Heparin is a complex glycosaminoglycan that inhibits vascular smooth muscle cell (SMC) growth in vitro and in vivo. To define the mechanism by which heparin exerts its antiproliferative effects, we asked whether heparin interferes with the activity of intracellular protein kinase C (PKC). The membrane-associated intracellular PKC activity increased following stimulation of cultured rat SMCs with fetal calf serum and was suppressed by heparin in a time- and dose-dependent manner. Heparin acted through a selective inhibition of the PKC-α since preincubation of the cells with a 20-mer phosphorothioate PKC-α antisense oligodeoxynucleotide (ODN) eliminated the heparin effect. In vivo, following balloon injury of the rat carotid artery, particulate fraction PKC content increased with a time course and to an extent comparable with the observed changes in vitro. Heparin, administered at the time of injury or shortly thereafter, inhibited the activity of the particulate PKC and suppressed the in situ phosphorylation of an 80-kDa myristoylated alanine-rich protein kinase C substrate (MARCKS), a substrate of PKC. The topical application of the phosphorothioate antisense ODN selectively suppressed the expression of the PKC-α isoenzyme in vivo but did not affect injury-induced myointimal proliferation. Topical application of the ODN also eliminated the antiproliferative activity of heparin. These results therefore suggest that heparin might block SMC proliferation by interfering with the PKC pathway through a selective direct inhibition of the PKC-α isoenzyme.

Proliferation of vascular smooth muscle cells (SMC) plays an important role in the pathogenesis of atherosclerosis (1) and is an important contributor to the formation of the fibrocellular lesion following surgical intervention such as percutaneous transluminal coronary angioplasty or bypass graft (2). Over the last decade, a number of growth-promoting factors have been described, and considerable progress has been made in understanding the signal transduction mechanisms utilized by these various growth factors. In contrast to our understanding of growth-stimulating factors, comparatively little is known about the mechanism of action of growth inhibitors. In particular, the highly sulfated glycosaminoglycan heparin acts as a potent modulator of SMC growth. In vivo, heparin inhibits the migration and the proliferation of the cells in response to vascular damage (3). Heparin also inhibits proliferation and promotes the induction of a contractile phenotype in cultured SMCs (4–6). However, although the antiproliferative activity of heparin has been acknowledged for a long time, the mechanisms by which it acts are not well understood. Heparin inhibits the induction or transcription of several genes associated with cell cycle progression, including c-myc, c-myb, and c-fos protooncogenes, the mitochondrial ATP/ADP carrier protein 2F1, and histone H3 (7–9). Consonant with growth inhibition, heparin has been demonstrated to act selectively on a PKC-dependent pathway (7, 10, 11), and modulation of kinase activity by heparin in vitro has been frequently observed (12–15), suggesting that heparin may have multiple targets. Although heparin interferes with a PKC-dependent pathway, it is not yet evident which of the PKC isoenzymes is affected. The PKC family of enzymes consists of at least 10 closely related gene products that are involved in the propagation of signals initiated at the cell surface (16). PKC is thought to be involved in inflammatory processes, immune responses, and cell proliferation. However, our knowledge of specific functions of individual enzymes is still limited (16).

To understand better the mechanism by which heparin inhibits SMC proliferation, we asked whether heparin modulated the activity of PKC in vitro in cultured rat SMC or in vivo in medial SMC of rat carotid arteries subjected to balloon injury.

**Materials and Methods**

**Chemicals**—[γ-32P]ATP (3000 Ci/mmol) was purchased from Amer sham (Les Ulis, France). 12-O-Tetradecanoylphorbol-13-acetate (TPA), histone H1 (type IIIS), ATP, 1,2-diolein, aprotinin, leupeptin, phosphatidylinerine, heparinase (from Clostridium histolyticum), and Triton X-100 were purchased from Sigma (Saint-Quentin Fallavier, France). Heparin (SR 80258), a non-anticoagulant heparin fraction, was from Sanofi Recherche (Toulouse, France). Phosphorothioate ODNs were synthesized by Appligene (Strasbourg, France). ODNs were depropyalted by ultrafiltration, with endotoxin below detectable levels. The sequence of the 20-mer phosphorothioate antisense ODN designed to specifically hybridize murine PKC-α mRNA (17) was 5′-CAGCCATG- GTTCCCCTCAAAC-3′ (which is complementary to positions 243–262 of the PKC-α mRNA (18). The sequence of the scrambled control ODN was 5′-CCAGTCACCTCCACCATGCG-3′. Tissue culture reagents were from Boehringer Mannheim (Mannheim, FRG). All of the other chemicals used in the present study were of reagent grade.

