Regulation of Vascular Smooth Muscle Proliferation by Heparin

INHIBITION OF CYCLIN-DEPENDENT KINASE 2 ACTIVITY BY p27kip1

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Uncontrolled proliferation of vascular smooth muscle cells (VSMCs) contribute to intimal hyperplasia during atherosclerosis and restenosis. Heparin is an antiproliferative agent for VSMCs and has been shown to block VSMC proliferation both in tissue culture systems and in animals. Despite the well documented antiproliferative actions of heparin, its cellular targets largely remain unknown. In an effort to characterize the mechanism of the antiproliferative property of heparin, we have analyzed the effect of heparin on cell cycle in VSMC. Our results indicate that the heparin-induced block in G1 to S phase transition is imposed by p27kip1-mediated inhibition of cyclin-dependent kinase 2 activity. Further analysis of p27kip1 mRNA levels showed that the increase in p27kip1 protein levels in heparin-treated VSMC occurs at posttranscriptional levels. We present evidence that heparin causes stabilization of p27kip1 protein during G1 phase and thereby prevents activation of cyclin-dependent kinase 2.

The proliferation of vascular smooth muscle cells (VSMCs) is a key event in the development of atherosclerotic lesions and postangioplasty restenosis (1). In a normal artery, the VSMCs are in a non-proliferative quiescent state and show a well differentiated contractile phenotype. After the vascular injury, there is a loss of differentiated phenotype and a shift to a synthetic phenotype, which is also accompanied by entry into the cell cycle and proliferation (2). Several cytokines, growth factors, vasoregulatory molecules, and extracellular matrix components exert their effects on the proliferation of VSMC. The development of an atherosclerotic lesion can be blocked in animals (7, 8). Despite the well documented antiproliferative effects of heparin in VSMC, the molecular mechanisms responsible for inhibition of cell cycle remain uncharacterized.

Cellular proliferation is regulated primarily by regulation of the cell cycle (9), which consists of four distinct sequential phases (G0/G1, S, G2, and M). This tightly regulated temporal order is controlled by the sequential activation of certain serine/threonine protein kinases known as cyclin-dependent kinases (Cdks) that phosphorylate the Rb protein (10). In quiescent cells, Rb exists in its hypophosphorylated state and is thus able to bind and sequester the members of E2F family of transcription factors (11). Phosphorylation of Rb at multiple sites by Cdks causes the release of E2F because hyperphosphorylated Rb cannot bind and sequester E2F factors, thus enabling them to activate transcription of genes whose products are absolutely essential for further cell cycle progression (12). The activity of Cdks is further regulated negatively by a number of Cdk inhibitors, which are grouped into two classes (13). The members of the INK4 family (p16INK4a, p15INK4b, p14INK4c, and p19INK4d) inhibit only Cdk4 and Cdk6 (14), and the members of Cip family (p21cip1, p27kip1, and p57kip1) inhibit all Cdks (15). It is unknown how heparin affects the activities of these proteins to regulate the cell cycle.

We have shown recently that heparin induces a block in G1 to S phase transition in VSMC (16). The interferon-induced protein kinase (PKR) activation in heparin-treated VSMC was essential in part for this cell cycle block. In this paper, we have extended our study to determine the mechanism of heparin-induced cell cycle block. Our results indicate that heparin causes the p27kip1 levels to remain elevated even in response to serum, thereby causing an inhibition of Cdk2 activity that results in deficient phosphorylation of Rb, leading to the block in S phase entry. Furthermore, the elevated levels of p27kip1 in heparin-treated cells were found to result from a posttranscriptional mechanism, mainly by stabilization of p27kip1 protein. This p27kip1 stabilization was found to be defective in cells treated with 2-aminopurine, an inhibitor of PKR activity. In addition, the p27kip1-null cells were found to be insensitive to heparin-induced cell cycle block, thereby demonstrating p27kip1 stabilization to be the main cause of the antiproliferative effects of heparin.

EXPERIMENTAL PROCEDURES

Cell Culture—The rat primary aortic vascular smooth muscle cells (RASMCs) were obtained from the thoracic aorta of male Sprague-Dawley rats. The cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. RASMCs were made quiescent in DMEM with 0.1% FCS for 72–96 h. For heparin treatment, the cells were shifted to 0.1% serum containing medium with 100 μg/ml heparin (Sigma, catalog number H-3149, lot 17H03885) for 2 h. After 2 h, the cells were serum-stimulated with DMEM containing 10% serum and 100 μg/ml heparin. As a control, RASMCs were subjected to identical treatment in the absence of heparin.

