:Control n = 4; p = 0.0134) in p21<sup>Cip1</sup> levels compared to Ad:Control infected cells, indicating that exogenous Skp2 promotes p21<sup>Cip1</sup> degradation in VSMC (Fig. 1A,B). Importantly, infection with Ad:ΔF-Skp2 resulted in a significant increase (to 272 ± 29.8% compared to Ad:Control, n = 4; p = 0.007) in p21<sup>Cip1</sup> levels (Fig. 1A,B), further supporting a role for endogenous Skp2 in regulating p21<sup>Cip1</sup> levels in VSMCs.

3.2. p21<sup>Cip1</sup> and Skp2 are both up-regulated in mitogen stimulated SMCs

We previously demonstrated that upregulation of Skp2 occurs at the same time as downregulation of p27<sup>Kip1</sup> levels in VSMCs [7]. To determine if p21<sup>Cip1</sup> is subject to a similar regulation we performed Western blotting and immunofluorescent staining for p21<sup>Cip1</sup> after mitogen stimulation of
VSMC. In contrast to p27\(^{kip1}\), expression of p21\(^{Cip1}\) was barely detectable, either by immuno-fluorescent staining or Western blotting, in quiescent unstimulated VSMCs. (Fig. 2A,B). However, p21\(^{Cip1}\) protein expression was strongly increased (Figs. 2A and 3A) after 16–24 h of mitogen stimulation (8.7 fold increase, \(p < 0.05; n = 3\) after 24 h stimulation compared to control). Because, p21\(^{Cip1}\) has recently been described to have both nuclear and cytoplasmic functions [9–11], we determined the cellular localisation of p21\(^{Cip1}\) expression after mitogen stimulation by immuno-fluorescent staining. This demonstrated that p21\(^{Cip1}\) was exclusively localised in the nucleus, demonstrated by intense nuclear fluorescence (Fig. 2B). No immuno-staining was evident with a non-immune mouse IgG control (Fig. 2B). Interestingly, Skp2 is also undetectable in unstimulated SMCs but also strongly increased between 16 and 24 h mitogen stimulation, at the same time as p21\(^{Cip1}\). Upregulation of both of these genes occurs concomitantly with hyper-phosphorylation of retinoblastoma protein (Fig. 2A), indicating transition through the G1 restriction point. Expression of GAPDH protein was used as a loading control and did not alter during the time course of mitogen stimulation. Expression of p21\(^{Cip1}\) and Skp2 mRNA was measured by semi-quantitative RT-PCR analysis. Whereas Skp2 mRNA was significantly increased by more than three fold \((n = 7, p = 0.0342)\) in response to 18 h mitogen stimulation no effect was observed on p21\(^{Cip1}\) mRNA detected by RT-PCR (Fig. 2C) or northern blot (data not shown). Hence the increase in p21\(^{Cip1}\) levels late in G1 appears to arise from post-transcriptional mechanisms.

### 3.3. Upregulation of p21\(^{Cip1}\) occurs in the face of ongoing degradation

Our data demonstrating that Skp2 promotes p21\(^{Cip1}\) downregulation but that both proteins are concomitantly upregulated is at first counterintuitive. It implies that accumulation of p21\(^{Cip1}\) occurs despite increased Skp2 mediated proteasomal degradation. To test this hypothesis, we treated cultures of SMCs with the 26S proteasome inhibitor MG-132 for the last 6 h of a 24 h mitogen stimulation. Consistent with our hypothesis, treatment with MG-132 resulted in a significant increase \((7.9 \pm 1.17 \text{ fold}, n = 3, p < 0.0231 \text{ compared to mitogen stimulation alone})\) in p21\(^{Cip1}\) protein levels (Fig. 2D). This confirms that the late G1mitogen upregulation of p21\(^{Cip1}\) occurs in the face of ongoing proteasomal-mediated p21\(^{Cip1}\) degradation. Importantly, levels of GAPDH were not affected by MG-132 treatment demonstrating that the increase in p21\(^{Cip1}\) after MG132 treatment was not due to a general inhibition of protein turnover (Fig. 2D).

