

# Contribution of Double-Stranded RNA-Activated Protein Kinase Toward Antiproliferative Actions of Heparin on Vascular Smooth Muscle Cells

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**Objective**—The proliferation of vascular smooth muscle cells (VSMCs) in blood vessels after endothelial injury contributes to the onset of atherosclerosis. Heparin is a potent antiproliferative agent for VSMCs in vivo and in vitro. Although heparin has shown promise in suppressing VSMC proliferation after invasive procedures in laboratory animals, the mechanism of its antiproliferative actions is largely unknown. Here, we present evidence for the first time that the antiproliferative action of heparin is in part mediated by its ability to activate double-stranded RNA-activated protein kinase (PKR), an interferon-induced protein kinase.

**Methods and Results**—We have analyzed the VSMC proliferation by cell-cycle analysis and correlated it to the kinase activity of PKR in the presence of heparin. Heparin treatment of VSMCs results in activation of PKR by direct binding and results in a block in G<sub>1</sub>- to S-phase transition. PKR-null cells are largely insensitive to the antiproliferative actions of heparin, and inhibition of PKR in VSMCs results in a partial abrogation of the antiproliferative effects of heparin.

**Conclusions**—These results invoke the involvement of novel PKR-dependent regulatory pathways in mediating the antiproliferative actions of heparin. (*Arterioscler Thromb Vasc Biol.* 2002;22:1439-1444.)

**Key Words:** vascular smooth muscle cell proliferation ■ heparin ■ RNA-activated protein kinase  
■ cell cycle ■ interferon

Proliferation of vascular smooth muscle cells (VSMCs) is a key step in the pathogenesis of atherosclerosis or restenosis after vascular interventions, such as angioplasty.<sup>1</sup> Several growth factors, such as platelet-derived growth factor, basic fibroblast growth factor, and epidermal growth factor, have a mitogenic effect on VSMCs in vitro.<sup>1</sup> Heparin, a component of the extracellular matrix, acts as potent antiproliferative agent for VSMCs in vivo (after invasive surgical procedures)<sup>2,3</sup> and in vitro (in tissue culture systems).<sup>4,5</sup> In spite of the well-documented antiproliferative effects of heparin on VSMCs, the molecular mechanisms that are involved have not yet been fully understood.

The double-stranded (ds) RNA-activated protein kinase (PKR) is a key mediator of the antiviral and antiproliferative effects of interferons.<sup>6</sup> The kinase activity of PKR stays latent until it is bound to an activator. In virally infected cells, PKR is activated by dsRNA. In addition to dsRNA, heparin is also known to activate PKR in vitro.<sup>7</sup> Binding to an activator causes a conformational change in PKR structure, thereby exposing its ATP-binding site, leading to its autophosphorylation and activation.<sup>7,8</sup> The best-studied cellular substrate for PKR activity is the  $\alpha$  subunit of the eukaryotic initiation factor eIF2 (eIF2 $\alpha$ ).<sup>9</sup> Phosphorylation of eIF2 $\alpha$  leads to

global inhibition of protein synthesis.<sup>10</sup> In addition to dsRNA and heparin, we have recently also identified PACT, the first known cellular PKR activator protein.<sup>11,12</sup> Overexpression of PKR is inhibitory to cell proliferation in yeast,<sup>13</sup> insect,<sup>14</sup> and mammalian<sup>15</sup> cells. Expression of *trans*-dominant negative mutants of PKR in NIH 3T3 cells results in a transformed phenotype.<sup>15,16</sup> Oncogenic Ras protein has also been reported to induce an inhibitor of PKR.<sup>17</sup>

In the present study, we have examined the involvement of PKR in mediating the antiproliferative actions of heparin, and our results indicate that the heparin-induced activation of PKR plays an important role in mediating the antiproliferative effects of heparin.

## Methods

### Cell Culture

Human aortic smooth muscle cells (HASMCs) were within passages 5 to 7. Rat primary aortic vascular smooth muscle cells (RASMCs) were obtained from the thoracic aortas of male Sprague-Dawley rats. Mouse embryonic fibroblasts (MEFs) from wild-type and PKR-null mice were kindly provided by Dr Bryan Williams (Department of Cancer Biology, Cleveland Clinic, Ohio) and have been characterized previously.<sup>18,19</sup> All cells were cultured in DMEM (Invitrogen) supplemented with 10% FCS, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin.

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### Proliferation Assay

HASMCs ( $3$  to  $5 \times 10^4$  per well) were plated in 6-well plates in DMEM with 0.1% serum. Seventy-two hours later, the cells were shifted to 0.1% serum-containing medium with  $100 \mu\text{g}/\text{mL}$  heparin (No. H-3149, lot 17H03885, Sigma Chemical Co) for 2 hours. After 2 hours, the cells were serum-stimulated with DMEM containing 10% serum and  $100 \mu\text{g}/\text{mL}$  heparin. As a control, HASMCs were subjected to identical treatment in the absence of heparin. The growth of both these sets of cells was compared daily by counting the cells in triplicate.

### PKR Activity Assays

The HASMCs were treated with heparin as indicated in the previous section. Cell extract preparation and PKR activity assays were performed as described previously.<sup>12</sup> Purified eIF2 was kindly provided by Dr William Merrick (Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio).