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Western Blot Analysis—The RASMCs grown in 100-mm plates were treated with heparin as indicated in the previous section for different lengths of time. Cell extracts were prepared in 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 200 mM NaCl, 4 mM MgCl₂, 2 mM dithiothreitol (DDT); 2% Triton X-100, 40% glycerol, 20 mM sodium fluoride, 20 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 20 mM sodium molybdate, 0.4 mM phenylmethylsulfonyl fluoride, and 2 μg/ml aprotinin. 100 μg of total cellular proteins were separated by SDS-PAGE and subjected to Western blot analysis using the ECL plus reagents (Amersham Biosciences) according to the manufacturer’s directions and following specific antibodies: cyclin D1 (Santa Cruz Biotechnology, sc-753, 1:1000); cyclin E (Santa Cruz Biotechnology, sc-481, 1:1000); cyclin A (Santa Cruz Biotechnology, sc-751, 1:1000); p21 (BD Biosciences, 65951A, 1:1000); Rb (BD Biosciences, 14001A, 1:1000); α-smooth muscle actin (Sigma, A2547 clone 1A4, 1:1000) and p27 (Santa Cruz Biotechnology, sc-1641, 1:1000).

Cell 2 Kinase Assays—Cell extracts were prepared from RASMCs treated with heparin as described in ice-cold lysis buffer (50 mM Hepes, pH 7.6, 150 mM NaCl, 2.5 mM EGTA, 20 mM sodium pyrophosphate, 2% Triton X-100, 40% glycerol, 20 mM sodium fluoride, and 2 μg/ml aprotinin). Lysates were centrifuged in cold temperature for 10 min in a microcentrifuge at maximum speed, and the supernatant was stored at −80 °C in small aliquots. Cell extracts containing 100 μg of total protein were incubated at 4 °C for 2 h under constant rotation in 500 μl of lysis buffer containing 250 ng of anti-Cdk2 antibodies (Santa Cruz Biotechnology) and 20 μl of protein A-Sepharose beads (Roche Applied Science). Immunoprecipitates were washed twice with 50 μl of kinase buffer (40 mM Tris-HCl, pH 7.6, 20 mM MgCl₂, and 2 mM dithiothreitol). The beads were then suspended in 25 μl of kinase buffer containing 2 μg of histone H1 (Sigma, 10 μM ATP, and 5 μCi of [γ-³²P]ATP. The reaction was incubated at 30 °C for 20 min and then stopped by the addition of 10 μl of 4× SDS-PAGE sample loading buffer and boiling for 10 min. The proteins were separated by 12% SDS-PAGE and visualized by phosphorimaging analysis of dried gels.

Northern Blot Analysis—Total RNA from RASMC was extracted by using the RNAzol D reagent (TelTest). 30 μg of total RNA from each sample was separated by Northern blot analysis according to Church and Gilbert protocol with a 1.5-kb EcoRI fragment of human p27kip1 cDNA (provided kindly by Sylvain Meleno, University of Montreal, Canada) as a probe. The signals were analyzed by phosphorimaging analysis. The same blot was then stripped and reprobed with a 28 S ribosomal RNA as a probe to ensure equal loading in all of the lanes.

Cloning the p27kip1 Internal Ribosome Entry Site (IRES) Element in the Eukaryotic Vector and Assaying Its Activity in VSMC—The pRL-null vector is composed of a fragment containing Renilla luciferase from pRL-null (Promega) as the first cistron fused to a fragment encoding the cytosolic firefly luciferase from pGL3-basic (Promega) as the second cistron. This vector and the pLVL construct containing the IRES element fused to a fragment encoding the p27kip1 protein was analyzed by SDS-PAGE followed by fluorography and phosphorimaging analysis.