**Smooth Muscle Cell Cultures**—Media fragments of Sprague-Dawley rat aortas were incubated for 16 h at 37 °C in Dulbecco’s modified Eagle medium (DMEM) containing 0.15% collagenase, 5% fetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 μg/ml), and glutamine (4 mM). After incubation, SMCs were sedimented by gentle centrifugation (400 × g for 10 min), resuspended in DMEM + 10% FCS, and grown at 37 °C in a humidified atmosphere of 5% CO2 in air. Culture medium (DMEM + 10% FCS) was changed every 3 days, and a confluent SMC monolayer was obtained after about 7 days. Cells were routinely used from the third to the sixth passage. For assays, cells were
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Preparation of Membrane and Cytosolic Fractions and Assays of PKC Activities—Membrane and cytosolic fractions of PKC were prepared according to Urrutia and Di Corleto (19). The pellet prepared from cultured SMC or from rat carotid arteries was resuspended and washed twice with ice-cold homogenizing buffer (20 mM Tris/HCl, pH 7.4, 10 mM 2-mercaptoethanol, 10 mM EGTA, 2 mM EDTA, 1 mM PMSF, and 0.25 mM sucrose) and homogenized using a Dounce homogenizer. The homogenate was centrifuged at 100,000 × g for 1 h at 4 °C. The supernatant was designated as the cytosolic fraction while the pellet was resuspended in 20 mM Tris/HCl, pH 7.4, 10 mM 2-mercaptoethanol, 10 mM EGTA, 2 mM EDTA, 1 mM PMSF, and 0.25 mM sucrose and homogenized using a Dounce homogenizer. The homogenate was centrifuged at 100,000 × g for 10 min. Cells were resuspended in phosphate-buffered saline and counted with a coulter counter (Coultronics, France) and centrifuged (1000 × g for 10 min). Supernatants were discarded and cell pellets were frozen at −20 °C for later use.

Determination of the Enzymatic Activity of the Various PKC Isozymes Present in SMC—Cell extracts prepared from cultured SMCs or from injured carotid arteries were suspended in ice-cold 20 mM Tris/HCl, pH 7.4, 10 mM 2-mercaptoethanol, 10 mM EDTA, 1 mM EGTA, 1 mM PMSF, and 2 µM leupeptin, and aprotinin (1 µg/ml) and homogenized using a Dounce homogenizer. The protein content of the various fractions was determined by a Bio-Rad DC protein assay using bovine serum albumin as standard. Aliquots were incubated for 2 h at 4 °C with 1 µg of anti-PKC-α, -β, -δ, and -ε polyclonal rabbit antisera (Boehringer Mannheim, France), sedimented with papsorbin A (Calbiochem, France), washed twice, and centrifuged at 100,000 × g for 1 h at 4 °C. Precipitated samples were then dissolved in buffer (20 mM Tris/HCl, pH 7.4, 10 mM 2-mercaptoethanol, 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM PMSF), and PKC was assayed as described above. Results were expressed as pmol of 32P incorporated into histone H1/min/105 cells or per mg of protein.

For the ODN treatments of Cells with ODNs—Rat aortic SMCs were grown in DMEM culture medium (10% FCS, 10 5 cells/well). After 3 days, cells were incubated with antisense ODNs (0.5 µg) and treated (20 µM 8-mercaptoethanol, 10 µM EGTA, 2 mM EDTA, 1 mM PMSF, and 0.25 M sucrose, 1 mM PMSF), snap-frozen in liquid nitrogen, stored at −80 °C, and later analyzed biochemically for PKC activity.