### Heparin-Binding Assay

The *in vitro*-translated  $^{35}\text{S}$ -labeled proteins were synthesized by using the TNT T7 (Promega) system.<sup>11</sup> Translation products ( $4 \mu\text{L}$ ) diluted with  $25 \mu\text{L}$  binding buffer (20 mmol/L Tris-HCl, pH 7.5, 0.3 mol/L NaCl, 5 mmol/L  $\text{MgCl}_2$ , 1 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulfonyl fluoride, 0.5% Igepal (Sigma), and 10% glycerol) were mixed with  $25 \mu\text{L}$  heparin-agarose (Sigma) and incubated at  $30^\circ\text{C}$  for 30 minutes with intermittent shaking. The beads were washed 4 times with  $500 \mu\text{L}$  binding buffer. The proteins remaining bound to the beads were analyzed by SDS-PAGE, followed by phosphorimager analysis.

### Heparin Internalization and PKR Binding

HASMCs were cultured in 6-well dishes in 0.1% serum-containing medium for 72 hours. The cells were treated with  $100 \mu\text{g}/\text{mL}$  heparin and  $100 \mu\text{Ci}/\text{mL}$   $^{35}\text{S}$ -heparin (NEN) in low serum. The cell extracts were prepared in immunoprecipitation buffer (20 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 1% Triton X-100, and 20% glycerol) and subjected to immunoprecipitation<sup>11</sup> with  $1 \mu\text{L}$  anti-PKR monoclonal antibody 71/10 (Ribogene) and  $10 \mu\text{L}$  protein A-agarose (Roche). Immunoprecipitations were also carried out by using an anti- $\alpha$ -actin monoclonal antibody (Sigma). The immunoprecipitates were washed 4 times with immunoprecipitation buffer, beads were collected on a glass fiber filter and dried, and the radioactivity associated with the beads was counted.

### Cell-Cycle Analysis

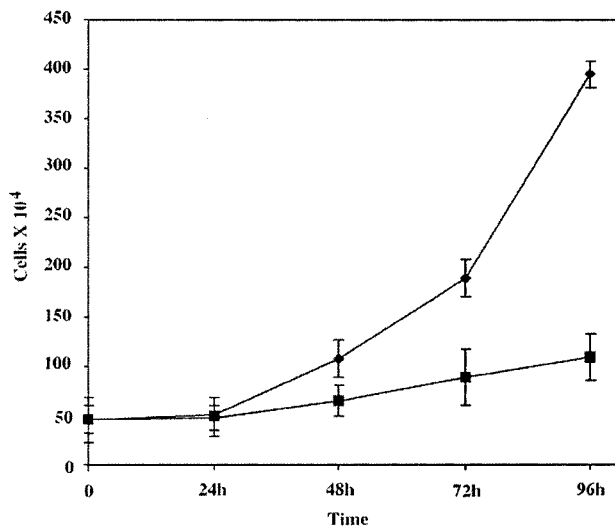
The RASMCs or MEFs were cultured and treated as described in the Figure 4 legend and analyzed by flow cytometry<sup>20</sup> with use of a Coulter Flow Cytometer.

### BrdU Incorporation Assay in Transfected RASMCs

RASMCs grown in 4-chamber slides were cotransfected by use of Effectene transfection reagent (Qiagen) with cytomegalovirus (CMV)- $\beta$ -galactosidase ( $\beta$ -gal) and empty vector pCB6<sup>+</sup> or K296R/pCB6<sup>+</sup>. K296R is a *trans*-dominant negative PKR mutant described previously.<sup>15,16,21</sup> Twelve hours after transfection, the cells were serum-starved for 36 hours before being serum-stimulated in either the presence or absence of heparin. Fourteen hours after serum stimulation, a BrdU incorporation assay was performed by using the BrdU labeling kit (Roche Biochemicals). The cells were incubated with BrdU for 4 hours, fixed, and stained for  $\beta$ -gal activity and nuclear BrdU incorporation.<sup>22</sup> The experiment was performed in triplicate for a total of 3 repeats, and the results were combined for statistical analysis by use of a *t* test.

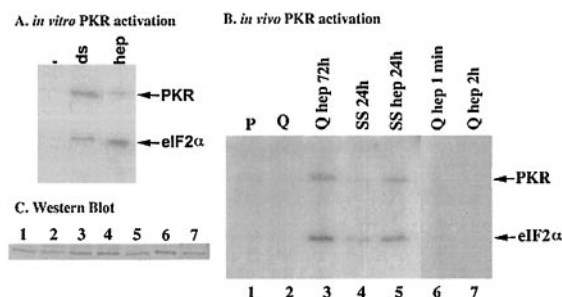
## Results

Heparin showed a strong antiproliferative effect on HASMCs in culture (Figure 1). Proliferation of heparin-treated cells was blocked  $\approx 70\%$  to  $75\%$  compared with control. To



**Figure 1.** Heparin inhibits the growth of HASMCs. HASMCs ( $3$  to  $5 \times 10^4$ , passage 6) were plated in 6-well plates in DMEM with 0.1% FCS. After 72 hours in low-serum medium,  $100 \mu\text{g}/\text{mL}$  heparin was added. Two hours later, the cells were serum-stimulated in the presence of  $100 \mu\text{g}/\text{mL}$  heparin. As a control, 1 set of cells was serum-starved without any heparin, and these cells were serum-stimulated without heparin. The growth of both these sets of cells was compared by counting the cells in triplicate, and the SE is shown by the error bars. Diamonds indicate control cells; squares, heparin-treated cells.

determine whether heparin activates PKR efficiently *in vitro*, we compared PKR kinase activity in the presence of heparin and the well-studied activator of PKR, dsRNA. dsRNA and heparin were both able to activate PKR (Figure 2A) efficiently. We next tested whether heparin treatment of VSMCs results in the activation of PKR *in vivo*. PKR activity is undetectable in extracts from proliferating cells or quiescent

