Heparin Inhibits Phosphorylation and Autonomous Activity of Ca^{2+} /Calmodulin-Dependent Protein Kinase II in Vascular Smooth Muscle Cells

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Vascular smooth muscle cell (VSMC) hyperproliferation is a characteristic feature of both atherosclerosis and restenosis seen after vascular surgery. A number of studies have shown that heparin inhibits VSMC proliferation *in vivo* and in culture. To test our hypothesis that heparin mediates its antiproliferative effect by altering Ca^{2+} regulated pathways involved in mitogenic signaling in VSMC, we analyzed the effect of heparin on multifunctional Ca^{2+} /calmodulin dependent protein kinase II (CaM kinase II) which is abundantly expressed in VSMC. Using activity assays, radioactive labeling, and immunoprecipitation it was found that heparin inhibits the overall phosphorylation of the δ -subunit of CaM kinase II which is consistent with inhibition of autophosphorylation-dependent, Ca^{2+} /calmodulin-independent CaM kinase II activity. This effect was less evident in heparin-resistant cells, consistent with a role for CaM kinase II in mediating the antiproliferative effect of heparin. Finally, the effects of pharmacological inhibitors of phosphatases like okadaic acid, calyculin, and tautomycin suggest that heparin inhibits CaM kinase II phosphorylation by activating protein phosphatases 1 and 2A. These findings support the hypothesis that alterations in calcium-mediated mitogenic signaling pathways may be involved in the antiproliferative mechanism of action of heparin. (*Am J Pathol* 2002, 161:1893–1901)

Diseases of the heart and blood vessels are the leading cause of death in the United States and are responsible for almost half the deaths recorded each year. Most of these are due to atherosclerosis and its ensuing complications, which include hypertension, myocardial infarction, and gangrene. Hyperplasia of vascular smooth muscle cells (VSMC) is the hallmark of early atherogenesis

and is considered the major cause of the high failure rate of vascular surgical procedures such as angioplasty, coronary artery bypass grafts, and heart transplants.¹ The severity and prevalence of the problems associated with VSMC hyperplasia have provided the impetus to develop and characterize inhibitors of VSMC proliferation.

Heparin and heparan sulfates are one such class of VSMC proliferation inhibitors.² Work in our laboratory and by others has supported the antiproliferative role of heparin in animals and in culture systems.³ Heparin suppresses VSMC and mesangial cell proliferation while most other cells are unaffected.⁴ Several studies point to the possibility that heparin blocks VSMC and mesangial cell proliferation via alterations in mitogenic signal transduction pathways.^{5–7} Heparin binds to specific, saturable high-affinity binding sites on VSMC and is internalized by receptor-mediated endocytosis.⁸ Heparin has also been shown to selectively block the PKC pathway of mitogenic signaling as well as the phosphorylation and activation of MAPK.^{5,9} This is followed by a rapid down-regulation of mRNA levels of genes involved in growth regulation (eg, *c-fos*, *c-jun*, *myb*, and *myc*), thus blocking cells in the G₁ phase of the cell cycle.¹⁰ Despite these studies a cause-effect relationship has not yet been established.

An important aspect of cellular signaling involves Ca^{2+} mobilization in response to various stimuli. Calcium regulates a number of important functions in cells including proliferation, migration, contraction, and transcription, and the ability of heparin to block calcium transients in SMC has been appreciated for more than a decade. Several studies using permeabilized VSMCs have shown that heparin can block IP_3 -induced calcium release.^{11–13} Himpens et al found a PKC-dependent correlation between nuclear and cytoplasmic calcium transients¹⁴ and showed that a rise in intracellular Ca^{2+} concentration induces the transcription of several eukaryotic genes, including transcription factors like *c-fos*.¹⁵ Heparin is also capable of blocking phase II calcium transients in mesangial cells that are sensitive to the antiproliferative effect of heparin.⁷ Other studies provide evidence that Ca^{2+} /

Supported by National Institutes of Health Grants HL49973 (to J.J.C.) and HL49426 (to H.A.S.).

Accepted for publication July 24, 2002.

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calmodulin-dependent protein kinase II (CaM kinase II) can activate MAPK in VSMC,¹⁶ and we have reported that both non-muscle myosin heavy chain and MAPK are rapidly and specifically tyrosine-dephosphorylated on heparin treatment of VSMC.¹⁷

Based on the work described above, we hypothesized that heparin could mediate its antiproliferative effect by modulating CaM kinase activity in VSMC. Here we report the effect of heparin on the phosphorylation state and activity of VSMC CaM kinase II. Our studies demonstrate that heparin inhibits both the overall phosphorylation of the CaM kinase II subunits and the autophosphorylation-dependent generation of Ca²⁺/calmodulin-independent (or "autonomous") kinase activity. Further, we provide evidence that heparin inhibits CaM kinase II phosphorylation by activating protein phosphatases. This effect was not observed in heparin-resistant VSMC or with non-antiproliferative glycosaminoglycans, supporting a role for CaM kinase II in mediating the antiproliferative effect of heparin.

