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Suppression of mitogen-activated protein kinase phosphatase-1 (MKP-1) by heparin in vascular smooth muscle cells

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Abstract

Heparin inhibits vascular smooth muscle cell (VSMC) proliferation, but mechanisms remain elusive. Because heparin inhibits signaling through multiple kinase cascades, we investigated the possibility that phosphatases could be involved. Mitogen-activated protein kinase phosphatase-1 (MKP-1) was the predominant MKP detected in VSMC lines. MKP-1 protein was increased by serum stimulation of quiescent cells, and this increase was diminished by heparin (1 μg/mL). Increased MKP-1 expression was dependent on the mitogen-activated protein kinase, Erk. Decreased Erk activity in the presence of heparin preceded, and may account for, decreased MKP-1. The antimitogenic effects of heparin are therefore unlikely to act through a shift in the kinase/phosphatase balance, but rather through direct kinase suppression. However, because MKP-1 is known to cause an increase in activity of kinases upstream of Erk, that may signal through additional pathways, the decrease in MKP-1 activity may paradoxically enhance heparin's antiproliferative effects. VSMC selected to grow in the presence of heparin express decreased levels of MKP-1 that are unresponsive to heparin, and Erk activity becomes unresponsive to heparin in one cell line. We conclude that phosphatase activation is not a direct mechanism of suppression of multiple kinase cascades by heparin.

Keywords: Smooth muscle; Protein kinases; Protein phosphorylation; Signal transduction; Heparin resistance

1. Introduction

Mitogen-activated protein kinases (MAPKs), a ubiquitous group of tyrosine and serine/threonine kinases, play a central role in transducing extracellular signals mediating cell growth, differentiation, and apoptosis [1–3]. The MAPKs Erk1/p44 and Erk2/p42 are activated in cells stimulated with serum, by phosphorylation on tyrosine and threonine residues mediated by MEK1 and MEK2 [4–6]. The phosphorylation of both residues is necessary and sufficient for activation. It is now clear that MAPKs are inactivated by members of a dual specificity MAPK phosphatase family named MKPs [7–9]. Approximately 10 members of this family have been identified to date [10]. It has been reported that MKPs show tissue and substrate specificities [11,12]. At least three are known to differentially regulate the mammalian MAPK pathways; MKP-2 is widely expressed in human tissues and specifically regulates the Erk and JNK pathways by dephosphorylating those kinases, whereas PAC1 dephosphorylates Erk and p38 [9,13]. MKP-1 dephosphorylates all three MAPKs [9].

Erk induces MKP-1 expression [14] and stabilizes the protein by phosphorylation [15]. Dephosphorylation of Erk by MKP-1 regulates Erk activity *in vitro* and *in vivo* [16], so MPK-1 acts as a negative feedback regulator to limit sustained Erk signaling [17]. In particular, MKP-1 regulates Erk in cultured VSMCs [7], where it is expressed at high levels [11,18,19]. Following balloon injury to the rat carotid artery, MKP-1 activity in the muscle layer is decreased while Erk activity is increased, consistent with the resultant VSMC proliferation [11]. On the other hand, MKP-1 is induced by subjecting VSMC to mechanical strain, and it then reduces VSMC proliferation by inactivating MAPK [20]. Thus, MKP-1 appears to play a crucial role in regulating the response of VSMC to proliferative stimuli.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; MAPK, mitogen-activated protein kinase; MKP, mitogen-activated protein kinase phosphatase; pNPP, *p*-nitrophenyl phosphate; VSMC, vascular smooth muscle cell.

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Heparin is well known to inhibit proliferation and migration of VSMC [21–23], and does so at concentrations well below those needed to suppress growth of other cells [24]. Protein kinase C-dependent induction of c-*fos* is inhibited by heparin [25]. In this Erk-dependent pathway, heparin has been shown to inhibit Erk activation [26], decrease activity of the Erk kinase MEK1 [27], and (or) suppress phosphorylation of Raf-1 [28], perhaps depending on the stimulus used to activate the Raf/MEK/Erk pathway. Our previous studies with VSMC and smooth muscle-like mesangial cells showed that following serum stimulation of quiescent cells, heparin inhibits c-*fos* induction and [^{3}H]thymidine incorporation through both Erk-dependent and -independent pathways [29,30], in particular by suppressing activity of the Ca^{2+}/calmodulin-dependent protein kinase pathway [31]. It has been postulated that heparin may decrease kinase activity generally by activating phosphatase(s), thus, shifting the kinase/phosphatase balance [32]. This would potentially account for effects on diverse signaling pathways, and recently Mishra-Gorur *et al*. [33] have presented pharmacological evidence for activation of protein phosphatase-2A by relatively high concentrations of heparin.