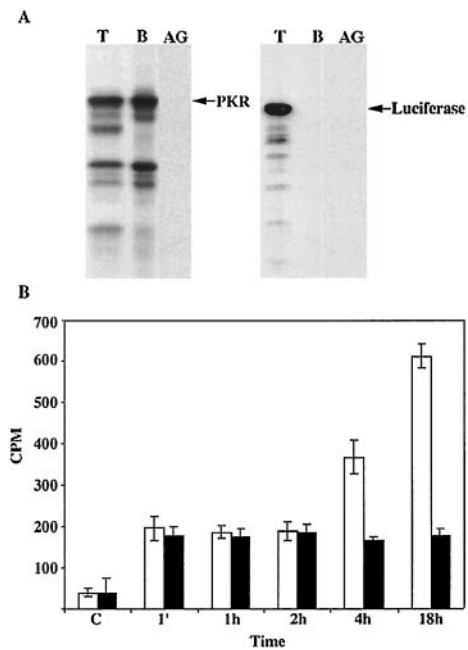


**Figure 2.** A, Heparin activates PKR *in vitro*. Cell extracts were prepared from HASMCs at 70% confluence. PKR was immunoprecipitated with a monoclonal antibody, and a PKR activity assay was performed. Lanes are as follows: -, no activator; ds,  $1 \mu\text{g}/\text{mL}$  poly(rI) · poly(rC); and hep,  $10 \text{ ng}/\text{mL}$  heparin. B, Heparin treatment of VSMCs activates PKR. HASMCs were treated with heparin as described, and PKR activity was assayed from the cell extracts. Lanes are as follows: 1, proliferating cells; 2, quiescent cells in low serum at 72 hours; 3, quiescent cells in low serum and heparin at 24 hours; 4, cells in lane 2 shifted to 10% serum 24 hours later; 5, cells in lane 3 shifted to 10% serum and heparin 24 hours later; 6, quiescent cells treated with heparin for 1 minute; and 7, quiescent cells treated with heparin for 2 hours. Lanes 6 and 7 are negative controls to show that activation occurs *in vivo* and not during extract preparation. C, Total protein ( $200 \mu\text{g}$ ) from each of the samples was analyzed by Western blot analysis with an anti-PKR monoclonal antibody.

cells in low serum (Figure 2B, lanes 1 and 2). The addition of heparin to low-serum medium activated PKR in quiescent cells (lane 3). This activity remained high when the cells were serum-stimulated in the presence of heparin (lane 5). In contrast, PKR activity in the serum-stimulated cells was very low (lane 4). To ensure that the activation of PKR does not occur during the extract preparation by the sticking of heparin nonspecifically to the cell membranes and association of heparin with PKR after the cells are lysed, we included a control in which heparin was added to the quiescent cells 1 minute before their lysis. No PKR activity was detected in this sample (lane 6), ensuring that the washing procedure removed most of the heparin sticking to the cell surface. No PKR activation was detected at 2 hours after heparin treatment (lane 7), further confirming that PKR activation occurs *in vivo*. The rationale behind the 2-hour time point was that neither heparin internalization nor its binding to PKR was detected 2 hours after treatment (Figure 3); therefore, it serves as a negative control. A Western blot analysis was performed to ensure that equal quantities of PKR protein were assayed for activity in each lane (Figure 2C).

PKR activation in response to heparin treatment of VSMCs could occur either by the uptake of heparin followed by direct binding to PKR or by a signaling cascade initiated by the binding of heparin to the cell surface. To analyze this, we tested the ability of PKR to bind heparin directly by using an *in vitro* heparin-binding assay. [<sup>35</sup>S]Methionine-labeled *in vitro*-translated PKR protein bound efficiently to heparin-agarose (Figure 3A). Under the same conditions, luciferase protein showed no binding, and PKR and luciferase proteins showed no binding to agarose beads alone, thereby confirming the specificity of the interaction. To determine whether heparin is internalized by VSMCs and whether it binds to PKR once internalized, we performed immunoprecipitation assays with PKR antibody after treatment of the VSMCs with <sup>35</sup>S-labeled heparin. Immunoprecipitation of PKR could bring down <sup>35</sup>S-heparin in a time-dependent manner (Figure 3B, open bars). Four hours after heparin treatment, there was a significant increase in PKR-associated <sup>35</sup>S-heparin counts, followed by a further increase at 18 hours. To ascertain that PKR-associated counts were due to internalization followed by a specific interaction, we measured PKR-associated counts after a short (1-minute) treatment. We noted that there was some increase in PKR-associated counts at 1 minute compared with control. However, there was no further increase for the next 2 hours in PKR-associated counts, indicating that these counts were due to nonspecific sticking of <sup>35</sup>S-heparin to the cell surface, which may have resulted in PKR association during extract preparation. After 2 hours, we observed a steady increase in PKR-associated <sup>35</sup>S-heparin counts, indicating that heparin is internalized by VSMCs in a slow process and associates with PKR after internalization. The same assay with an anti- $\alpha$ -actin monoclonal antibody (solid bars, Figure 3B) showed no counts above background, thereby confirming that the <sup>35</sup>S-heparin and PKR interaction was specific.