Effect of Heparin on the Activity of Intracellular PKC in Vitro—PKC exists in an inactive and soluble or an active and membrane-bound state, and these states are interconvertible (18). Changes in the bimodal distribution of PKC induced by diacylglycerol or tumor-promoting phorbol esters have been observed.
Fig. 1. Effect of heparin on FCS-induced activation of PKC in SMCs. Confluent quiescent rat SMCs in 24-well cluster plates were incubated with 5% FCS with (circles) or without (squares) heparin (100 

Fig. 2. Effect of heparin on FCS-induced activation of PKC in SMCs. Confluent quiescent rat SMCs in 24-well cluster plates were incubated with 5% FCS. Increasing concentrations of heparin (0.3 to 100 

proposed to regulate the activity of this kinase (16). As shown in Fig. 1, PKC was preferentially associated with the soluble fraction of quiescent rat SMCs, but the addition of 5% FCS led to a dramatic loss of the activity of PKC present in the cytoplasmic (soluble) fraction. Almost all of the cytoplasmic PKC disappeared 4 h after the beginning of the incubation with FCS, whereas shortly after the addition of FCS, there was a rapid increase of the activity of PKC present in the particulate extract of SMCs indicating that translocation of PKC had occurred. This result is in general agreement with other observations on other cell types with different agonists (16, 19, 21, 22).

Incubation of cells with heparin (100 

TABLE I

| PKC activity | Particulate | Soluble |
|--------------|-------------|---------|
| pmol/min/10^6 cells | | |
| 1. Resting SMCs | 1.9 ± 0.7 | 16.6 ± 3.9 |
| 2. SMCs, 16 h after the addition of 5% FCS | 15.2 ± 4.9 | 3.2 ± 1.9 |
| 3. SMCs, 16 h after the addition of 5% FCS in the presence of 100 

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Fig. 3. Effect of heparinase on the activity of heparin on FCS-induced activation of PKC in SMCs. Confluent quiescent rat SMCs in 24-well cluster plates were incubated with 5% FCS in the presence of heparin (100 

fraction in a time- and dose-dependent manner (Figs. 1 and 2). The ED_{50} value (dose causing 50% inhibition of the activity of particulate PKC) was 1.5 ± 0.4 

In order to eliminate any heparin bound to the cells that would inhibit PKC nonspecifically during the extraction process, SMCs were treated with heparinase prior to homogenization. In these experimental conditions, we observed exactly the same inhibitory effect (Fig. 3), therefore demonstrating that heparin is specifically interacting with the intracellular PKC and showing that this effect is not due to heparin carried over during preparation of the extracts. Using [3H]heparin in the same experimental conditions, we found that the heparinase treatment was able to eliminate 92% of the heparin initially added (data not shown). The heparinase-resistant, cell-associated fraction therefore represented a very small amount of the extracellular concentration (less than 0.8 

When added in vitro to cell extracts (particulate PKC prepared from untreated SMCs), such a small quantity of heparin only reduced PKC activity by 12%. Heparin added to particulate PKC showed an IC_{50} value of 32 

Heparinase treatment of heparin (100 

At various times, cells were rinsed and treated with heparinase (10 IU/ml at 37 °C for 15 min) (solid symbols) or saline (open symbols). Soluble (circles) and particulate (squares) extracts were prepared, and the PKC-specific activity was assessed as described under “Materials and Methods.” Each value is the mean ± S.D. of determinations from triplicate wells.

Note that, under the same experimental conditions, heparin inhibited FCS-induced proliferation of SMC in the same range of concentrations (ED_{50} = 2.8 ± 0.4 

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Effect of Heparin on FCS-induced PKC phosphorylation of MARCKS. Quiescent rat SMCs were incubated with saline (lanes 1, 2, and 4) or heparin (100 µg/ml) (lanes 3 and 5) in the presence of saline (lane 1), 5% FCS (lanes 2 and 3), or TPA (100 nM) (lanes 4 and 5). Cell extracts were prepared 24 h later, immunoprecipitated with anti-MARCKS antibodies, probed with a mixture of anti-phosphoserine and anti-phosphothreonine antibodies, and detected with anti-mouse IgG conjugated to horseradish peroxidase. MARCKS phosphorylation was quantitated by densitometric measurement. These results are from a typical experiment that has been reproduced three times without noticeable modifications.

Effect of Heparin on the Activity of the Various PKC Isoenzymes Present in Rat SMCs—Quiescent rat SMCs contained at least five PKC isoenzymes (Fig. 5A). PKC-β predominated and represented about 40% of the total PKC activity, although other PKC isoenzymes such as PKC-ζ, -ε, -δ, and -β were detected. Treatment with heparin (100 µM) dramatically inhibited total PKC activity but also totally abrogated the activity of all PKC isoenzymes (Fig. 5B).