The present study was undertaken in order to evaluate the effect of heparin on phosphatase activities in both naïve VSMC and VSMC selected for growth in the presence of heparin. The latter have been found to differ in their capacity to induce c-*fos* in response to serum stimulation, and therefore potentially have distinct signaling responses [34]. The results indicate that phosphatases are not involved in growth suppression by heparin through MAPK pathways. Rather, MKP-1 activity is itself diminished by heparin, and this is secondary to effects on upstream kinases.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS), culture media, and TRIzol reagent were obtained from Gibco BRL Life Technologies. NuSerum IV^{TM} was from BD Biosciences. Antibodies against MKP-1, MKP-2, PAC1, and Erk2 were obtained from Santa Cruz Biotechnology. Secondary antibody (donkey anti-rabbit IgG), enhanced chemiluminescence (ECL) kits, and protein A-Sepharose CL-4B were from Amersham Pharmacia Biotech. Bovine lung heparin was from Sigma (lot # 36H0659 was used throughout). Nitrocellulose membrane was from Bio Rad and Hybond N membrane from Amersham Pharmacia Biotech. Microcystin-leucine-arginine was from Calbiochem, and protease inhibitors, *p*-nitrophenyl phosphate (pNPP), and myelin basic protein were from Sigma. PD98059 was a product of New England Biolabs. A random primed DNA labeling kit was from Roche Diagnostics GmbH.

2.2. Cell culture

Two VSMC lines with differing sensitivity to heparin were used, reflecting the sensitivity of these cells *in vivo*. A10 cells from embryonic rat thoracic aorta [35] were obtained from the American Type Culture Collection (product no. CRL-1476) at passage 17. PAC-1 cells from rat pulmonary artery [36] were obtained at passage 40 from Dr. Catherine Prody (Hospital for Sick Children, Toronto). All cells were maintained in 10 cm petri dishes in a 5% CO_2 environment at 37°, in medium containing 10% FBS, and passaged by trypsinization. The basal medium was M199 for PAC-1 cells and DMEM for A10 cells. For experiments, cells were passaged 1:3 by trypsinization and allowed to grow overnight before being made quiescent by growth for 48 hr in 0.4% FBS. The resultant near confluent cultures were then stimulated by addition of the appropriate medium (M199 or DMEM) containing 5% NuSerum, with or without bovine lung heparin (1 μg/mL). The addition of NuSerum is defined as time 0 in all experiments. To select cells for growth in heparin, cultures were passaged at least five times at a 1:5 split ratio in the continual presence of 10 μg/mL heparin. Heparin was omitted when these heparin-selected cells were passaged for use in individual experiments.

2.3. Western blotting and immunoprecipitation

Cells were lysed in 0.5 mL RIPA buffer (50 mM Tris–HCl/pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mg/mL pepstatin, 1 mM Na_3VO_4, and 1 mM NaF), scraped from the dishes, and the cell lysate was centrifuged for 5 min in a microcentrifuge at 4°. Protein concentration was measured by Peterson's method [37]. Equal amounts of total protein in loading buffer (50 mM Tris–HCl/pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) were separated by 10% SDS–polyacrylamide gel electrophoresis. Proteins were transferred to 0.2 μm nitrocellulose membrane. Non-fat milk (5%) in TTBS solution (10 mM Tris–HCl/pH 7.4, 150 mM NaCl, 0.01% Tween 20) was used overnight to block non-specific binding. Primary antibody against the target protein was diluted in 1% non-fat milk in TTBS and incubated with the membrane for 2 hr at room temperature. The membrane was washed three times with TBS (10 mM Tris–HCl/pH 7.4, 150 mM NaCl), then probed with secondary antibody conjugated to horseradish peroxidase for 1 hr at room temperature. The blot was washed in TTBS (three times) and TBS (twice) in sequence. Immunoreactive bands were visualized by ECL detection. Immunoprecipitation was achieved by incubating cell lysate and antibody with protein A-Sepharose CL-4B beads overnight, then collecting the immunoprecipitate by centrifugation.