### 3.4. The increase in p21\(^{Cip1}\) and Skp2 levels depends on PI 3-kinase activity

Proliferation of SMCs and downregulation of p27\(^{kip1}\) have previously been shown to be dependent on signalling through the PI 3-kinase pathway [12]. Consistent with this, stimulation of rat VSMC with serum mitogens resulted in increased phosphorylation of AKT, a downstream effector of PI 3-kinase (Fig. 3A). Therefore, we sought to determine
if signalling through PI 3-kinase was involved in the regulation of Skp2 and p21Cip1 levels after mitogen stimulation in VSMC. Treatment of SMC with the PI 3-kinase inhibitor LY294002 resulted in a significant inhibition of Skp2 protein levels (to 15.2 ± 1.1% compared to mitogens alone, n = 3; p = 0.0166) without affecting cell viability (98.7 ± 0.56% viable in controls versus 97.8 ± 1.3% viable when treated with LY294002) (Fig. 3B). Treatment with LY294002 also significantly inhibited p21Cip1 protein levels (to 16.5 ± 6.8% compared to mitogens alone, n = 3; p = 0.0066). Inhibition of AKT, a downstream effector of PI 3-kinase, had no effect on p21Cip1 or Skp2 expression (Fig. 3C). However, treatment with rapamycin resulted in a 50% inhibition of both Skp2 and p21Cip1 expression (Fig. 3C).

Interestingly, inhibition of PI 3-kinase activity had no effect on either p21Cip1 or Skp2 mRNA expression quantified by real-time PCR (Fig. 3D). Furthermore, treatment with LY294002 had no effect on the stability of p21Cip1 or Skp2 protein (Fig. 4) implying that PI 3-kinase signalling regulates p21Cip1 and Skp2 protein levels via a translational mechanism in VSMC. Taken together, these data demonstrates that Skp2 and p21Cip1 are both increased in SMCs in response to mitogen stimulation in a PI 3-kinase and mTOR dependent but AKT independent manner in VSMC.

3.5. Biphasic effect of p21Cip1 on SMC proliferation

To further explore the role of p21Cip1 in regulating SMC proliferation, we used an adenovirus vector expressing p21Cip1. SMCs were infected with an adenovirus expressing p21Cip1 and then treated with 10% FCS and cyclohexamide for 48 h. Western blots were performed to assess p21Cip1 and Skp2 levels. A significant increase in p21Cip1 and Skp2 levels was observed in the p21Cip1 expressing group compared to the control group (Fig. 4). These results suggest that p21Cip1 can promote SMC proliferation through upregulation of Skp2 levels.
p21Cip1 (Ad:p21Cip1) to elevate p21Cip1 levels. Infection of rat SMC cultures with 200 pfu/cell of Ad:p21Cip1 but not Ad:Control resulted in elevated expression of p21Cip1 protein in the nucleus and cytoplasm, detected by Western blotting and immuno-fluorescent staining (Fig. 5A,B). The blots were under exposed so as to clearly visualise the exogenous p21Cip1 protein and hence the lower levels of endogenous p21Cip1 are barely visible. Infection with Ad:p21Cip1 resulted in a significant inhibition (1.34 \pm 0.54 \times 10^5 / \text{C} \times 10^5 \text{ cells infected with Ad:control, } n = 4 ; \ p = 0.0052) of cell number 72 h post infection compared to Ad:Control infected cells (Fig. 5C). Infection with Ad:Control had no effect on cell number compared to uninfected controls. Infection of SMCs with Ad:p21Cip1 also resulted in a significant inhibition in the number of cells staining for incorporation of 5-bromo-2-deoxyuridine (BrdU) compared to Ad:Control infected cells (18.26 \pm 3.53\% for Ad:Control cells compared to 6.48 \pm 2.3\% for Ad:p21Cip1 infected cells, \ n = 5 ; \ p = 0.004) (Fig. 5D). This data demonstrates that elevated levels of p21Cip1 inhibit SMC proliferation.