Effect of Inhibition of PKR Activity by 2-Aminopurine—RASMCs were made quiescent in DMEM with 0.1% FCS for 72–96 h. The cells were then treated with 10 mM 2-aminopurine (2AP) for 2 h prior to the addition of heparin. After this treatment, 100 μg/ml heparin was added to the cells in 0.1% serum containing medium for 2 h. After 2 h, the cells were serum-stimulated with DMEM containing 10% serum and 100 μg/ml heparin. As a control, RASMCs were subjected to identical treatment in the absence of 2AP. Cell extracts were prepared 20 h after the start of heparin treatments, and a Western blot analysis was performed with an anti-p27kip1 monoclonal antibody. The blot was then stripped and reprobed with anti-a-actin antibody to ascertain that an equal amount of protein from different cell extracts was analyzed.

Effect of Heparin on p27kip1-null Cells—The p27kip1 and isogenic p27kip1-negative embryonic fibroblast (MEF) cell lines were provided by J. M. Roberts (Fred Hutchinson Cancer Research Center). The p27kip1 and p27kip1−/− MEFs were made quiescent in 0.1% serum for 36–48 h and were stimulated with DMEM containing 10% serum. The cells were pretreated for 2 h with 100 μg/ml heparin in low serum medium before the addition of serum-containing medium with 100 μg/ml heparin. As a control, the same treatments were performed without any heparin. 24 h after serum stimulation, the cells were permeabilized using the Vindelov’s solution (17) and stained with propidium iodide and analyzed by flow cytometry. The data are representative of three separate experiments.

RESULTS

It has been shown previously that heparin induces a block in G₁ to S transition in VSMC (16). To characterize the mechanism of this cell cycle block, we have analyzed the effect of heparin on defined cell cycle events that occur in response to serum. As a first step toward characterizing the effect of heparin on cell cycle, we performed Western blot analysis to examine the expression of cyclin D and E in the presence of heparin. Heparin showed no effect on the expression of cyclins D and E. Cyclin D was barely detectable in quiescent VSMCs and was induced within 4 h after addition of serum, both in presence and absence of heparin (Supplementary Fig. 1A, lanes 1–8). Cyclin E was detected at low levels in serum-starved quiescent VSMCs and was up-regulated by the addition of serum at 8 h, both in presence and absence of heparin (Supplementary Fig. 1B, lanes 1–12). We next examined whether the phosphorylation of Rb in response to serum was affected by the addition of heparin. In quiescent cells, most of the Rb protein was present in the faster migrating hypophosphorylated form (Supplementary Fig. 2, lanes 1 and 2). The addition of serum showed a prominent induction of Rb phosphorylation with slower migrating phosphorylated Rb first appearing at 18 h (Supplementary Fig. 2, lane 8) and then the phosphorylated form completely prevailing at 24 and 30 h (Supplementary Fig. 2, lanes 10 and 12). However, in the presence of heparin, there was no phosphorylated form of Rb at 18 h (lane 7) with only a marginal induction of 24 and 30 h. In the presence of heparin, the phosphorylated form of Rb protein never prevailed completely, even at 24 and 30 h after serum addition, as in the absence of heparin. These results indicate that heparin prevents Rb phosphorylation in response to serum.

Induction of cyclin A synthesis in response to serum occurs as cells enter S phase, and it is abruptly degraded before metaphase during mitosis (18, 19). The synthesis of cyclin A is mainly controlled at the transcriptional level, involving E2F and other transcription factors (20). Enzymatic activation of cyclin A is responsible for up-regulating gene expression required for progression through the S, G2, and M phases (21). Therefore, the regulation of p27kip1 by heparin during late G1 phase was examined. After this treatment, 100 μg/ml heparin was added to the cells in 0.1% serum containing medium for 2 h. After 2 h, the cells were serum-stimulated with DMEM containing 10% serum and 100 μg/ml heparin. As a control, RASMCs were subjected to identical treatment in the absence of 2AP. Cell extracts were prepared 20 h after the start of heparin treatments, and a Western blot analysis was performed with an anti-p27kip1 monoclonal antibody. The blot was then stripped and reprobed with anti-a-actin antibody to ascertain that an equal amount of protein from different cell extracts was analyzed.
cells (compare the middle bar with the vertically hatched bar). In the presence of heparin, the Cdk2-bound p27kip1 levels (horizontally hatched bar) remained high and were 2.1-fold more than the levels in the quiescent cells (vertically hatched bar). These results clearly indicate that the elevated levels of p27kip1 contribute to the inhibition of Cdk2 activity in the presence of heparin.