Experimental Procedures

Cell Culture

Rat aortic SMC from Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were isolated, cultured and characterized as previously described.⁴ Briefly, the abdominal segment of the aorta was removed and the fascia cleaned away under a dissecting microscope. The aorta was cut longitudinally and small pieces of the media were carefully stripped from the vessel wall. Two or three such strips were placed in 60-mm dishes. Within 1 to 2 weeks, VSMC migrated from the explants; they were capable of being passaged approximately 1 week after the first appearance of cells. They were identified as smooth muscle cells by their "hill and valley" growth pattern and indirect immunofluorescence staining for VSMC specific α -actin. VSMC cultures were maintained in RPMI 1640 plus 10% fetal calf serum at 37°C in a 5% CO₂/95% air incubator. Primary cultures were used for 8 to 9 passages. Cells were counted using a Coulter counter (Coulter Counter Corporation, Hialeah, FL). Cells were routinely grown in RPMI 1640 containing 10% fetal calf serum (FCS). SV40 large T antigen transformed heparin resistant and sensitive cells¹⁸ were grown and maintained under similar conditions.

Heparin-Resistant Cells

Rat aortic VSMC were transfected with SV40 large T antigen in a retroviral vector that also contained the neomycin resistance gene. Stable transfectants that were resistant to heparin were isolated and cultured as previously described.¹⁸

Growth Arrest of Cells

Cells were routinely plated at 5–6 × 10⁵/100 cm² dish, washed with RPMI, and placed in RPMI containing 0.2 to

0.4% FCS for 72 hours.⁹ Flow microfluorometry and termination of [³H]thymidine-labeled nuclei indicated that greater than 95% of the cells were arrested in G₀(G₁). Cells were released from quiescence by replacing the low serum medium with normal growth medium (ie, RPMI 1640 containing 10% FCS). The cells were approximately 40 to 60% confluent at the time of harvest.

Protein Analysis

Quiescent (G₀) cells were treated with 10% FCS/RPMI ± heparin for various time intervals. Most experiments reported here were performed with 500 μ g/ml heparin or chondroitin sulfate, experiments performed with 100 μ g/ml of heparin or chondroitin sulfate gave quantitatively identical results. Proteins were harvested with 100 μ l of lysis buffer (TBS + 1% NP40, 10% glycerol, 100 mmol/L NaF, 2 mmol/L PMSF, 20 μ g/ml aprotinin, 1 μ g/ml leupeptin, 2 mmol/L vanadate), rocked for 20 minutes/4°C, and then spun at 12,000 rpm for 10 minutes. Supernatants were stored at –20°C until use. Protein estimations were done by the Pierce BCA method. 50 μ g of protein lysate was boiled with 1X SDS sample loading buffer, resolved by SDS-PAGE, and blotted onto nitrocellulose membranes in Towbin buffer.

Western Blot Analysis

Nitrocellulose membranes were blocked with 5% milk in 1X TBS and immunostained with anti-CaM kinase II antibody and HRP-coupled anti-rabbit IgG antibody. The affinity purified anti-CaM kinase II antibody used was previously described¹⁹ (C26, anti-CaM kinase II δ isoform).²⁰ The antibody was raised in rabbits against a peptide (KPPCIPNGKENFSGGTSLSWQNI) corresponding to the unique C terminus of δ -subunit variants. The proteins were visualized by the ECL (Dupont, Wilmington, DE) Renaissance detection system.

Radioactive Labeling and Immunoprecipitation of CaM Kinase II

Quiescent cells were rinsed in phosphate-free RPMI + 0.1% FCS and incubated in this medium for 2 hours in the presence of 200 μ Ci/ml of [γ -³²P]phosphate.²¹ Cells were rinsed with RPMI and then treated with 10% FCS/RPMI + heparin or with 1 μ mol/L ionomycin/RPMI ± heparin. Proteins were harvested in buffer A (50 mmol/L MOPS pH 7.4, 2 mmol/L EGTA, 100 mmol/L NaF, 100 mmol/L Na pyrophosphate, 2 mmol/L DTT, 0.2 mmol/L PMSF, 1% NP-40 and 0.4 unit/ml aprotinin), allowed to sit on ice for 5 minutes and the lysates cleared by centrifuging at 10,000 rpm/10 minutes at 4°C. The supernatant was used for immunoprecipitation with anti-CaM kinase II δ -subunit specific antibodies¹⁹ for 90 minutes at 4°C followed by protein A+G agarose beads (Oncogene Science, Cambridge, MA) for 60 minutes at 4°C. The immunoprecipitates were resolved by SDS-PAGE, blotted onto nitrocellulose and differential phosphorylation analyzed after overnight exposure on a PhosphorImager.