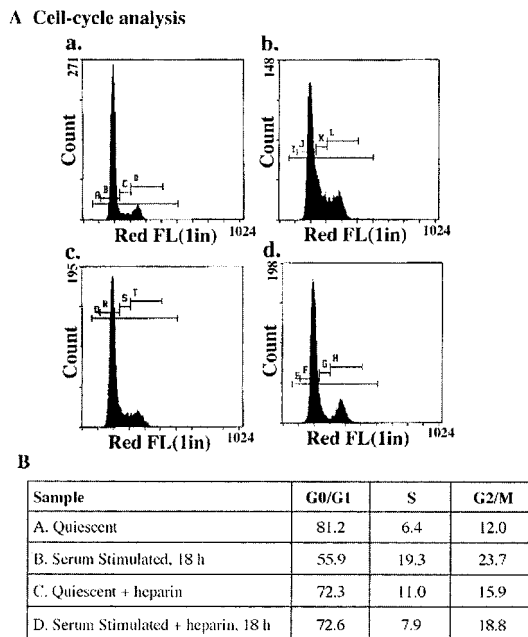
To gain insight into the mechanism of the antiproliferative effects of heparin on VSMCs, we performed a cell-cycle analysis after heparin treatment. As represented in Figure 4A



**Figure 3.** A, Heparin binds directly to PKR. Four microliters of *in vitro*-translated [<sup>35</sup>S]methionine-labeled proteins were bound to heparin agarose in binding buffer at 30°C for 30 minutes. The beads were washed with binding buffer, and the protein bound to the beads was analyzed by SDS-PAGE, followed by fluorography. Lanes are as follows: T, total protein from reticulocyte lysate; B, protein bound to heparin-agarose beads; and AG, proteins bound to agarose beads only. The multiple bands corresponding to PKR lanes are known to be the products of translation initiation from internal methionines *in vitro*. B, Direct binding of heparin to PKR *in vivo* is shown. VSMCs were grown in 6-well plates and were treated with 10  $\mu$ Ci/mL <sup>35</sup>S-heparin mixed with 100  $\mu$ g/mL nonradioactive heparin in 0.5 mL of 0.1% serum-containing DMEM for 2 hours. Two hours later, 0.5 mL of 10% serum-containing DMEM was added to the cells without the removal of <sup>35</sup>S-heparin-containing medium. Cell extracts were prepared at times indicated after the addition of <sup>35</sup>S-heparin, and PKR was immunoprecipitated from half of the extract from each well by using the anti-PKR monoclonal antibody and protein A-sepharose. Counts associated with the beads after washing were determined by scintillation counting (open bars). The other half of the extract was immunoprecipitated with anti- $\alpha$ -actin monoclonal antibody, and counts associated with the beads were determined (solid bars). Each time point was analyzed in triplicate, and the SD is indicated by error bars.

and 4B, serum starvation introduced a G<sub>0</sub>/G<sub>1</sub> arrest in VSMCs with 81.2% cells in the G<sub>0</sub>/G<sub>1</sub> phase and 6.4% cells in the S phase of the cell cycle. Eighteen hours after serum stimulation, 19.3% of the VSMCs were in S phase, with a corresponding decrease in the percentage of cells in the G<sub>0</sub>/G<sub>1</sub> phase. Heparin treatment of serum-starved VSMCs did not change the cell-cycle distribution of cells compared with serum-starved VSMCs. However, in heparin-treated samples, only 7.9% of the VSMCs were in S phase 18 hours after serum stimulation. These results strongly indicate that heparin treatment causes a block in the G<sub>1</sub>- to S-phase transition of VSMCs. We also performed BrdU incorporation assays to confirm a G<sub>1</sub>-phase arrest in response to heparin (please refer to online Figure I, which can be accessed at <http://atvb.ahajournals.org>).





**Figure 4.** Heparin blocks serum-induced G<sub>1</sub> to S transition of VSMCs. A, Quiescent RASMCs (0.1% serum for 72 hours) were stimulated with serum. The cells were pretreated for 2 hours with 100 μg/mL heparin in low-serum medium before addition of serum-containing medium with 100 μg/mL heparin. As a control, the same treatments were performed without any heparin. Eighteen hours after serum stimulation, the cells were analyzed by flow cytometry: a indicates quiescent RASMCs; b, RASMCs stimulated with 10% serum; c, quiescent RASMCs in the presence of 100 μg/mL heparin for 2 hours; and d, RASMCs pretreated with heparin stimulated with serum and 100 μg/mL heparin. The x- and y-axes represent the intensity of propidium iodide fluorescence and cell number, respectively. The data are representative of 3 separate experiments. B, Quantitative analysis of cell-cycle profiles in panel A is shown.

If heparin-induced PKR activation contributes to its antiproliferative actions, the PKR-null cells are expected to show abrogation of the antiproliferative effects. PKR-null mice and the MEFs established from them have been characterized and

Sample PKR +/+	G0/G1	S	G2/M
Quiescent	81.1	6.5	8.0
Serum Stimulated, 18 h	42.5	38.8	16.3
Quiescent + heparin	74.1	15.7	5.2
Serum Stimulated + heparin, 18 h	61.5	20.6	15.4
Sample PKR null	G0/G1	S	G2/M
Quiescent	78.5	10.1	4.3
Serum Stimulated, 18 h	51.8	26.9	19.7
Quiescent + heparin	81.3	6.4	12.0
Serum Stimulated + heparin, 18 h	52.9	22.3	23.7