It is known that p27kip1 protein levels are regulated primarily by a posttranscriptional mechanism either at the level of translation or protein stability (23-30). To examine whether the p27kip1 levels were regulated at a posttranscriptional level in VSMC, we performed Northern blot analysis of p27kip1 expression. As seen in Fig. 4A, p27kip1 mRNA levels were the highest in serum-starved cells and dropped somewhat in serum-stimulated cells, both in presence and absence of heparin to the same extent. These results indicate that heparin treatment seems to regulate p27kip1 expression at the posttranscriptional level in VSMC. The regulation of p27kip1 protein levels occurs mainly by regulation of its translation and protein stability (12). The 5'-UTR region of p27kip1 mRNA has been shown to contain an IRES element (25) and is shown to regulate its translation (31). Some IRES elements from VEGF, platelet-derived growth factor, and c-Myc mRNAs function to enhance their translation under conditions when eIF2α is phosphorylated in cells (32). We have shown previously that heparin treatment of VSMC resulted in PKR activation and eIF2α phosphorylation (16). Thus, it is possible that heparin enhances p27kip1 protein synthesis via the IRES element because it also causes eIF2α phosphorylation. To examine whether heparin was enhancing p27kip1 synthesis via the IRES element because it also causes eIF2α phosphorylation. To examine whether heparin was enhancing p27kip1 synthesis via the IRES element because it also causes eIF2α phosphorylation.
Regulation of p27kip1 by Heparin

To determine whether heparin affected the p27kip1 protein stability, we performed the pulse-chase experiments. As seen in Fig. 5A (0 h lanes), a 2-h labeling period resulted in the labeling of p27kip1 to equal levels, both in the absence and presence of heparin. These results are in agreement with the results shown in Fig. 4 and further confirm that the rate of p27kip1 synthesis is same in the absence or presence of heparin. As seen during the chase, labeled p27kip1 protein is degraded to undetectable levels within 12 h in the absence of heparin. However, in the presence of heparin, the stability of p27kip1 protein is significantly enhanced, thereby indicating that heparin causes stabilization of p27kip1 protein during the G1 phase. The radioactivity present in the p27kip1 bands was quantified on a PhosphorImager and was plotted as a percentage of initial amount at 0 h (Fig. 5B). Thus, heparin causes a block in G to S phase transition in VSMC by stabilizing the p27kip1 protein and a consequent inhibition of Cdk2 activity.

To determine the role of PKR in enhancing p27kip1 protein stability in the presence of heparin, we examined the effect of inhibiting PKR activity in VSMC by its known inhibitor 2-aminopurine (2AP) (34–36). As shown in Fig. 6A, VSMCs treated with 2AP and heparin show lower amount of p27kip1 protein than the VSMC treated only with heparin. These results indicate that PKR activation by heparin is essential at least in part for the observed increase in p27kip1 stability. The levels of p27kip1 protein in cells treated with heparin and 2AP are intermediate between those in control and heparin-treated VSMC. Thus, the p27kip1 stabilization in response to heparin seems to occur both by PKR-dependent and independent pathways. Similar results were obtained with PKR-null fibroblasts where PKR-null MEFs did not show a significant increase of p27kip1 protein levels in response to heparin (data not shown). We have previously shown (16) that these PKR-null fibroblasts do not show a complete block of G1 to S phase transition in response to heparin. The results presented here show that the lack of a cell cycle block is due to a defect in p27kip1 stabilization in response to heparin in the absence of PKR activity.