Kinase Assays

Proteins were harvested as described above. Total CaM kinase II activity was assayed as previously described.¹⁹ Briefly, reactions were carried out in 25- μ L volume containing 10 mmol/L MOPS (pH 7.4), 10 mmol/L MgCl₂, 3 mmol/L EGTA, 4 mmol/L CaCl₂, 400 nmol/L calmodulin, 0.2 mmol/L [γ -³²P]ATP (400–1000 cpm/pmol), 20 μ mol/L autocamtide-2 (KKALRRQETVDAL) as substrate,¹⁶ and 0.5 to 2.5 μ g of lysate protein. Determination of the Ca²⁺/CaM-independent (autonomous) activity in the same lysates was performed by omitting CaCl₂ and CaM from the kinase assay mixture. The reactions were carried out at 30°C for 2 minutes and terminated by precipitation of the phosphorylated peptide on Whatman P-81 paper. The papers were rinsed in 75 mmol/L phosphoric acid and the adherent radioactivity quantified by liquid scintillation counting. As controls, autonomous CaM kinase II activity was measured in quiescent (G₀) cells; blanks containing the peptide substrate with no cell lysates were used as controls. Kinase activities were calculated as nanomoles of P_i transferred to the substrate/min/mg lysate protein and autonomous activity is expressed as percentage of total activity.

Inhibitor Studies

Okadaic Acid

Quiescent VSMC were radioactively labeled as described above. Ninety minutes into the incubation with [γ -³²P]phosphate, okadaic acid was added to the plates at 0.1 nmol/L or 15 nmol/L final concentration for 30 minutes. The cells were then stimulated with heparin + 1 μ mol/L ionomycin for 5 minutes. Cells were harvested and used for immunoprecipitation with anti-CaM kinase II δ -specific antibodies as described above and the immunoprecipitates resolved by SDS-PAGE. The proteins were blotted onto nitrocellulose and phosphorylation detected by overnight exposure on a PhosphorImager cassette.

Calyculin and Tautomycin

Quiescent VSMC were radioactively labeled with [γ -³²P]phosphate as described above. Ninety minutes into the incubation, calyculin or tautomycin was added at a final concentration of 1 nmol/L. The cells were then treated with heparin + 10% FCS for 5 minutes. The cells were harvested and lysates used for immunoprecipitation as described above.

Results

A number of studies have suggested that heparin may mediate its antiproliferative effect on VSMC by altering calcium regulated signaling pathways.^{7,22} It is important to note that most of the studies establishing heparin as a pharmacological inhibitor of Ca²⁺ channels have been performed using permeabilized cells.^{11–13} In addition, studies involving measurement of [Ca²⁺]_i by Miralem et

al²² suggest that heparin does not mediate its antiproliferative effect by altering Ca²⁺ transients. Further, previous observations in our laboratory as well as other studies^{22,23} have suggested that heparin affects the CaM kinase II signaling pathway in VSMC. Based on these observations and the recent reports of CaM kinase II subunit expression and activation by Ca²⁺-dependent stimuli in VSMC¹⁹ we reasoned that CaM kinase II may be a target of heparin. To test this hypothesis, we performed kinase assays to estimate the activation of CaM kinase II in VSMC in cells treated with 10% FCS in the presence or absence of heparin. The assay is based on a specific peptide substrate (autocamtide-2) and takes advantage of the unique property of CaM kinase II to undergo an activation dependent autophosphorylation¹⁶ (on Thr^{286/287})²⁴. This autophosphorylation results in the generation of Ca²⁺/calmodulin-independent or “autonomous” activity which can be measured in cell lysates.¹⁹ Autocamtide-2 has been found to be a relatively specific substrate for CaM kinase II as compared to other multifunctional kinases such as protein kinase C and cAMP-dependent protein kinases.²⁵

Heparin Inhibits CaM Kinase II Activity

Quiescent VSMC were treated with 10% FCS/RPMI $\pm$ heparin for 5 minutes at 37°C, after which the cells were harvested. VSMC treated with 1 μ mol/L ionomycin/RPMI were used as a positive control since ionomycin, a calcium ionophore, strongly activates CaM kinase II in VSMC.¹⁹ Quiescent cells were pretreated with heparin (10–20 minutes), rinsed and then treated with ionomycin to ensure that the heparin-effect observed on ionomycin induced CaM kinase activity was not a result of heparin binding to ionomycin. Total CaM kinase II activity measured in these cells was 6.5 ± 0.3 pmol/min/mg protein. Autonomous CaM kinase II activity was calculated as a percentage of the total activity present in the cell. In resting or quiescent (G₀) cells autonomous CaM kinase II activity ranged from 7 to 12% of total activity, similar to values previously reported in VSMC.¹⁹ A 40% reduction was seen in the autonomous CaM kinase II activity in cells treated with heparin + 10% FCS as compared to cells treated with 10% FCS for 5 minutes (Figure 1). A 60% reduction in autonomous activity was seen in cells pretreated with heparin for 10 minutes followed by ionomycin treatment for 30 seconds as compared to cells treated with ionomycin alone for 30 seconds (Figure 1; note the different scales on the two y axes). Thus, heparin inhibits the generation of autonomous CaM kinase II induced by both serum and ionomycin.