Effect of Heparin on SMC Proliferation Following Down-regulation of PKC-α Expression—As already described (17), the antisense ODN that hybridizes the AUG translation initiation codon consistently inhibited PKC-α expression in SMCs (Fig. 5C). A single treatment of SMCs with 1 µM antisense ODN was found to decrease PKC-α protein by 87% after 4 h. In the same experimental conditions, the level of PKC-α activity was not affected by a scrambled sequence control phosphorothioate ODN or by the corresponding sense phosphorothioate ODN. As already shown by Dean and McKay (17), under conditions where the antisense ODN decreased PKC-α activity by 90%, levels of PKC-ζ, -ε, -δ, and -β were unchanged (Fig. 5C). We have extended these studies to determine the ability of heparin to inhibit SMC proliferation following selective down-regulation of PKC-α with the antisense ODN. As shown in Fig. 6, preincubation of SMC with the antisense ODN abrogated the antiproliferative effect of heparin, whereas preincubation of SMC with the corresponding sense or scrambled ODNs did not affect its ability to reduce SMC growth. IC₉₀ values were close to the IC₅₀ values obtained in the corresponding controls. It is noteworthy that none of the phosphorothioate ODNs (1 µM) affected 5% FCS-induced SMC proliferation (not shown).

Effect of Heparin on the Activity of Intravascular PKC in Vivo—High levels of PKC activity were found in the soluble fraction of tissue extract prepared from rat carotid arteries (Fig. 7). Following balloon injury, the amount of PKC activity present in the soluble fraction decreased dramatically at the same time the PKC activity detected in the particulate fraction increased. PKC translocation reached its maximum 48 h after the injury. By 1 week, the cytosolic and particulate PKC activities returned to the initial levels, therefore indicating that such a process was reversible and occurred as an early event, shortly after the injury. Similar to what was observed in vitro, heparin strongly inhibited PKC activity in both fractions. Moreover, it strongly reduced PKC activity in the soluble fraction, at late times after the vascular injury. Analysis of these fractions by a heparin-Sepharose chromatography column indicated that this effect was not due to an inhibition of the
As already shown, the artery induced a 4-fold increase of phosphorylated MARCKS. As shown in Fig. 9, balloon injury of the rat carotid artery (90% inhibition).

In order to go further into the details of the nature of such an effect, several additional control experiments were performed (Table II). 1) An intravenous bolus injection of heparin (1 mg/kg) did not affect the activity of PKC measured in the cytosolic fraction 10 min after the administration. 2) A 48-h infusion of heparin (0.1 mg/kg/h) to uninjured arteries did not inhibit PKC significantly in uninjured arteries. 3) Heparin (0.1 mg/kg/h), infused starting 18 h after the injury, only slightly reduced the activity of PKC measured in the particulate fraction, whereas when infused starting 6 h after balloononing, a strong inhibitory effect was observed at 48 h. 4) Heparin, administered as a bolus 48 h after the injury (1 mg/kg), did not affect PKC in the particulate fraction. This effect was specific for heparin since chondroitin 6-sulfate, infused for 48 h (0.1 mg/kg/h), did not affect either the translocation of PKC or its activity in the particulate fraction. This effect was specific for heparin since chondroitin 6-sulfate, infused for 48 h (0.1 mg/kg/h), did not affect either the translocation of PKC or its activity in the particulate fraction.

Translocation of the enzyme but was rather due to an effect on the enzyme itself (Fig. 8). Indeed, after elimination of the heparin bound on the enzyme, the levels of PKC activity in the particulate and cytoplasmic fractions isolated from treated or nontreated arteries were the same. The specificity of such an effect was further confirmed by the in vivo measurement of phosphorylated MARCKS. As shown in Fig. 9, balloon injury of the artery induced a 4-fold increase of phosphorylated MARCKS. As already shown in vitro, a 48-h treatment with heparin strongly reduced the level of phosphorylated MARCKS in injured artery (90% inhibition).