To determine the role of endogenously expressed p21Cip1 in regulating SMC proliferation, we employed small interfering RNAs (siRNA) to specifically silence endogenous p21Cip1 expression. Cultures of rat SMCs were transfected with 100 nM siRNA targeting either rat p21Cip1 (si-p21) expression or an equimolar amount of siRNA with an identical GC ratio, targeting luciferase (si-Luc) as a negative control. p21Cip1 protein expression was analysed 72 h post-transfection by Western blotting. Transfection with si-p21 resulted in a significant silencing (to 14.85 \pm 7.9\% relative to si-Luc, \ n = 4 ; \ p = 0.0017) of endogenous p21Cip1 expression compared to si-Luc transfected cells (Fig. 6A,B). Importantly, transfection with si-p21 did not significantly affect the expression of the housekeeping gene GAPDH, confirming the specificity of gene silencing. Silencing of p21Cip1 expression did not result in any change in expression of Cyclin D1 or Cyclin E (Fig. 6A). A small reduction in p27Kip1 protein level was observed in cells treated with p21Cip1 siRNA. However, this inhibition of p27Kip1 expression did not occur at the mRNA level (data not shown) indicating that this occurs as a result of altered p21Cip1 expression rather than non-specific silencing of p27Kip1. Transfection with si-p21 resulted in a significant inhibition of cell number (4.6 \times 10^5 \pm 0.48 \times 10^5 \text{ for si-p21 compared to } 8.7 \times 10^5 \pm 0.6 \times 10^5 \text{ for si-Luc, } \ n = 4 ; \ p = 0.0297) determined 72 h post-transfection, (Fig. 6C). Furthermore, si-p21 transfection significantly inhibited incorporation of BrdU (from 23 \pm 9.2\% for si-Luc to 9.6 \pm 7.4\% for si-p21, \ n = 4 ; \ p = 0.0218 (Fig. 6D). Taken together, this data demonstrates that endogenous p21Cip1 expression is required for SMC proliferation.

3.6. Silencing of endogenous p21Cip1 induces SMC apoptosis

An increase in VSMC cell death, characterised by cell rounding, shrinkage and detachment, was observed 72 h after transfection with si-p21 but not si-Luc (Fig. 7A,C). To determine if this cell death was the result of increased VSMC apoptosis we performed immuno-staining with an antibody that recognises the active form of caspase-3. Transfection of cells with si-p21 resulted in a significant increase in the number of cells staining for active caspase-3 (12.5 \pm 4.5\% for si-p21 compared to 1.1 \pm 0.8\% for si-Luc, \ n = 6 ; \ p = 0.0419) compared to cells transfected with si-Luc.
No immunostaining was evident with a non-immune rabbit IgG control, confirming the specificity of the staining (data not shown). Transfection with si-p21 also resulted in a significant increase ($0.81 \pm 0.23$ O.D. units compared to $0.1 \pm 0.04$ O.D. units, $n=6$; $p=0.419$) in the formation of nucleosomal DNA fragments, indicative of an

Fig. 6. p21Cip1 is required for maximal VSMC proliferation. Rat VSMC were transfected with 100 nM si-Luc or si-p21 siRNA using calcium phosphate followed by BrdU labelling 48 h later, for a further 18 h. p21Cip1 and GAPDH expression was analysed 72 h post-transfection by Western blotting (A) and densitometric analysis (B). VSMC proliferation was analysed by cell counting (C) and immuno-staining for BrdU incorporation (D). *$p<0.05$ two-tailed paired $t$ test.