Heparin Inhibits CaM Kinase II Phosphorylation

An increase in intracellular calcium is detected by calmodulin which binds to CaM kinase II. The binding of the Ca²⁺/calmodulin complex results in activation of CaM kinase II and subsequent autophosphorylation and generation of autonomous CaM kinase II activity.²⁶ In our experiments, serum and ionomycin-stimulated increases in autonomous CaM kinase II activity are inhibited by

Figure 1. Heparin inhibits CaM kinase II activity in sensitive cells. Quiescent heparin-sensitive cells were treated with 10% FCS/RPMI in the presence (Hep+FCS) or absence (FCS) of heparin; or pretreated with heparin for 10 minutes, rinsed and then treated with 1 μ mol/L ionomycin for 30 seconds (H+Iono) or treated with 1 μ mol/L ionomycin alone for 30 seconds (Iono). Cells were harvested and lysates cleared by centrifugation at 10,000 rpm for 10 minutes. The lysates were then used to perform kinase assays using autocalmitide-2, a specific CaM kinase II substrate and γ - 32 P. The specific activity was measured as the picomoles of ATP transferred to the substrate/min/mg protein. The amount of calcium/calmodulin-independent (autonomous) activity is reported as a percentage of the total CaM kinase II activity in the cell. Student's *t*-test indicates *P* < 0.02 for FCS $\pm$ heparin treated cells; *P* < 0.01 for ionomycin $\pm$ heparin-treated cells.

heparin. To understand the mechanism of how heparin inhibits CaM kinase II activity, the effect of heparin on the overall phosphorylation state of CaM kinase II was tested by radioactive labeling of cells followed by immunoprecipitation of the enzyme with CaM kinase II antibodies that specifically recognize the C terminus of the δ -subunits, the predominant isozymes expressed in VSMC.¹⁹

Quiescent VSMC were equilibrated with [γ - 32 P]phosphate in phosphate-free RPMI. The cells were then treated with 10% FCS/RPMI in the presence or absence of heparin for 5 minutes. This time point was chosen because previous work in other laboratories confirms it as an appropriate length of time for detection of changes in CaM kinase II.^{19,20} Cell lysates were prepared as before and used for immunoprecipitation with antibodies specific for the δ -subunit of CaM kinase II subunit. SDS-PAGE analysis of the immunoprecipitates followed by autoradiography shows that heparin inhibits the overall phosphorylation of CaM kinase II subunits induced by FCS treatment in a dose-dependent manner (Figure 2).

To test the specificity of the effect of heparin on CaM kinase II phosphorylation, quiescent VSMC were labeled with [γ - 32 P]phosphate and then treated with 10% FCS/RPMI containing chondroitin sulfate, a non-antiproliferative glycosaminoglycan, for 5 minutes (Figure 3, top panel). Cells were harvested and lysates used for immunoprecipitation as described above. Autoradiography of the immunoprecipitates indicates that chondroitin sulfate does not affect phosphorylation of CaM kinase II. The slight attenuation of phosphorylation compared to 10% FCS/RPMI is probably due to heparin-like contaminants in the chondroitin sulfate preparation used. Thus, heparin inhibition of autonomous CaM kinase II activity appears to be mediated at the level of the autophosphorylation reaction or proximally at the level of Ca^{2+} or calmodulin.

Figure 2. Heparin inhibits phosphorylation of CaM kinase II in a dose-dependent manner. Quiescent VSMC were radioactively labeled with [γ - 32 P]phosphate for 2 hours in phosphate-free medium followed by stimulation with 10% FCS/RPMI alone (F) or containing heparin at 500 μ g/ml (H) or 100 μ g/ml (H100) for 5 minutes. The cells were harvested and lysates used for immunoprecipitation with anti-CaM kinase II antibodies. The immunoprecipitates were analyzed by SDS-PAGE, blotted onto nitrocellulose, and differential phosphorylation detected by overnight exposure on a PhosphorImager cassette. Densitometric analysis was performed using background subtraction methods to normalize the different background exposure levels. Compared to the serum-treated control cells, CaM kinase II phosphorylation was reduced 49% and 68% at 100 μ g/ml and 500 μ g/ml heparin, respectively.

Heparin Inhibits Ionomycin Induced Phosphorylation of CaM Kinase II

Since heparin was found to inhibit generation of autonomous CaM kinase II activity in response to ionomycin, we determined if heparin would also block ionomycin induced phosphorylation of CaM kinase II (Figure 3, bottom panel). As described above, quiescent VSMC were labeled with [γ - 32 P]phosphate for 2 hours. The cells were then treated with 1 μ mol/L ionomycin in the absence or presence of heparin for 5 minutes, cells were harvested and lysates used for immunoprecipitation with anti-CaM kinase II antibodies as described above. Quiescent, untreated VSMC were used to determine the phosphorylation of CaM kinase II in resting cells. Autoradiography shows that heparin blocks ionomycin induced phosphorylation of CaM kinase II. To ensure that this was not an effect of heparin chelating ionomycin, making ionomycin unavailable to induce CaM kinase II, cells were pretreated with heparin for 15 minutes, the medium washed off and then treated with RPMI containing 1 μ mol/L ionomycin. Prior studies in our laboratory have shown that the washing conditions used here effectively removes >95% of exogenously added heparin from the cells. Heparin also inhibited the effect of ionomycin on CaM kinase II phosphorylation under these conditions indicating that the heparin effect on ionomycin-induced autonomous CaM kinase II is not due to binding to ionomycin (Figure 3, bottom panel). Thus, like serum, the reduction in ionomycin-induced CaM kinase II autonomous activity is mirrored by the reduction in phosphorylation of CaM kinase II.