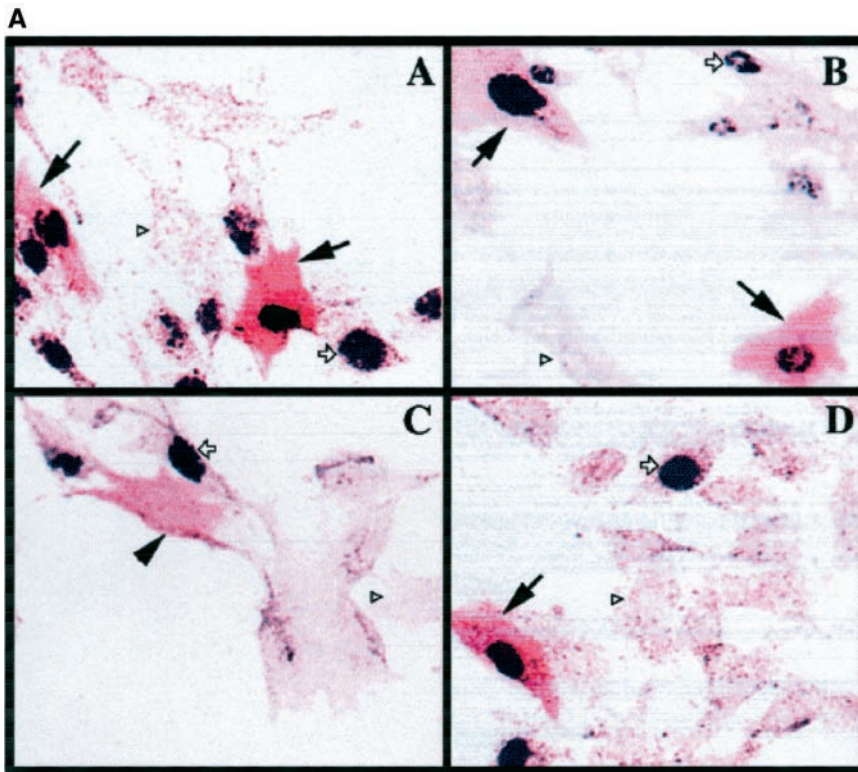
**Figure 5.** PKR-null cells show abrogation of antiproliferative actions of heparin. Quiescent MEFs (0.5% serum for 36 hours) were stimulated with 10% serum-containing DMEM. The cells were pretreated with 100 μg/mL heparin in low-serum medium for 2 hours before the addition of 10% serum medium also containing 100 μg/mL heparin. As a control, the same treatments were performed without any heparin. Eighteen hours after serum stimulation, the cells were analyzed by flow cytometry. Quantitative analysis of the cell-cycle profiles is shown. The data shown are representative of 3 separate experiments.

studied extensively.<sup>18,19</sup> As represented in Figure 5, heparin treatment caused a block in the G<sub>1</sub>- to S-phase transition in wild-type MEFs. The PKR-null MEFs were resistant to heparin-induced block of the G<sub>1</sub>- to S-phase transition. In the absence of heparin, 18 hours after serum stimulation, 38.8% of the wild-type MEFs were in S phase, and this value dropped to 20.6% in the presence of heparin. In contrast, 26.9% of the PKR-null cells were in S phase in the absence of heparin, and in the presence of heparin, 22.3% of the cells still entered the S phase, thereby indicating that the antiproliferative effect of heparin was largely abolished because of the absence of PKR. BrdU incorporation assays to monitor DNA synthesis during S phase confirmed the G<sub>1</sub> arrest (online Figure IIA, which can be accessed at <http://atvb.ahajournals.org>). To ensure that heparin was internalized by MEFs and activated PKR by direct binding, we also performed PKR activity assays and <sup>35</sup>S-heparin-binding assays with MEFs (online Figures IIB and IIC).

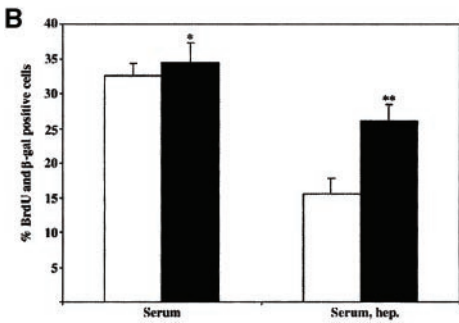
To establish the role of PKR in mediating the antiproliferative actions of heparin in VSMCs, we assayed the effect of inhibiting the PKR activity by the *trans*-dominant negative PKR mutant K296R. The K296R mutation is at the ATP-binding site of PKR,<sup>21</sup> and an overexpression of this mutant has been shown previously to inhibit the endogenous PKR activity.<sup>16</sup> We cotransfected the RASMCs with CMV-β-gal plasmid and either the empty vector (negative control) or the K296R expression construct. The cells were made quiescent after transfection and were then serum-stimulated in either the presence or absence of heparin, and their entry into S phase was monitored by BrdU labeling. The β-gal activity staining was performed to identify the transfected cells, and the nuclei that incorporated BrdU were detected by immunostaining with an anti-BrdU antibody. The percentage of nuclei undergoing DNA synthesis within the transfected population was obtained by counting the number of cells showing red cytoplasmic β-gal staining that were also BrdU positive, as indicated by dark purple nuclear staining (Figure 6A, black arrows). Quantification of these data appears in Figure 6B. As shown in Figure 6B, the percentage of cells with positive BrdU staining was similar for vector-transfected (≈33%, open bars) and K296R-overexpressing cells (≈34%, solid bars) in the absence of heparin. In the presence of heparin, only ≈16% of the cells showed positive BrdU staining (open bars) for the vector-transfected population, indicating a heparin-induced block in the cell cycle. In contrast to this, the K296R-overexpressing cells (solid bars) showed that ≈26% of the cells were positive for BrdU, indicating a partial release from the block in G<sub>1</sub> to S transition. These results confirm that the antiproliferative effects of heparin in VSMCs are mediated at least in part via the activation of PKR.