If heparin-induced block in the cell cycle operates primarily because of a rise in p27kip1 protein levels, it is expected that cells lacking p27kip1 would be insensitive to heparin. To establish a direct causal relationship between the higher p27kip1 levels in heparin-treated cells and the cell cycle block, we examined the effect of heparin on p27kip1-null cells (37). We have previously shown that MEFs respond well to the antiproliferative actions of heparin (16). We compared the effect of heparin on MEFs isolated from wild type and p27kip1-null mice. Heparin treatment caused a block in G to S phase transition in wild type MEFs. The p27kip1-null MEFs were resistant to heparin-induced block of G1 to S phase transition. As represented in Fig. 6B, 39.4% wild type MEFs were in S phase at 24 h after serum stimulation. This percentage dropped to 19.6% in heparin-treated wild type MEFs, thereby confirming that heparin effectively blocked the S phase entry of MEFs. In addition, there were 15.2% cells in G2/M phase in the absence of heparin and this percentage dropped to 6.3% in the presence of heparin. The p27kip1-null MEFs seemed to progress through the cell cycle much faster than their wild type counterparts. At 24 h after serum stimulation, p27kip1-null cells had progressed through the S phase and thus most of the cells that entered cell cycle were in the G2/M phase at this time point. 22.5% p27kip1-null cells were in G2/M phase at 24 h after serum stimulation in the absence of heparin as compared with only 15.2% in wild type MEFs. Nevertheless, in the case of p27kip1-null cells, clearly no effect of heparin treatment was observed on the cell cycle progression. The percentages of p27kip1-null cells in S and G2/M
phases after serum stimulation either in the absence or presence of 100 μg/ml heparin, and total RNA was isolated from quiescent cells and 12- and 18-h posttreatments. The RNA was analyzed by Northern blot analysis using a p27kip1 cDNA probe. The same blot was stripped and rehybridized to cDNA probe for 28 S RNA to normalize the loading. The time points are as indicated at the top of the panels. B, a schematic representation of the bicistronic constructs. The 502-bp long p27kip1 5'-UTR was inserted between the Renilla and firefly luciferase coding regions. CMV: cytomegalovirus. C, p27kip1 5'-UTR functions as IRES element to enhance translation but is unresponsive to heparin. Rat VSMCs grown in 6-well plates were transfected in triplicates with 1 μg of pLL or M502/pLL using Lipofectamine reagent. 24 h after transfection, the cells were treated with 100 μg/ml heparin. 18–24 h after heparin treatment, the cells were harvested and the luciferase activities in the extract were determined. The data shown are representative of three separate experiments, each one performed in triplicates. The error bars shown represent the mean ± S.D. C, represents untreated cells.

**DISCUSSION**

We have previously shown that heparin inhibits the VSMC proliferation by blocking the G1 to S transition. The interferon-induced protein kinase PKR was shown to be involved at least partly in mediating this cell cycle block in response to heparin (16). In PKR-null MEFs and in VSMCs expressing the transdominant negative K296R mutant of PKR, there was a marked but not a complete loss of the antiproliferative effects of heparin, indicating that pathways in addition to PKR activation

![Fig. 4.](image)

**Fig. 4.** A, p27kip1 mRNA levels are not affected by heparin (hep). Quiescent VSMCs (Q) were stimulated with serum either in absence or presence of 100 μg/ml heparin, and total RNA was isolated from quiescent cells and 12- and 18-h posttreatments. The RNA was analyzed by Northern blot analysis using a p27kip1 cDNA probe. The same blot was stripped and rehybridized to cDNA probe for 28 S RNA to normalize the loading. The time points are as indicated at the top of the panels. B, a schematic representation of the bicistronic constructs. The 502-bp long p27kip1 5'-UTR was inserted between the Renilla and firefly luciferase coding regions. CMV: cytomegalovirus. C, p27kip1 5'-UTR functions as IRES element to enhance translation but is unresponsive to heparin. Rat VSMCs grown in 6-well plates were transfected in triplicates with 1 μg of pLL or M502/pLL using Lipofectamine reagent. 24 h after transfection, the cells were treated with 100 μg/ml heparin. 18–24 h after heparin treatment, the cells were harvested and the luciferase activities in the extract were determined. The data shown are representative of three separate experiments, each one performed in triplicates. The error bars shown represent the mean ± S.D. C, represents untreated cells.

![Fig. 5.](image)

**Fig. 5.** Heparin treatment of VSMC increases the half-life of p27kip1 protein. A, the rat VSMC in G0/G1 phase (6 h after serum stimulation) were pulse-labeled for 2 h with [35S]methionine and chased in fresh medium containing 10% serum for the indicated times. Cell lysates were subjected to immunoprecipitation with anti-p27kip1 monoclonal antibody, and the labeled immunoprecipitated p27kip1 protein was analyzed by SDS-PAGE followed by fluorography and PhosphorImager analysis. B, quantification of the data shown in A was plotted as percentage of original amount of radioactivity present in p27kip1 band at 0 h.

phases after serum stimulation either in the absence or presence of heparin were very similar. In the absence of heparin, 22.5% cells were in G2/M phase (8.5% in S phase) and in the presence of heparin and 19.5% cells were in G2/M phase (6.6% in S phase) at 24 h after serum stimulation. Thus, the cell cycle inhibitory effect of heparin was clearly absent in p27kip1-null MEFs.