Heparin Inhibits CaM Kinase II Phosphorylation by Activating Protein Phosphatases

Inhibition of serum as well as ionomycin-induced CaM kinase II autonomous activity and phosphorylation could be due to inhibition of Ca^{2+} transients or Ca^{2+} /calmod-

Figure 3. Top panel: Heparin but not chondroitin sulfate inhibits phosphorylation of CaM kinase II. Quiescent VSMC were radioactively labeled with [γ^{32} P]phosphate for 2 hours in phosphate free medium followed by stimulation with 10% FCS/RPMI alone (F) or containing chondroitin sulfate (C) or heparin (H) for 5 minutes. The cells were harvested and lysates used for immunoprecipitation with anti-CaM kinase II antibodies. Quiescent, untreated (G_0) cells were used as controls. The immunoprecipitates were analyzed by SDS-PAGE, blotted onto nitrocellulose and differential phosphorylation detected by overnight exposure on a PhosphorImager cassette. Densitometric analysis was performed as described above. Compared to the serum-treated control cells, CaM kinase II phosphorylation was reduced 59% in heparin-treated VSMC, and 22% in chondroitin-treated VSMC. **Bottom panel:** Heparin inhibits ionomycin induced phosphorylation of CaM kinase II. Quiescent VSMC were radioactively labeled as before and treated with 1 μ mol/L ionomycin (Iono) for 5 minutes. Cells pretreated with heparin for 10 minutes (H+Iono) were rinsed and then stimulated with 1 μ mol/L ionomycin for 5 minutes. Quiescent, untreated (G_0) cells were used as controls. Lysates were used for immunoprecipitation as described in the top panel and phosphorylation detected by exposure to a PhosphorImager cassette. Densitometric analysis was performed as above; heparin was found to inhibit ionomycin-induced phosphorylation by 82%.

ulin complexes that activate CaM kinase II or by the stimulation of phosphatase activities that act to reverse the autophosphorylation event. Phosphatases have been implicated in the regulation of CaM kinases in the brain, where protein phosphatase 2A (PP-2A) and protein phosphatase 1 (PP-1) have been found to play a major role in the dephosphorylation of soluble and postsynaptic density associated CaM kinase II.²⁷ To test the hypothesis that heparin activates phosphatases, we used okadaic acid, a protein phosphatase inhibitor. Okadaic acid inhibits PP-2A (IC_{50} = 0.1 nmol/L) and PP-1 (IC_{50} = 10–15 nmol/L).^{28,29} Quiescent VSMC were labeled with [γ^{32} P]phosphate for 2 hours. Ninety minutes into the incubation, different concentrations of okadaic acid were added to the medium for 30 minutes. The cells were then treated with 10% FCS/RPMI in the presence or absence of heparin for 5 minutes. Cells were harvested and used for immunoprecipitation as described before. Ionomycin stimulated CaM kinase II phosphorylation was potentiated by okadaic acid pretreatment and heparin was

Figure 4. 0.1 nmol/L and 15 nmol/L okadaic acid block the effect of heparin on CaM kinase II phosphorylation. Quiescent VSMC were radioactively labeled as described in Figure 2 for 2 hours. Ninety minutes into the incubation okadaic acid was added at the indicated concentrations. After 30 minutes of treatment, cells were treated with 1 μ mol/L ionomycin in the presence (H+Iono) or absence (Iono) of heparin for 5 minutes. The cells were then harvested and used for immunoprecipitation with anti-CaM kinase II antibodies as described in Figure 2. Phosphorylation of CaM kinase II was visualized by exposure on a PhosphorImager cassette. Cells treated with okadaic acid alone are indicated as (-). Densitometric analysis was performed as above; in the presence of 0.15 nmol/L and 15 nmol/L okadaic acid, heparin reduced ionomycin-induced phosphorylation by 13% and 8%, respectively.

found to have no effect on CaM kinase II phosphorylation in the presence of 0.1 or 15 nmol/L okadaic acid. These data (Figure 4) suggest that PP-2A and possibly PP-1 activity dephosphorylates CaM kinase II and that heparin inhibits the phosphorylation of CaM kinase II by activating these phosphatases.