(Fig. 7B,D,E). No immunostaining was evident with a non-immune rabbit IgG control, confirming the specificity of the staining (data not shown). Transfection with si-p21 also

Fig. 7. Silencing of p21Cip1 expression induces VSMC apoptosis. Rat VSMC were transfected with 100 nM si-Luc or si-p21 by calcium phosphate-mediated transfection. Cell death was analysed 72 h later by phase-contrast microscopy (A, C) and immuno-fluorescent staining for active caspase-3 (B, D, E). Cell lysates were prepared 72 h post-transfection and analysed for nucleosomal DNA fragments using a nucleosome ELISA (F). *$p<0.05$ two-tailed paired $t$ test.
apoptotic mode of cell death, detected by a nucleosome ELISA (Fig. 7F). This data demonstrates that endogenously expressed p21\textsuperscript{Cip1} plays an anti-apoptotic role in VSMCs.

4. Discussion

In this study, we sought to elucidate the complex mechanisms underlying control of VSMC proliferation by different levels of the cyclin-dependent kinase inhibitor, p21\textsuperscript{Cip1}, and the role of the F-box protein, Skp2, in regulating these levels. We established that over expressing wild-type Skp2 decreases and dominant negative Skp2 increases the level of p21\textsuperscript{Cip1}. Nevertheless, endogenous p21\textsuperscript{Cip1} and Skp2 levels rise concomitantly during late G1 in VSMC, even though there is increased proteasomal degradation of p21\textsuperscript{Cip1}. Activation of PI 3-kinase contributes to increased levels of p21\textsuperscript{Cip1} and Skp2 by post-transcriptional mechanisms. Together, this data implies that p21\textsuperscript{Cip1} is maintained at intermediate levels in late G1 by the balance between PI 3-kinase driven up-regulation and Skp2 mediated degradation. We show that high p21\textsuperscript{Cip1} levels are inhibitory but that moderate levels of p21\textsuperscript{Cip1} are permissive for VSMC proliferation and protect against apoptosis. Skp2, an F-box protein component of the SCF\textsuperscript{Skp2} ligase, was originally identified as a protein interacting with cyclin A and cdk2 [13]. Several recent studies have demonstrated that Skp2 is a key regulator of cell-cycle progression in a number of different cell-types [14–17]. This is mediated, at least in part, by increased ubiquitination and proteasome-mediated degradation of the CDK1, p27\textsuperscript{Kip1}. For example, we demonstrated that Skp2 controls p27\textsuperscript{Kip1} levels and proliferation in VSMC in a mitogen and adhesion dependent manner [7]. Recently, Skp2 has also been shown to promote the ubiquitination of other substrates, including p21\textsuperscript{Cip1}, p57\textsuperscript{Kip2} and p130 [18,19]. Although Skp2 has been shown to directly promote the ubiquitination of p21\textsuperscript{Cip1} in a cell-free system, a recent paper by Chen et al. suggests that this does not occur in intact embryonic fibroblasts [20]. Rather, p21\textsuperscript{Cip1} stability in these cells is dependent on levels of cyclin E, which is directly ubiquitinated by Skp2. In this study, we clearly demonstrate that Skp2 regulates the levels of p21\textsuperscript{Cip1} in the late G1 phase of the cell cycle in VSMC. Whether this regulation occurs via a direct or indirect mechanism remains to be determined. However, our data clearly demonstrates that Skp2 mediates its effects on VSMC proliferation by controlling the levels of multiple CDKIs, including p21\textsuperscript{Cip1}.

Numerous studies have implicated p21\textsuperscript{Cip1} as an inhibitor of cell-cycle progression by associating the growth inhibitory effects of anti-proliferative agents and stimuli as nitric oxide, paclitaxel treatment and irradiation, with increased expression of p21\textsuperscript{Cip1} [21–23]. This has lead to the generally accepted paradigm that p21\textsuperscript{Cip1} acts as an inhibitor of the cell cycle. Indeed, we confirm here that elevated levels of p21\textsuperscript{Cip1} are growth inhibitory in VSMC. However, our other results challenge this paradigm. Firstly, we show that endogenous p21\textsuperscript{Cip1} is elevated in late G1, more consistent with a positive role in G1-S phase progression. Secondly, we demonstrate directly using siRNA-mediated gene silencing that endogenous p21\textsuperscript{Cip1} expression is required for maximal cell-cycle progression. This is consistent with the recent finding that early G1 phase expression of p21\textsuperscript{Cip1} can act as an assembly factor for cyclinD1:cdk4 complexes [24]. Taken together, our findings demonstrate that p21\textsuperscript{Cip1} regulates VSMC proliferation in a biphasic manner: high levels of p21\textsuperscript{Cip1} are growth inhibitory but the moderate levels of p21\textsuperscript{Cip1} that are expressed in late G1 by VSMCs are growth permissive. It follows, therefore, that the regulation of p21\textsuperscript{Cip1} levels during late G1 are critical for maximal VSMC proliferation.