### Discussion

Although PKR has been shown to be activated by heparin *in vitro*, no direct link had been established so far between the antiproliferative effects of heparin and PKR activation. Our results in the present study demonstrate that heparin treatment of VSMCs results in PKR activation by direct interaction. By using immunoprecipitation assays, we could detect heparin binding to PKR after treatment of VSMCs with <sup>35</sup>S-labeled



**Figure 6.** A, Inhibition of PKR activity results in partial abrogation of antiproliferative activity of heparin in RASMCs. RASMCs were cotransfected with the pCB6+ and CMV- $\beta$ -gal or K296R/pCB6+ and CMV- $\beta$ -gal plasmids. Twelve hours after transfection, the cells were serum-starved for 36 hours, after which they were serum-stimulated in either the presence or absence of heparin. Fourteen hours after serum stimulation, the cells were labeled with BrdU, fixed, and stained for  $\beta$ -gal and BrdU incorporation. Transfected cells show red cytoplasmic staining and are either BrdU negative (black arrowhead, no nuclear staining) or BrdU positive (black arrows, dark purple nuclear staining). Nontransfected cells (cells lacking red  $\beta$ -gal staining) are also BrdU positive (open arrows) or BrdU negative (open arrowheads). A, Cells transfected with CMV- $\beta$ -gal and pCB6+, untreated. B, Cells transfected with K296R/pCB6+ and CMV- $\beta$ -gal, untreated. C, Cells



transfected with CMV- $\beta$ -gal and pCB6+, treated with heparin. D, Cells transfected with K296R/pCB6+ and CMV- $\beta$ -gal, treated with heparin. B, Quantitative analysis of the BrdU incorporation data is shown. Among the cells showing red cytoplasmic staining, the numbers of cells either positive or negative for BrdU nuclear staining were counted. A minimum of 300 cells were counted, and each experiment was performed in triplicate. The percentage of BrdU-positive cells among the transfected population was calculated as follows: % BrdU-positive cells =  $100 \times (\text{number of cells with red cytoplasmic staining and BrdU nuclear staining} / \text{total number of cells with red cytoplasmic staining})$ . Open bars indicate cells transfected with pCB6+ and CMV- $\beta$ -gal; solid bars, cells transfected with K296R/pCB6+ with CMV- $\beta$ -gal. K296R-mediated PKR inhibition resulted in significant reduction in the antiproliferative effects of heparin (15% vs 26%,  $**P=0.0047$ ). Expression of K296R without heparin treatment did change the percentage of cells in S phase (32% versus 34%,  $*P=0.485$ ).

heparin. These results are in agreement with previous reports of heparin internalization by VSMCs with the use of fluorescent-tagged heparin.<sup>23</sup> Although our results demonstrate activation of PKR by direct interaction with heparin, additional involvement of any signaling events triggered by the binding of heparin on the cell surface cannot be ruled out.

Heparin has been thought to inhibit the VSMC proliferation by arresting the G<sub>1</sub> to S transition<sup>24</sup> and to block the expression of immediate-early genes.<sup>25</sup> Our data clearly supported the notion that the cell-cycle progression of heparin-treated VSMCs was blocked at the G<sub>0</sub>/G<sub>1</sub> to S transition. The PKR-null MEFs were markedly insensitive to the antiproliferative actions of heparin, strengthening the role of PKR activation in introducing the cell-cycle block. PKR activity is highest during the G<sub>1</sub> phase; it peaks twice, once in early G<sub>1</sub> and then at G<sub>1</sub>/S boundary, and then declines during S phase.<sup>26</sup> PKR overexpression has been shown to result in a slow passage through the G<sub>1</sub> to S transition.<sup>27</sup> On the contrary, PKR has also been implicated in signal transduction in response to platelet-derived growth factor.<sup>28</sup> In agreement with the role of PKR in mitogenic signaling, several breast

cancer cell lines have been shown to possess elevated levels of PKR protein and activity.<sup>29</sup> These apparently opposite effects of PKR on proliferation are not well understood at present. However, it is clear from our results that the antiproliferative actions of heparin were greatly diminished in PKR-null cells, confirming our hypothesis that PKR activation by heparin leads to a cell-cycle block. These findings were further strengthened by abrogation of the heparin-induced cell-cycle block in RASMCs after inhibition of endogenous PKR by the overexpression of the trans-dominant negative K296R mutant.

In PKR-null MEFs and also in K296R-overexpressing RASMCs, we observed a marked but not a total loss of the antiproliferative actions of heparin, indicating that additional pathways also contribute to heparin-mediated growth inhibition. Other documented effects of heparin on VSMCs include inhibition of the immediate-early genes,<sup>30</sup> matrix-degrading proteases,<sup>31–33</sup> mitogen-activated protein kinase activation,<sup>34</sup> matrix molecules,<sup>35,36</sup> and extracellular signal-regulated kinases ERK1 and ERK2<sup>37</sup> and also thrombin-induced VSMC migration via inhibition of epidermal growth factor receptor

transactivation.<sup>38</sup> The tyrosine kinase receptor EphB2 mRNA levels are also downregulated by heparin treatment of VSMCs.<sup>39</sup> The results of the present study describe for the first time a relationship between PKR activation and the antiproliferative actions of heparin. Thus, we have identified PKR as a novel component of the antiproliferative actions of heparin on VSMCs.