To further test the role of PP-2A and PP-1 in mediating the effect of heparin on CaM kinase II, calyculin and tautomycin, were used (Figure 5). Calyculin has relative selectivity for PP-2A at a concentration of 1 nmol/L while tautomycin has relative selectivity for PP-1 at a concentration of 1 nmol/L. The treatment protocol followed was identical to that used for okadaic acid. Calyculin pretreatment markedly potentiated CaM kinase II phosphorylation. In the presence of either calyculin or tautomycin, heparin did not inhibit the phosphorylation of CaM kinase II. However, the selectivity of the phosphatase inhibitors is relative at low concentrations. Hence, the effect of

Figure 5. Heparin activates PP-2A and PP-1: Quiescent VSMC were radioactively labeled as described in Figure 3 for 2 hours. Ninety minutes into the incubation 1 nmol/L calyculin or tautomycin was added. After 30 minutes of treatment, cells were stimulated with 1 nmol/L ionomycin in the presence (H+Iono) or absence (Iono) of heparin for 5 minutes. The cells were then harvested and used for immunoprecipitation with anti-CaM kinase II antibodies as described in Figure 3. Phosphorylation of CaM kinase II was visualized by autoradiography. Cells treated with calyculin or tautomycin alone are indicated by (-). Densitometric analysis indicated that in the absence of calyculin or tautomycin, heparin reduced ionomycin-induced phosphorylation by 91%, whereas in the presence of these phosphatase inhibitors heparin had no effect (phosphorylation was increased by 26% in the presence of calyculin and 15% in the presence of tautomycin).

Figure 6. A: Heparin does not inhibit CaM kinase II activity in resistant cells. Quiescent heparin-resistant VSMC were treated with 10% FCS/RPMI in the presence (5 H) or absence (5 F) of heparin for 5 minutes. The lysates were then used to determine CaM kinase II activity as described in Figure 1. The amount of Ca^{2+} /calmodulin-independent kinase activity is reported as a percentage of the total activity in the cells. **B:** Heparin does not affect CaM kinase II phosphorylation in heparin-resistant cells. Quiescent heparin-resistant and -sensitive cells were radioactively labeled and treated with 10% FCS/RPMI in the presence (H) or absence (F) of heparin. Cells were harvested and lysates used for immunoprecipitation with anti-CaM kinase II antibody as described in Figure 2. Differential phosphorylation was detected on a Phosphorimager. The very low levels of protein required relatively long exposure times for the resistant cells and necessarily overexpose the G_0 and sensitive cell lanes. Densitometric analysis indicated that heparin-treated sensitive cells had 68% less phosphorylation than serum-treated controls, whereas heparin-treated resistant cells showed only a 31% reduction. **C:** Heparin-resistant cells contain reduced levels of CaM kinase II δ -subunit. Quiescent heparin-resistant and sensitive cells were treated with 10% FCS/RPMI in the presence (Hep) or absence (FCS) of heparin for 5 minutes. The cells were harvested, proteins resolved by SDS-PAGE, blotted onto nitrocellulose membranes and CaM kinase II detected by immunostaining using affinity purified anti-CaM kinase II δ -subunit specific antibodies. The proteins were visualized using HRP-conjugated secondary antibodies and the ECL detection system. Using densitometric analysis, resistant cell lysates contain only 17% as much CaM kinase II protein as sensitive cells; no differences were seen in protein levels in either sensitive or resistance SMC exposed to heparin.

okadaic acid and calyculin is consistent with PP-2A; the tautomycin effect may be as a result of the effect on PP-2A or PP-1. Thus, the results strongly suggest an important role for phosphatases in regulating CaM kinase II autophosphorylation and autonomous activity in VSMC and support the hypothesis that heparin inhibits the phosphorylation of CaM kinase II by activating PP-2A and perhaps PP-1.

Heparin Does Not Inhibit CaM Kinase II Activity in Resistant Cells

Heparin-resistant cells, ie, cells that continue to proliferate in the presence of heparin, have recently been isolated and characterized¹⁸ and serve as an important tool to screen proteins/genes that could be involved in mediating the antiproliferative effect of heparin. It was reasoned that if inhibition of CaM kinase II is required for heparin to mediate its antiproliferative effect, then CaM kinase II activation by serum should be less affected by heparin in heparin-resistant VSMC. To test this, CaM

kinase activity was determined in cell lysates prepared from heparin-resistant VSMC treated with 10% FCS/RPMI $\pm$ heparin. Heparin did not affect the serum induced increase in autonomous kinase activity in resistant cells (Figure 6A). These data are consistent with the idea that heparin mediates its antiproliferative effect, at least in part, by inhibiting CaM kinase II in VSMC. Interestingly, the total amount of CaM kinase activity (1.1 ± 0.5 pmol/min/mg protein) in these cells was substantially lower than in control VSMC (6.5 ± 0.3 pmol/min/mg protein).

Heparin Does Not Alter Phosphorylation of CaM Kinase II in Heparin-Resistant Cells