To examine the effect of heparin on cell cycle in p27kip1-null VSMC, experiments were also carried out to induce p27kip1 down-regulation by small interfering RNA transfections with three different small interfering RNAs. Although this reduced the p27kip1 mRNA levels to barely detectable levels, it did not completely knock out p27kip1 protein levels in quiescent VSMC (data not shown). In addition, the transfected small interfering RNA did not persist for the entire duration of the experiment due to the fact that VSMCs need to be maintained in serum-starved conditions for a minimum of 72 h to induce a G0/G1 arrest. We have shown previously that heparin-induced cell cycle block is also present in MEFs and that PKR-null MEFs are insensitive to this block in G1 to S phase transition (16). The results shown in Fig. 6B with p27kip1-null MEFs further show that this heparin-induced block in G1 to S phase transition is dependent on p27kip1.

Thus, we have shown that the cell cycle block imposed by heparin in VSMC occurs by up-regulation of p27kip1 protein levels. Heparin causes stabilization of p27kip1 protein without enhancing its transcription or translation. In addition, the p27kip1 protein stabilization occurs by both PKR-dependent and independent pathways. The p27-null cells are markedly resistant to G1 to S phase block imposed by heparin, indicating that p27kip1 is essential for heparin-induced cell cycle block.

**DISCUSSION**

We have previously shown that heparin inhibits the VSMC proliferation by blocking the G1 to S transition. The interferon-induced protein kinase PKR was shown to be involved at least partly in mediating this cell cycle block in response to heparin (16). In PKR-null MEFs and in VSMCs expressing the transdominant negative K296R mutant of PKR, there was a marked but not a complete loss of the antiproliferative effects of heparin, indicating that pathways in addition to PKR activation...
FIG. 6. A, PKR kinase activity is essential for p27kip1 stabilization in response to heparin (hep). Rat VSMCs were made quiescent in DMEM with 0.1% FCS for 72–96 h. The cells were then treated with 10 μM 2AP for 2 h prior to the addition of heparin. After this treatment, 100 μg/ml heparin was added to the cells in 0.1% serum containing medium for 2 h. After 2 h, the cells were serum-stimulated with DMEM containing 10% serum and 100 μg/ml heparin. As a control, VSMCs were subjected to an identical treatment in the absence of 2AP. C lanes represent serum-stimulated cells without any treatment. Cell extracts were prepared 20 h after the start of heparin treatments, and a Western blot analysis was performed with an anti-p27kip1 antibody. The blot was then stripped and reprobed with anti-α-actin antibody to ascertain that equal amounts of protein from different cell extracts were analyzed. B, the p27kip1-null cells are unresponsive to the antiproliferative effects of heparin. Quiescent (Q) MEFs (0.1% serum for 24 h) were stimulated with 10% serum containing DMEM. A majority of the serum-starved wild-type and p27kip1-null MEFs were in G1 phase as judged by the cell cycle profile. These were pretreated with 100 μg/ml heparin in low serum medium for 2 h prior to the addition of 10% serum medium also containing 100 μg/ml heparin. As a control, the same treatments were done without any heparin. At 24 h after the serum stimulation, DNA was stained with propidium iodide and the cells were analyzed by flow cytometry. Quantitative analysis of the cell cycle profiles is shown. The data shown are representative of three separate experiments. The error bars represent the mean ± S.D.

may also contribute to growth inhibition. Other documented effects of heparin on VSMCs include inhibition of immediate-early gene expression (38, 39), matrix-degrading proteases (40–42), mitogen-activated protein kinase activation (43), matrix molecules (44, 45), and extracellular signal-regulated kinases, ERK1 and ERK2 (46). Thrombin-induced VSMC migration via inhibition of epidermal growth factor receptor transactivation has also been documented (47). The tyrosine kinase receptor EphB2 mRNA is also down-regulated by heparin (48).