Our data demonstrates that p21\textsuperscript{Cip1} upregulation in VSMC is dependent on signalling through PI 3-kinase, which is known to be involved in the regulating of VSMC proliferation and G1-S phase transit in many cell types both in vitro and in vivo [12,25,26]. Consistent with this, we demonstrate that inhibition of PI 3-kinase signalling in rat VSMC blocks Rb-hyperphosphorylation, a marker of G1-S phase transition. Furthermore, inhibition of PI 3-kinase completely blocked p21\textsuperscript{Cip1} expression in late G1. Upregulation of p21\textsuperscript{Cip1} appears to be mediated via increased translation of p21\textsuperscript{Cip1} mRNA, as PI 3-kinase inhibition had no effect on the steady state p21\textsuperscript{Cip1} mRNA levels or the stability of p21\textsuperscript{Cip1} protein. This is consistent with previous findings that signalling through the PI 3-kinase pathway regulates protein synthesis [27,28].

Our data suggests that this PI 3-Kinase-dependent increase in p21\textsuperscript{Cip1} protein synthesis is countered by the concomitant PI 3-kinase-dependent upregulation of Skp2. The upregulation of Skp2 limits the PI 3-kinase-driven accumulation of p21\textsuperscript{Cip1} by promoting its ubiquitination and subsequent proteasome-mediated degradation in late G1 phase. The balance between these two opposing forces presumably limits the final level of p21\textsuperscript{Cip1} expression in late G1 phase to that which is permissive for progression into S-phase.

In addition we demonstrate another important role for intermediate levels of p21\textsuperscript{Cip1} as an anti apoptotic factor in these cells. We demonstrated that silencing of p21\textsuperscript{Cip1} gene expression in rat VSMC significantly increased the rate of apoptotic cell death, even in the absence of an additional apoptotic stimulus. This is consistent with previous reports demonstrating that inhibition of p21\textsuperscript{Cip1} expression enhances the apoptotic response to various apoptotic stimuli [29–32]. Furthermore, deletion of the p21\textsuperscript{Cip1} gene in apoE−/− mice also leads to increased apoptosis and results in a more stable and smaller plaque phenotype, implying that this anti-apoptotic mechanism has important implications for disease progression in vivo [33].

Taken together our results imply that p21\textsuperscript{Cip1} plays multiple roles in VSMC. High levels of p21\textsuperscript{Cip1} are clearly involved in growth arrest in response to growth inhibitory
stimuli, whereas moderate levels of p21\textsuperscript{Cip1} are required for maximal proliferation in response to growth factor stimulation and promote survival. Cell death is thought to be the default pathway during the cell cycle from which the cell has to be constantly rescued. This is likely to be an important mechanism in preserving genetic fidelity, particularly during S-phase and mitosis. Furthermore, we have highlighted the role of Skp2 as a regulator of p21\textsuperscript{Cip1} levels in these cells. In addition to promoting degradation of p27\textsuperscript{Kip1} in VSMC, Skp2 also regulates VSMC proliferation by controlling p21\textsuperscript{Cip1} expression, preventing its accumulation to growth inhibitory levels.

Acknowledgements

This work was funded by the British Heart Foundation. We thank Gillian Tarlton for her help in preparing adenoviruses and Prof. E. Nabel for providing the p21\textsuperscript{Cip1} adenovirus vector.

References