2.4. Phosphatase assays

Cells starved for 48 hr were stimulated with 5% NuSerum with or without 1 μg/mL heparin, and then washed with PBS after different time periods. Cell lysate buffer (500 μL) was then added directly to the plate. For measuring total protein, total tyrosine phosphatase activity, or individual tyrosine phosphatases, either 20 μg cell lysate protein or immunoprecipitate from 500 μg of total protein was mixed in 80 μL of 50 mM HEPES/pH 7.2 containing 10 mM dithiothreitol, and 5 mM EDTA. When measuring phosphotyrosine phosphatase activity, 20 nM microcystin-leucine-arginine (inhibitor of PP1 and PP2A) was included, and the phosphotyrosine phosphatase inhibitors Na_3VO_4 (1 mM) and $ZnCl_2$ (100 μM) were included in some experiments. After 5 min on ice, 20 μL of pNPP substrate was added at a final concentration of 10 mM. Reactions were incubated at 30° for 45 min (whole cell lysates) or 6 hr (immunoprecipitates). The reaction was stopped by adding 0.9 mL 0.2 M NaOH and the absorbance was measured at 405 nm.

2.5. Assay of Erk activity

Cells were scraped into lysis buffer 30 min after treatment and processed for immunoprecipitation with anti-Erk2 antibody as previously described [30]. Immunoprecipitates were recovered with protein A-Sepharose, and Erk activity was determined by phosphorylation of myelin basic protein in the presence of [γ-^{32}P]ATP. Incorporation of ^{32}P into myelin basic protein was determined by electrophoresis and autoradiography.

2.6. Northern blotting of MKP-1

For RT–PCR, 2–4 μg total RNA extracted from PAC-1 cells with TRIzol was used to synthesize the first-strand cDNA of rat MKP-1 with Moloney murine leukemia virus reverse transcriptase. The primer sequences for rat MKP-1 were designed based on the published sequences (GenBank accession no. NM_053769.1): upper primer, 5′-GCC TTG GGT ATC ACT GCT TTG-3′ (nucleotides 589–609); lower primer, 5′-TAC TCC GCC TCT GCT TCA CG-3′ (nucleotides 880–861). PCR (30 cycles) was carried out as follows: denaturing at 94° for 30 s, annealing at 55° for 45 s, and extension at 72° for 1 min. The 292 bp band of PCR product (MKP-1) was cut from a 2% agarose gel and purified with phenol and chloroform, precipitated with 100% ethanol and 3 M sodium acetate (pH 5.2). Fifty micrograms of MKP-1 fragment was labeled with [α-^{32}P]dCTP by random primed DNA labeling for use as a