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# Arteriosclerosis, Thrombosis, and Vascular Biology



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## Contribution of Double-Stranded RNA-Activated Protein Kinase Toward Antiproliferative Actions of Heparin on Vascular Smooth Muscle Cells Rekha C. Patel, Indhira Handy and Chandrashekhar V. Patel

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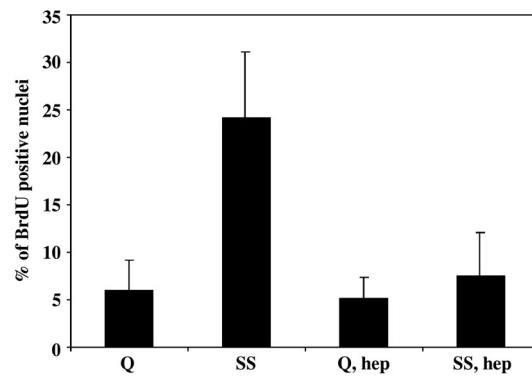
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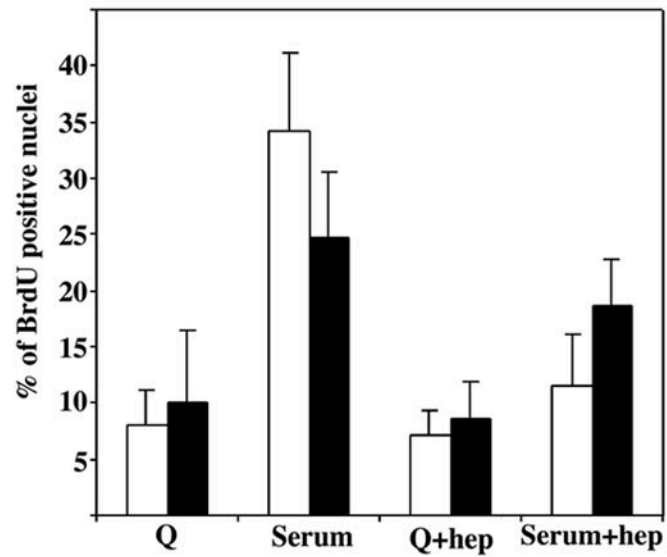
### BrdU labeling analysis



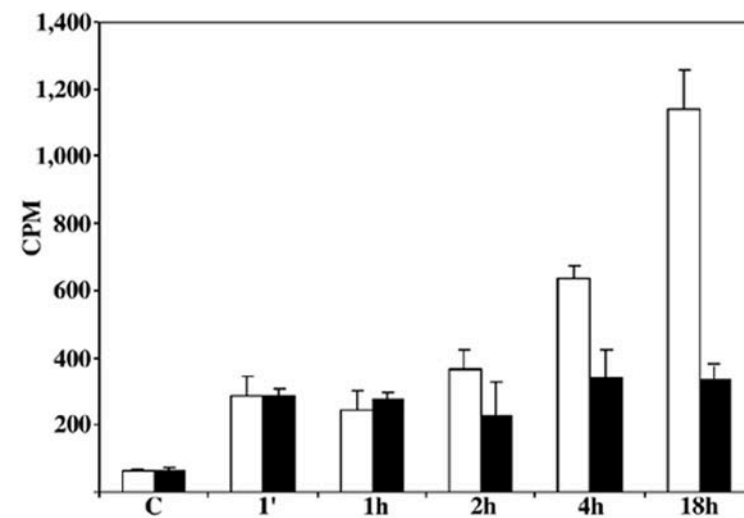
**Fig. I**



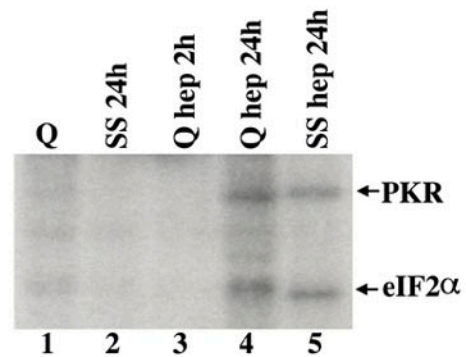
**A. BrdU labeling analysis**



**C. Heparin internalization and PKR binding**



**B. *in vivo* PKR activation**



**Fig. II**

### **Methods:**

**BrdU incorporation assay:** The RASMCs or MEFs were cultured in chamber slides in DMEM containing 0.1% serum for 48 h before addition of 100 µg/ml heparin. Two h after heparin addition, the cells were serum stimulated. 14 h after the serum stimulation, BrdU incorporation assay was performed using the BrdU labeling kit (Roche Biochemicals) according to the manufacturer's instructions. The nuclei that incorporated BrdU fluoresce green because of the fluorescein-conjugated anti-BrdU antibody used for detection. The cells were counterstained with DAPI which stains all nuclei. At least 300 nuclei from each sample were counted for BrdU staining using a fluorescence microscope equipped with appropriate filter sets. Percentage of BrdU positive nuclei was calculated as (number of green fluorescent nuclei/ total number of nuclei) X 100.

**PKR activity assays:** The PKR activity assays from PKR<sup>+/+</sup> MEFs were performed in a similar manner as described in the main text by using the anti-murine PKR monoclonal antibody from Transduction Labs.

**Heparin internalization assay:** The heparin internalization assays from PKR<sup>+/+</sup> MEFs were performed in a similar manner as described for HASMCs in the main text by using the anti-murine PKR monoclonal antibody from Transduction Labs.

### **Results:**

As seen in Fig. I, the heparin treated VSMC showed that only 7.5% of nuclei labeled positive for DNA synthesis as compared to 24.1% of positive nuclei in serum stimulated cells. These results further confirmed a block in G1 to S phase transition in heparin treated samples.