In order to go further into the details of the nature of such an effect, several additional control experiments were performed (Table II). 1) An intravenous bolus injection of heparin (1 mg/kg) did not affect the activity of PKC measured in the cytosolic fraction 10 min after the administration. 2) A 48-h infusion of heparin (0.1 mg/kg/h) to uninjured arteries did not inhibit PKC significantly in uninjured arteries. 3) Heparin (0.1 mg/kg/h), infused starting 18 h after the injury, only slightly reduced the activity of PKC measured in the particulate fraction, whereas when infused starting 6 h after balloononing, a strong inhibitory effect was observed at 48 h. 4) Heparin, administered as a bolus 48 h after the injury (1 mg/kg), did not affect PKC in the particulate fraction. This effect was specific for heparin since chondroitin 6-sulfate, infused for 48 h (0.1 mg/kg/h), did not affect either the translocation of PKC or its activity in the particulate fraction.

**Effect of Heparin on the Myointimal Proliferation Following Down-regulation of PKC-α**—Because PKC-α antisense ODN abrogated the inhibitory activity of heparin with regard to FCS-induced SMC growth in vitro, we sought to determine whether the local application of this ODN modified the effect of heparin on the vessel wall response to injury. Two weeks after injury of the carotid artery of saline-treated rats, the intima had grown substantially and was 21 ± 6% of the tunica media area. Neointimal area in the control group 14 days after endothelial injury was 0.27 ± 0.08 mm² (n = 10). In the right carotid artery (which had not been subjected to topical application of the antisense ODN), heparin (1 mg/kg/h, intravenously) strongly inhibited intimal proliferation (59 ± 16% inhibition, p < 0.001) (Fig. 10). As shown in Fig. 10, topical application of the antisense ODN (500 µg/artery) to the left carotid artery significantly reduced the antiproliferative activity of heparin.
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Attempts to define a mechanism of action that accounts for all these effects of heparin have not as yet been successful. Recent observations provide evidence that heparin may exert its effect in late G1 phase by inhibiting a serum-dependent step in the mitogenetic pathway (10, 24), but the precise location of the block has not yet been determined. Consonant with growth inhibition, heparin has been shown to inhibit the expression of early proto-oncogenes c-fos and c-myc in fibroblasts and SMCs (7, 8) by acting selectively on a protein kinase C (PKC)-dependent pathway (7, 11, 16). Moreover, modulation of kinase activity by heparin in vivo has been frequently observed, e.g. PKC (12, 13), casein kinase II (15), phosphatidylinositol 4-kinase (14), but none of these observations have been extended conclusively to intact cells. Since the modulation of a PKC-dependent pathway by heparin has been frequently suggested (7, 10, 13), we have examined the heparin sensitivity of PKC in vitro in cultured rat SMC and in vivo following balloon injury of the rat carotid artery.

The present study clearly demonstrates an agonist-induced PKC redistribution in cultured SMC but also in vivo following endothelial injury of the rat carotid artery. To our knowledge, our study is the first to show that PKC redistribution occurs as a response to growth factors that are thought to induce SMC proliferation both in vitro but also in vivo after mechanical manipulation of the vascular wall. This is supported by the observation that the translocation of PKC to the vicinity of the surface membrane coincided with peak cell proliferation observed in the same experimental conditions (10, 13, 25). The redistribution of PKC that was essentially complete during a period of 24–48 h following exposure with FCS or after balloon injury was a reversible process. Most interesting was the observation that the translocation between the PKC translocation observed in FCS-stimulated cultured rat SMCs and in medial SMCs following balloon injury of the rat carotid artery.

Although a number of studies have been performed, the role of PKC in mitogenesis remains unclear. It has been suggested that PKC promotes the G1/G0 transition, since phorbol esters and diacylglycerol can replace competence factors (25–27) and stimulate the expression of immediate early genes such as c-fos and c-myc (28, 29). However, several studies also showed that the PKC pathway operates as a negative regulator in SMC proliferation, even in the absence of exogenous PKC activators such as phorbol esters or diacylglycerol, a physiological activator of PKC (25, 30, 31), but it remains to be clarified whether these effects are due to activation of PKC or to down-regulation that occurs as a result of prolonged stimulation of PKC.