Cellular proliferation is controlled by multiple Cdkks that are regulated by different cyclin proteins during distinct cell cycle phases (49). Cdk activity is also negatively regulated by the interaction with specific Cdk inhibitors, which cause a cell cycle block when overexpressed artificially in several cell lines (50–52). Two classes of Cdk inhibitors have been defined: the INK proteins (p16INK4a, p15INK4b, p18INK4c, and p19INK4c) and the Cip/Kip proteins (p21Cip1, p27kip1, and p57kip2) (53). The INK proteins interact with the Cdk4 and Cdk6 and thus inhibit them. The Cip/Kip proteins are thought to inhibit all Cdkks, and p27kip1 is thought to be the primary modulator of the proliferative status in most cell types where it is thought to cause and maintain the quiescent state (54). p27kip1 accumulates in serum-starved or density-arrested cultures, and the addition of mitogens to cultures rapidly down-regulates the levels of p27kip1 (13). Conditions that block p27kip1 down-regulation prevent quiescent cells from entering the cell cycle in response to mitogens (55). These conditions include treatment of cells with rapamycin (56–58), hypoxia-inducing agents (59), and cAMP analogs (60). Changes in p27kip1 protein levels in normal cells usually do not correlate with changes in the transcription of the p27kip1 gene (for review, see Ref. 12). The p27kip1 protein levels in normal cells are most commonly regulated by changes in the rate of proteasome-mediated p27kip1 degradation or the rate of translation of p27kip1 mRNA (27, 61, 62). Consistent with the cell cycle regulatory functions of p27kip1, the mice lacking p27kip1 exhibit large size because of enhanced growth and multiple organ hyperplasia (63). p27kip1 also functions as a tumor suppressor, and reduced expression of p27kip1 has been correlated with poor cancer patient survival (64, 65).

Accumulating evidence implicates p27kip1 as an important regulator of the phenotypic response of VSMCs to mitogenic or hypertrophic stimuli. At late time points after the balloon angioplasty, p27kip1 up-regulation has been shown to limit the growth of VSMCs (52, 66). In accordance with this finding, overexpression of p27kip1 can efficiently block mitogen-induced induction of cyclin A promoter activity in cultured VSMCs in vitro (52). Adenovirus-mediated overexpression of p27kip1 also effectively blocks vascular exclusive lesion formation in balloon-injured arteries in rats (67). In addition, p27kip1 has also been implicated in determining hypertrophic versus hyperplastic growth (26, 68). The protective role of p27kip1 against neointimal thickening has been also demonstrated in hypercholesterolemic apolipoprotein-deficient mice in which genetic inactivation of one or both p27kip1 alleles shows progressively accelerated atherosclerosis (56, 69, 70). The migratory response exhibited by VSMC in response to mitogens has been shown to be inhibited by overexpression of p27kip1 (58, 67, 71, 72). Inhibition of VSMC proliferation by salicylate has also been shown to be mediated by up-regulation of p27kip1 and p21 (73). Contrary to this finding, neointimal hyperplasia after mechanical damage of the artery wall was comparable in both wild-type and p27kip1-null mice (56, 74). In addition, although rapamycin-
dependent growth arrest in cultured cells has been shown to result from stabilization of p27kip1. Rapamycin does not prevent the down-regulation of p27kip1 seen after balloon injury (57). The neointimal formation after balloon injury was also found to be similar in wild-type and p27kip1-null mice in response to rapamycin where rapamycin inhibited VSMC proliferation in both wild-type and p27kip1-null mice to similar levels (74). These results indicate that the antiproliferative actions of rapamycin in VSMC are not mediated by p27kip1 but via other mechanisms.