As shown in Fig. II A, in heparin treated wild type MEFs (white bars) only 11.5% of nuclei labeled positive for DNA synthesis as compared to 34.2% in the absence of heparin. In contrast to this, in PKR null cells (black bars), 18.6% nuclei scored positive in the presence as compared to 24.7% in the absence of heparin. These results show that PKR plays an important and essential role in heparin's antiproliferative actions. In order to ensure that heparin treatment of PKR<sup>+/+</sup> MEFs does result in PKR activation, we performed PKR activity assays without the addition of any exogenous activator. As shown

in Fig. II B, quiescent MEFs showed no PKR activation (lane 1). Heparin treatment for 2 h also showed no PKR activation, thereby ruling out PKR activation during extract preparation (lane 3). However, heparin treatment of MEFs for 24 h showed marked PKR activation (lane 4), thereby confirming that heparin treatment results in PKR activation in MEFs. Serum stimulated cells showed no PKR activity at 24 h after serum addition (lane 2), however, in the presence of heparin, serum stimulated MEFs showed PKR activation comparable to the quiescent cells with heparin (lane 5). These results confirmed that heparin treatment of MEFs results in PKR activation. To ensure that PKR activation in response to heparin treatment resulted from direct binding of heparin with PKR, we repeated the heparin internalization experiment on MEFs. As seen in Fig. II C,  $^{35}\text{S}$ -heparin counts could be immunoprecipitated with PKR in a time-dependent manner (white bars). We observed some increase in PKR-associated  $^{35}\text{S}$ -heparin counts at 1', 1 h, and 2 h over the control. However, these counts are due to non-specific sticking of heparin, since similar counts are immunoprecipitated with the anti- $\beta$  actin monoclonal antibody (black bars). We observed a specific, steady increase in PKR associated  $^{35}\text{S}$ -heparin counts both at 4 h and 18 h post-treatment, which was not observed with the anti-actin antibody. This indicated that heparin internalization and PKR binding in MEFs follows a time course very similar to VSMC (Fig. 3 B). These results confirm that heparin treatment of MEFs results in internalization of heparin, and PKR activation by direct binding.

### Figure Legends:

**Fig. I. Heparin treatment blocks the DNA synthesis in response to serum.** The RASMC were plated in four-chambered slides and were treated as in A. 14 h after the serum stimulation, BrdU incorporation assays were carried out as described in the methods section. The percentage of BrdU positive nuclei among the total number of nuclei was calculated and plotted. Each assay was done in triplicate and the error bars indicate standard error. Q: quiescent RASMC; SS: serum stimulated RASMC; Q,hep: quiescent RASMC treated with 100  $\mu\text{g/ml}$  heparin for 2 h; SS, hep: RASMC serum stimulated in the presence of heparin.

**Fig. II A. Heparin-induced block in the DNA synthesis is abrogated in PKR null cells.**

The MEF cells were plated in four-chambered slides and were treated as in A. 14 h after the serum stimulation, BrdU incorporation assays were carried out as described in the methods section. The percentage of BrdU positive nuclei among the total number of nuclei was calculated and plotted. Each assay was done in triplicate and the error bars indicate standard error. Q: quiescent MEFs; Serum: serum stimulated MEFs; Q+hep: quiescent MEFs treated with 100 µg/ml heparin for 2 h; Serum +hep: MEFs serum stimulated in the presence of heparin. Open bars represent data from PKR<sup>+/+</sup> cells and the black bars represent data from PKR<sup>-/-</sup> cells. **B. Heparin treatment of PKR<sup>+/+</sup> MEFs activates PKR.**

The PKR<sup>+/+</sup> MEFs were treated as described in the Fig. 5 A legend. PKR was immunoprecipitated with a monoclonal antibody (Transduction Labs) from 400 µg of total protein and incubated without any exogenously added activator in kinase assay buffer containing 0.1 µCi of  $\gamma$  <sup>32</sup>P-ATP, 250 ng of purified eIF2 for 5 min at 30°C. The phosphorylated proteins were analyzed by SDS-PAGE followed by autoradiography. Lane 1: quiescent cells, lane 2: cells shifted to 10% serum for 24 h, lane 3: quiescent cells in low serum and heparin for 2 h, lane 4: quiescent cells in low serum and heparin for 24 h, lane 5: cells in 3 shifted to 10% serum and heparin for 24 h. Lane 3 is a negative control to show that activation occurs *in vivo* and not during extract preparation. **C. Direct binding of heparin to PKR *in vivo* after treatment of MEFs with <sup>35</sup>S-heparin.**

PKR<sup>+/+</sup> MEFs were grown in 6-well plates and were treated with 10 µCi/ml of <sup>35</sup>S-heparin mixed with 100 µg/ml of non-radioactive heparin in 0.5 ml of 0.1% serum containing DMEM for two hours. Two hours later, 0.5 ml of 10% serum containing DMEM was added to the cells without the removal of <sup>35</sup>S-heparin containing medium. Cell extracts were prepared at times indicated after the <sup>35</sup>S-heparin addition and PKR was immunoprecipitated from half of the extract from each well using the anti-PKR monoclonal antibody and protein A-sepharose. The counts associated with the beads after washing the beads were determined by



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scintillation counting (Open bars). The other half of the extract was immunoprecipitated with anti- $\beta$  actin monoclonal antibody and the counts associated with the beads were determined (Black bars). Each time point was analyzed in triplicates and the standard deviation is indicated by error bars.