As before, it was reasoned that if the dephosphorylation of CaM kinase II and inhibition of autonomous activity by heparin is essential for mediating its antiproliferative effect, then the phosphorylation of CaM kinase II should not be affected by heparin in resistant cells. To test this, radioactively labeled quiescent heparin-resistant cells were treated with 10% FCS/RPMI in the presence or

absence of heparin. Quiescent, untreated cells and heparin-sensitive primary VSMC were analyzed alongside as controls. Interestingly, almost no phosphorylated CaM kinase II was recovered from the heparin-resistant cells, consistent with the low kinase activities in the cells described above (Figure 6B). Densitometry analysis with software capable of sophisticated background subtraction indicated that heparin reduced CaM kinase II phosphorylation in sensitive cells by 58%, compared to only 21% in resistant cells. Western analysis performed on heparin-resistant and -sensitive cell lysates confirmed that the reduced levels of CaM kinase II activity and phosphorylation corresponded to reduced levels of CaM kinase II protein and was not an artifact of immunoprecipitation in the resistant cells (Figure 6C). Although the antibody used recognizes the carboxyl terminus of the CaM kinase II δ -subunit only, it is important to note that the total CaM kinase II activity is reduced in the resistant cells suggesting that the δ -isoform represents most of the CaM kinase II activity in these cells. Abraham et al¹⁹ have previously shown that immunoprecipitation of cell lysates with the CK2-DELTA antibody results in the depletion of Ca^{2+} /CaM-dependent and -independent autocalcineurin activity by 80 to 90%. This not only confirms the specificity of autocalcineurin-2 for CaM kinase II in VSMC lysates but also supports the observation that the δ -isoform is the major isoform CaM kinase II in cultured VSMC.²⁰

Discussion

In this study, we have examined the effect of heparin on CaM kinase II in VSMC. The new observations reported here are: 1) heparin inhibits the generation of autonomous CaM kinase II activity; 2) suppression by heparin of autonomous CaM kinase II activity correlates with inhibition of phosphorylation of the CaM kinase II δ -subunit, consistent with the known requirement for Thr^{286/287} autophosphorylation in the generation of autonomous activity; 3) the effect is specific for heparin since chondroitin sulfate, a non-antiproliferative glycosaminoglycan, does not elicit a similar effect on CaM kinase II phosphorylation; 4) at a biochemical level, heparin appears to alter the phosphorylation of CaM kinase II primarily by activating protein phosphatases PP-2A and PP-1; 5) neither the phosphorylation nor the activity of CaM kinase II is affected by heparin treatment of SV40-transformed heparin-resistant VSMC; furthermore, heparin-resistant cells express very low levels of CaM kinase II. Taken together, these observations strongly implicate CaM kinase II in the antiproliferative mechanism of heparin.

Attachment of cells to substrates leads to the generation of PIP_2 by the activation of PIP kinase. This PIP_2 is used as a substrate by phospholipase C to generate IP_3 and diacylglycerol. The generation of IP_3 or phospholipid hydrolysis in response to extracellular stimuli is an important event leading to the release of intracellular calcium in VSMC. The release of intracellular calcium plays a pivotal role in both the contractile as well as growth and differentiation responses of VSMC.³⁰ CaM kinase II is activated by calmodulin binding. Calmodulin, a cytoplasmic

protein extremely sensitive to changes in $[\text{Ca}^{2+}]_i$, binds to CaM kinase II leading to the autophosphorylation of CaM kinase II on Thr^{286/287} and the generation of a calcium/calmodulin-independent activated form of the enzyme.³¹ CaM kinase II is widespread in nature and has been implicated in the regulation of a number of major cellular responses including contraction,³² VSMC migration,³³ cell proliferation,³⁴ oocyte fertilization,³⁵ gene expression,^{36–39} and other responses to calcium mobilizing agents.^{40,41} CaM kinase II is composed of 8 to 10 subunits of 54 to 60 kd each.³¹ There are four known isoforms of CaM kinase II subunits (α , β , δ , γ) and a number of differentially spliced variants.¹⁹ The δ_2 -subunit variant is the major form expressed in cultured VSMC.²⁰ In our studies, heparin inhibits the overall phosphorylation of the CaM kinase II δ_2 -subunit.

A recent report by Miralem et al²² has also shown that heparin inhibits CaM kinase II activity in mesangial cells, which like VSMC are sensitive to the antiproliferative effects of heparin. In the study reported here we provide significant insight into the molecular mechanism of action of heparin. We show that heparin inhibits CaM kinase II phosphorylation induced by serum and Ca^{2+} -mobilizing stimuli and consequently generation of Ca^{2+} /calmodulin independent activity. This inhibitory effect of heparin on CaM kinase II activity cannot be due to "carryover" of heparin from the cell lysate to the kinase assay because: 1) our past studies have shown that the rinsing conditions used in these studies successfully removes almost all of the exogenously added heparin;⁴² and 2) Miralem et al²² have shown that addition of 1 $\mu\text{g/ml}$ heparin directly to the *in vitro* kinase assay mixture does not inhibit CaM kinase II activity. Further, the inhibition of phosphorylation is primarily achieved by the activation of protein phosphatase PP-2A. Thr²⁸⁶ can be dephosphorylated *in vitro* by purified catalytic subunits of PP1, PP2A, and PP2C.^{24,43,44} However, the catalytic subunits of PP1 and PP-2A do not exist freely in the cells, instead they are found associated with a number of different proteins resulting in differentially regulated/targeted holoenzymes.^{45–47} Despite these studies, the exact role of the phosphatase holoenzymes in regulating CaM kinase II phosphorylation is not well understood. The present studies with heparin provide an added tool to dissect this pathway. The abundant phosphorylation of CaM kinase II in the presence of low concentration of okadaic acid and calyculin suggests that PP-2A and possibly PP-1 may play major roles in the regulation of CaM kinase II in VSMC. In the brain,⁴⁸ neuronal CaM kinase II translocates to postsynaptic densities when activated by phosphorylation on Thr²⁸⁶. PP-2A is thought to play a major role in regulating the phosphorylation of soluble neuronal CaM kinase II on Thr²⁸⁶, while PP1 plays an important role in the dephosphorylation of postsynaptic density-associated CaM kinase II.^{27,49} Since the CaM kinase II phosphorylation was analyzed after 5 minutes of treatment, it is possible that there is a differential temporal localization of CaM kinase II and it is possible that PP-2A plays an important role in dephosphorylating this differentially localized form of CaM kinase II. Although the pharmacological data suggests a more prominent role for PP-2A