Effects of heparin on cell cycle in VSMC have been analyzed previously by Castellot et al. (75). It was reported that heparin had a dual effect on G0 to S transition and that it delayed entry into S phase and also reduced the number of cells entering the cycle from G0. Through simple and elegant studies, this work further demonstrated that there were two points during G1 phase when the cells were sensitive to heparin, one was during early G1 and the other was during mid-late G1. Our data agree well with these early observations, because our results demonstrate that p27kip1 stability is increased by heparin during mid-late G1 phases. The early G1 block observed in this work may result from effects of heparin on cell cycle regulatory molecules other than p27kip1. For example, the induction of c-Fos and c-Myc in murine fibroblasts (76) and c-Fos induction in mesangial cells (77, 78) is blocked by heparin. In addition, heparin has been shown to inhibit cell cycle by inhibition of multiple pathways such as mitogen activated protein kinase (MAPK), calmodulin-dependent protein kinase II, and protein kinase C (PKC). Although seemingly separate, all of these pathways have been shown to affect cellular p27kip1 levels in recent years. Ottlinger et al. (79) documented that MAPK activation in response to FCS and phorbol 12-myristate 13-acetate but not in response to epidermal growth factor was inhibited by heparin in VSMC. Although not tested in this work, the MAPK pathway has been shown to have effect on cellular p27kip1 levels in VSMC. Pharmacological inhibition of MAPKs increased p27kip1 expression and attenuated VSMC proliferation and migration (72). MAPK cascade has also shown to be required for p27kip1 down-regulation and S phase entry in fibroblasts and epithelial cells (80). Inhibitory effects of heparin on activation of calmodulin-dependent protein kinase II have been documented by Mishra-Gorur et al. (81, 82) in VSMC. Recently, it has been shown that inhibition of calmodulin-dependent protein kinase II in NIH 3T3 cells enhances association of p27kip1 with Cdk2 and thus causes a G1 arrest (83). Our results are in agreement with these findings, because heparin-treated VSMC did exhibit a higher amount of p27kip1 associated with Cdk2 compared with the quiescent VSMC. Herbert et al. (84) showed that heparin blocks VSMC proliferation by interfering with the PKC pathway through a selective direct inhibition of the PKC-α isoenzyme. Pukac et al. (85) also showed that heparin inhibits activation of PKC by platelet-derived growth factor and serum but not by epidermal growth factor. Any connection between the inhibition of PKC-dependent pathways to the antiproliferative effects of heparin remains intriguing. This is especially true because, in various cell systems, the major effect of PKC activation appears to be an inhibition of cell cycle due to a reduced activity of Cdk2 as a result of increased levels of p27kip1 (86).

The levels of p27kip1 protein are regulated by changes in the protein stability during the cell cycle (12). Two different mechanisms have been shown to be responsible for this regulation, one operating during the G1/S phase and the other operating during the S phase (87). p27kip1 protein is much more stable during G0/G1 phase than during S phase. In cells exposed to mitogens, p27kip1 levels fall before Cdk2 becomes active and the decreases in p27kip1 levels are required for Cdk2 activation. Thus, Cdk2-independent processes mediate the down-regulation of p27kip1 levels during G1 phase (12). The phosphorylation of p27kip1 at threonine 187 by Cdk2/cyclinE has been shown to destabilize it and cause a rapid decline in its levels via the Skp2-dependent ubiquitin-proteasome pathway in the nucleus during the G1/S transition (88–91). The degradation of p27kip1 during G1/G0 transition takes place by a distinct pathway in the cytoplasm that is independent of both the threonine 187 phosphorylation and Skp2-mediated proteasome pathway (92). During the G0 phase, p27kip1 is phosphorylated by Mirk/Dyrk1B at serine 10 and this has been shown to increase its stability in the nucleus (93). Mirk/Dyrk1B levels rapidly fall after serum stimulation of G0-arrested cells. Another kinase, kinase-interacting stathmin, is induced in response to serum and maintains the serine 10 phosphorylation of p27kip1 during the G1 phase (94). Nuclear export of p27kip1 has been shown to occur during G1 phase, and phosphorylation at serine 10 has been shown to be required for binding to CRM1 via Jab1/CSN5 and subsequent nuclear export (95). However, because serine 10 is already phosphorylated during G0 phase, an additional signal must be required for causing the nuclear export of p27kip1 during G1 phase (96, 97). Evidence has also been presented that, although required for its cytoplasmic localization, serine 10 phosphorylation of p27kip1 is not a prerequisite for its proteolysis (98). Thus, although the threonine 187 phosphorylation-dependent proteasome pathway has been well established, the exact mechanism(s) regulating the p27kip1 destabilization during G1 phase has not been elucidated as yet.

An earlier report showed that heparin inhibits proliferation of myometrial and leiomyomalous smooth muscle cells through the induction of α-smooth muscle actin, calponin h1, and p27kip1 (99). However, no mechanistic experiments were presented in this study. Our results presented here indicate that p27kip1 protein is stabilized in VSMC during G1 phase by heparin treatment of VSMC via both PKR-dependent and independent pathways. In addition, the p27kip1-null cells show no effect of heparin treatment on cell cycle, thereby indicating p27kip1 stabilization to be a major mechanism for heparin-induced cell cycle arrest.

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