The present study demonstrates that a heparin treatment of SMCs in culture and in the rat carotid artery selectively inhibits PKC activity following mitogenic stimulation. This effect of heparin occurred at concentrations that have already been reported to affect PKC activity (13) and to inhibit SMC growth in vitro (7–11) but also at doses that have been reported several times to reduce intimal hyperplasia that occurred as a result of vascular injury (3, 5). While inhibiting both membrane-associated PKC and PKC remaining in the cytosol, heparin did not appear to affect the translocation process itself. Both in vitro and in vivo, this effect was associated with an inhibition of the in situ phosphorylation of MARCKS, a well known intracellular substrate of PKC (23). This latter observation is of considerable importance since it shows that the effect of heparin occurs in situ on intact cells and could not be attributed to an artifact caused by cell or matrix-bound heparin contamination of the extract. Since the inhibitory effect of heparin could not be overcome by a treatment with heparinase, our results demonstrate that heparin affected the intracellular activity of PKC directly. This effect upon PKC was specific for heparin and was

Table II

| Experimental conditions | PKC activity |
|-------------------------|-------------|
|                         | Particulate | Cytosolic |
| Controls (10 min after injury) | 7.4 ± 3.8 | 112.7 ± 21.9 |
| Heparin (iv bolus 10 min before injury) | 6.0 ± 1.6 | 127.8 ± 14.9 |
| Uninjured arteries: controls | 11.8 ± 8.1 | 115.1 ± 27.0 |
| Uninjured arteries: heparin (infused for 48 h) | 18.1 ± 4.8 | 88.9 ± 37.3 |
| Controls (48 h after injury) | 132.0 ± 21.5 | 15.3 ± 6.7 |
| Heparin (infused for 48 h, starting immediately after injury) | 8.1 ± 2.6 | 12.4 ± 4.2 |
| Heparin (infused for 12 h, starting 6 h after injury) | 27.2 ± 3.7 | 12.9 ± 2.9 |
| Heparin (infused for 30 h, starting 18 h after injury) | 78.2 ± 12.6 | 19.0 ± 5.9 |
| Heparin (administered as a bolus 48 h after injury) | 121.6 ± 21.0 | 9.2 ± 2.2 |
| Chondroitin 6-sulfate (infused for 48 h) | 127.2 ± 7.4 | 13.1 ± 8.9 |

Fig. 10. **Effect of an antisense ODN on the antiproliferative activity of heparin in vitro.** Rat carotid arteries were treated with F127 pluronic gel containing either saline or 500 μg of sense, scrambled, or antisense ODNs. 24 h later, balloon injury was performed, and animals were treated by a continuous infusion of saline (shaded bars) or heparin (1 mg/kg/h, intravenously) (empty bars). After 14 days, arteries were removed, and the intimal and medial cross-sectional areas were measured with an image analyzer as described under "Materials and Methods." Data are expressed as mean % inhibition ± S.D. of myointimal proliferation (n = 11). Statistical significance: ***p < 0.001 (Mann-Whitney test).
not observed with chondroitin sulfate, a glycosaminoglycan related to heparin.

These observations therefore raise a discrepancy with earlier experiments that showed that heparin did not inhibit the phorbol 12-myristate 13-acetate-stimulated phosphorylation of a Mr 80,000 protein in BALB/c cells (7) or PKC activity as measured in cell extracts (11). The basis of this discrepancy is unknown but may be related to differences in cell types, culture, or assay conditions. It can also be attributed to the mode of stimulation of PKC with phorbol 12-myristate 13-acetate that induces a strong and rapid increase of the total PKC activity (16). Indeed, in our experiments, serum or balloon injury, while inducing a rapid and sustained translocation of PKC from the cytosol to the plasma membrane, did not significantly modify the total amount of intracellular PKC activity.

It is noteworthy that the pharmacological kinetics and specificity of heparin on PKC paralleled its effects on SMC proliferation both in vitro and in vivo. For example, heparin treatment after balloon injury could be delayed 6–18 h and still inhibit PKC and SMC proliferation.

PKC is a complex family of isoforms, which include calcium-dependent PKCs (PKC-α, β, and γ) and calcium-independent PKC-related enzymes (PKC-ε, ζ, δ, η, θ, and λ) (16, 32–34). Each isoform shows a slightly different mode of activation, substrate specificity, sensitivity to phorbol ester-induced down-regulation, and intracellular and tissue distribution. Little is known about the isoform-specific action of PKC on SMC proliferation in vitro and in vivo. We therefore studied the effect of heparin on the activity of the various PKC isoenzymes found in cultured SMCs.