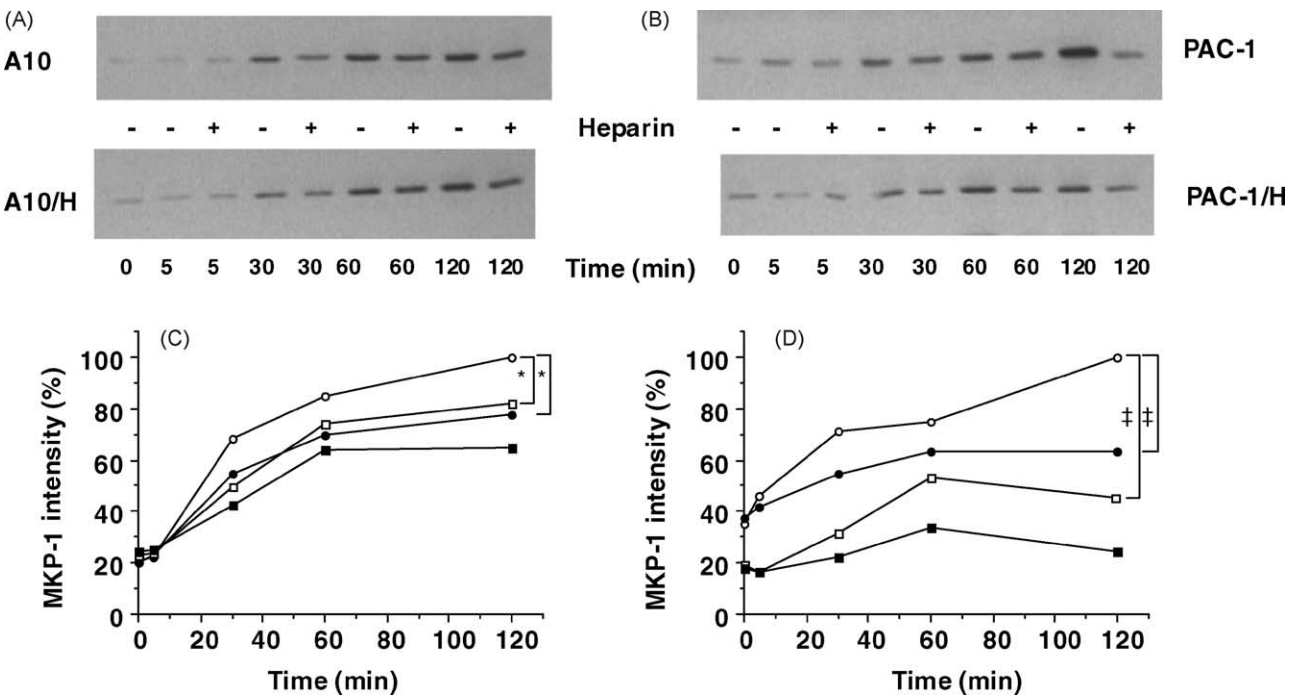

Fig. 1. Induction of MKP-1 expression by serum stimulation in smooth muscle cell lines. The photographs show representative Western blots of MKP-1 in A10 (A) or PAC-1 (B) cells, as indicated. The designation '/H' in the second row photographs refers to cells selected for growth in the presence of heparin (10 μg/mL) for between 5 and 10 passages. In all cases, cells were rendered quiescent by serum starvation in heparin-free medium as described in Section 2, then stimulated with 5% serum at time = 0 min, with or without heparin, as indicated under the top row of photographs. Cell extracts were prepared for Western blotting at 5, 30, 60, and 120 min as indicated. The line graphs (panel C, A10; panel D, PAC-1) show the mean signal intensities from three independent experiments, expressed relative to the value in native, heparin-unexposed cells at 2 hr taken as 100%. Error bars are omitted for clarity. Circles show results for native cells stimulated in the absence (open circles) or presence (filled circles) of 1 μg/mL heparin. Squares show results for heparin-selected cells, again stimulated in the absence (open squares) or presence (filled squares) of 1 μg/mL heparin. In the absence of added heparin (open symbols), all increases above $t = 0$ are significant in A10 cells, heparin-selected A10 cells, and PAC-1 cells by 30 min, and in heparin-selected PAC-1 cells at 60 min ($P < 0.05$). Significant decreases below control cells with heparin treatment or heparin selection are indicated at 120 min ($^{*}P < 0.05$; $^{‡}P < 0.01$).

probe for Northern blot hybridization. For Northern blotting, cells were starved for 48 hr in 0.4% FBS, then treated with 5% NuSerum with or without 1 μg/mL heparin for 15 min. After extraction with TRIzol, 15 μg of total RNA were loaded on a 1.2% formaldehyde gel, transferred to Hybond N membrane, hybridized with [α-^{32}P]dCTP-labeled MKP-1 cDNA fragment and plasmid coding 18S rRNA. Signals are corrected for 18S intensity as a loading control.

2.7. Statistical analysis

Results from multiple experiments are expressed as mean ± SD and single comparisons were made with the unpaired Student's *t*-test. For multiple experimental groups, significance was checked with one-way ANOVA and P values calculated by multiple comparison testing with Tukey's test. Values with $P < 0.05$ are considered significant.

3. Results

Initial experiments to assess potential global effects of heparin on phosphatase activity were conducted with total PAC-1 cell lysates using pNPP as a substrate. (Note that PAC-1 designates the cell line and PAC1 the phosphatase, consistent with terminology in the literature.) When quiescent cells were stimulated with 5% serum, there was no early effect on total phosphatase activity. However, the inclusion of heparin (1 μg/mL) decreased activity by 8.3 ± 4.4% (mean ± SD) over the first 30 min in three independent experiments. Inclusion of vanadate, a tyrosine phosphatase inhibitor, in the reaction buffer caused a decrease to 22.2 ± 1.2% ($P < 0.01$) of the activity seen in the absence of vanadate, and eliminated any difference between serum-stimulated samples with or without heparin in two separate experiments. This suggested an effect of heparin on phosphotyrosine or mixed function phosphatases, and we examined in more detail the involvement of specific MAPK phosphatases.