than PP-1, it is not possible to exclude a PP-1 effect without further experimentation.

Despite the well-documented effects of heparin on Ca^{2+} transients, the data presented in this paper strongly suggest that the effect of heparin on autonomous CaM kinase II is not due to an inhibitory effect of heparin on increases in $[\text{Ca}^{2+}]_i$. Although there is a distinct possibility that heparin could affect ionomycin-induced calcium transients, there is no data to support this scenario. Instead, the data presented here show that in the presence of phosphatase inhibitors the phosphorylation of CaM kinase II is significantly increased, even in the presence of ionomycin. This strongly suggests that the inhibitory effect of heparin on autonomous CaM kinase II activity is due to the activation of phosphatases rather than due to the inhibition of Ca^{2+} transients. In other words, the effect of heparin on CaM kinase II appears independent of its effect on Ca^{2+} transients. This would imply a novel role for the autonomous form of CaM kinase II in VSMC proliferation.

We and others have earlier reported that heparin inhibits MAPK activation, which is known to play a key role in mitogenic signaling.⁵⁰ Our present study presents a potentially important insight into the regulation of MAPK by heparin. Abraham et al¹⁶ have recently reported that CaM kinase II is involved in the activation of MAPK in response to calcium mobilizing agents like ionomycin. Since serum is a mixture of many mitogens as well as Ca^{2+} mobilizing factors, we postulate that heparin inhibits both signaling cascades and results in the inhibition of VSMC proliferation; ie, heparin can inhibit MAPK activation in response to growth factors as well as ionomycin. It is possible that CaM kinase II provides an important second signal (in addition to PKC and PKA) required for the activation of MAPK in VSMC.

A number of studies support a role for CaM kinase II in proliferation; for example, a recent study by Tombes et al³⁴ has shown that inhibition of CaM kinase II by KN-93 blocks cells in late G_1 . Also, Lorca et al³⁵ have shown that CaM kinase II is required for the G_2/M progression in oocytes. KN-93 has also been found to inhibit VSMC proliferation (unpublished results). Further, Morris et al⁵¹ have shown that CaM kinase II inhibition results in reduced cyclin D1 levels and enhances the association of p27^{kip1} with Cdk2 to cause G_1 arrest. Since heparin inhibits cyclin D1 expression⁵² as well as blocks cells in the late G_1 phase of the cell cycle,⁴ it is tempting to speculate that the inhibition of CaM kinase II by heparin causes the cells to block in the late G_1 phase of the cell cycle. This idea is supported by our observation that heparin did not affect either the phosphorylation or activity of CaM kinase II induced on serum treatment of heparin-resistant VSMC. The data also indicate that, compared to primary VSMC, the SV40 large T-transformed heparin-resistant cells contain reduced amount of the δ -subunit of CaM kinase II protein. This is very interesting because, if a target protein for CaM kinase II is important in delivering the negative signal for growth, then lack of CaM kinase II could contribute to transformed cell growth and be one mechanism for the resistance of these cells to heparin. Although the importance of CaM kinase II in

proliferation is consistent with the above observations in resistant cells, we note that the data must be viewed with caution as these cells are SV40-transformed and may have deficient or modified regulatory mechanisms. Overexpressing CaM kinase II in heparin-resistant VSMC and examining the effects on proliferation will help provide further insights, and these experiments are underway.

In summary, the data presented here suggest a potential mechanism for the antiproliferative effect of heparin on VSMC. The generation of autonomous CaM kinase II in response to Ca^{2+} mobilizing stimuli is inhibited specifically by heparin, and this effect is not observed in heparin-resistant cells. Mechanistically, heparin inhibits generation of autonomous CaM kinase II activity by inhibiting net (auto)phosphorylation. Furthermore, the inhibition of phosphorylation appears to be mediated by the activation primarily of protein phosphatase PP2A. Experiments are in progress to determine whether inhibition of autonomous CaM kinase II activity is an important step leading to growth arrest of VSMC, and to elucidate the physiological substrates for CaM kinase II involved in this effect.

Acknowledgments

We gratefully acknowledge Benjamin Caleb for assistance with cell culture and Laurie Delmolino, Roman Ginan, and Nancy Stearns for helpful discussions. We thank Emanuel Zycband for help in the preparation of figures for this manuscript.

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