This study revealed that five isoforms of PKC, i.e., PKC-α, β, ε, ζ, and δ, are expressed in SMCs. This result is in agreement with already published observations (35, 36). PKC-α appears to be the principal PKC isoform expressed in late G1. This PKC isoform has been previously suggested as a possible mediator of the G1/S transition in SMCs (25). The exact role of PKC-α in SMC growth has not yet been characterized, but a recent study suggests that it may be associated with differentiation of SMCs in culture (37).

When incubated with SMCs in culture, heparin reduced the activity of all PKC isoenzymes therefore suggesting that the action of heparin was not specific for one PKC isoenzyme in particular. Since PKC-α is the principal PKC isoform in rat SMCs, we used a phosphorothioate antisense ODN recently designed to inhibit PKC-α expression in vitro and in vivo (17) to evaluate the effect of this down-regulation on the overall anti-proliferative activity of heparin.

We observed that, both in vitro and in vivo, the antisense ODN achieved isoenzyme-selective inhibition of PKC-α as already shown by Dean and McKay (17) in other experimental systems. We were surprised to observe that this selective inhibition of PKC-α by the antisense ODN did not affect either FCS-induced SMC proliferation in vitro or myointimal proliferation in vivo.

This observation is remarkable since PKC expression in general and PKC-α expression in particular has been associated with SMC growth or cell differentiation (25, 37). Our data are consistent with the studies of Haller et al. (37) demonstrating that PKC-α declines in cycling cells and that PKC-α overexpression induces differentiation and cell growth. If PKC-α activity is associated with cell differentiation, then blockade or elimination of PKC-α with antisense ODNs should promote de-differentiation and perhaps cell growth. Our finding that the antisense ODNs to PKC-α eliminated the effect of heparin without further stimulation of SMC growth is certainly compatible with Haller’s conclusions.

How are we to understand the apparent paradoxical effects of heparin on PKC-α? Heparin, like PKC-α, induces α-actin expression in rat SMC (37). If they both are differentiation and growth inhibiting factors, then they should act cooperatively or possibly synergistically. In fact, heparin inhibits PKC-α phosphorylation activity but requires the presence of PKC-α for inhibition of SMC growth. The observations seem mutually incompatible. However, we note that heparin inhibits all of the PKC isoforms, some of which might be needed for cell cycle progression. The effect of heparin is not irreversible since PKC activity can be recovered after the extracts of heparin-treated cells have been passed over a heparin-Sepharose column. Heparin may be binding to PKC-α and thereby altering its function. It could be that minute amounts of heparin complexed to PKC-α could not only decrease kinase activity but also increase some other activity (phosphatase activity?) or block other kinases by occupying critical sites on the membrane. In short, heparin-PKC-α might act like a “dominant negative mutant” and thereby interfere with the overall kinase cascade associated with PKC.

Since little is known about the exact role of PKC-α in the overall mitogenic activity of serum and in the effect of the initial vascular injury on the subsequent myointimal response, it is difficult to find the link between the numerous effects of heparin described on SMC in response to various stimuli and the present observation of a selective effect of heparin with regard to PKC-α. In particular, one will have to determine how this effect relates to other effects of heparin on SMCs such as the selective inhibition of the expression of the early proto-oncogenes c-fos and c-myc (7, 8), the AP-1-mediated transactivation of the phorbol ester responsive element (38), or the inhibition of the induction or transcription of other genes associated with cell cycle progression, including c-myc, the mitochondrial ATP/ADP carrier protein 2F1, histone H3 (11), t-PA (39), collagenase (40), and ornithine decarboxylase (41).

In conclusion, our present observations provide evidence for a role of PKC-α in the anti-proliferative effect of heparin for SMCs. Our data suggest that this effect on PKC-α plays an important role in the regulation of SMC proliferation and cell cycle progression, but because PKC-α is present in many cell types, it may therefore be important to determine if heparin also exhibits a similar effect with regard to other events where the activation of PKC-α occurs.
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Protein Kinase C α Expression Is Required for Heparin Inhibition of Rat Smooth Muscle Cell Proliferation in Vitro and in Vivo
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