Neither MKP-2 nor PAC1 was detected by Western blotting extracts from either cell line, and phosphatase activity was low or absent in attempted immunoprecipitates of both phosphatases (data not shown). MKP-1 protein levels increased in all cells in a time-dependent manner after treatment with serum (Fig. 1). The increase is significant by 30 min in both A10 and PAC-1 cells ($P < 0.05$). Inclusion of 1 μg/mL heparin with the serum significantly decreased the level of MKP-1 attained at 2 hr in both A10 ($P < 0.05$) and PAC-1 ($P < 0.01$) cells. Selection of the cells by growth in the presence of 10 μg/mL heparin for at least five passages blunted the response of MKP-1 to serum, but the increase above basal levels is still significant in heparin-selected A10 cells at 30 min, and in heparin-selected PAC-1 cells at 60 min. Although the inclusion of 1 μg/mL heparin also resulted in lower MKP-1 levels at all times in both heparin-selected cell lines, these differences did not reach significance.

We confirmed our previous finding [34] that heparin partially inhibits serum-dependent Erk activation in both A10 and PAC-1 cells (Fig. 2). After heparin selection, both cell lines retain the ability to activate Erk in response to serum, and do so to a similar degree as prior to selection. However, A10 cells, but not PAC-1 cells, become insensitive to heparin following heparin selection; inclusion of 1 μg/mL heparin during serum stimulation has no effect on Erk activation in heparin-selected A10 cells (Fig. 2). Because Erk induces and stabilizes MKP-1 [14,15], we considered that decreased Erk activation could lead to the decreases in MKP-1 levels observed in the presence of heparin. The MEK1/2 inhibitor, PD98059, significantly decreased the level of MKP-1 in response to 60 min of serum treatment, in both A10 and PAC-1 cells (Fig. 3A and B). This effect is more pronounced in heparin-selected cells (Fig. 3C and D). Note that the absolute intensities are not comparable from panel to panel. The representative blots shown are from different experiments, and thus at different exposures, and are normalized to the 60 min control within each experiment. The relative amounts of basal MKP-1 in heparin-selected and unselected cells can be compared in Fig. 1, where they are seen to be similar within experimental error.

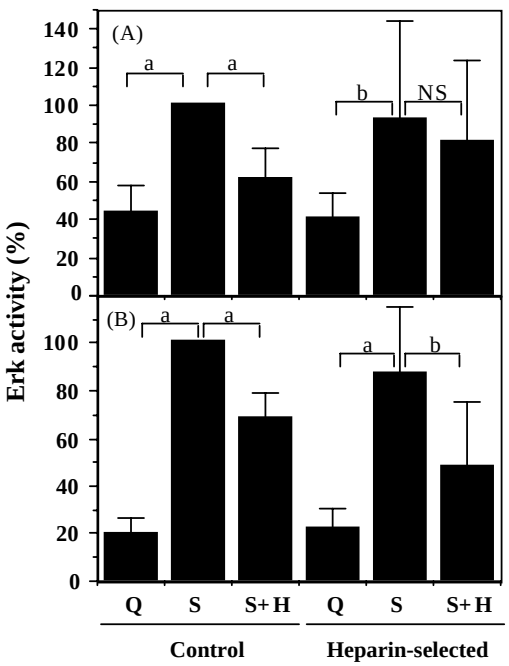

Fig. 2. Effect of heparin on Erk activity. Cells (panel A, A10 cells; panel B, PAC-1 cells) were left quiescent (Q) or stimulated with 5% serum (S) alone or with 1 μg/mL heparin (S + H). Cells not previously exposed to heparin (Control) are compared to those grown for five passages in the presence of 10 μg/mL heparin (Heparin-selected). Erk activity was determined after 30 min as described in Section 2 and expressed relative to serum-stimulated control cells taken as 100%. Values are mean ± SD (N = 7). Significant differences are indicated: a: $P < 0.0001$, b: $P < 0.03$, NS—not significant.

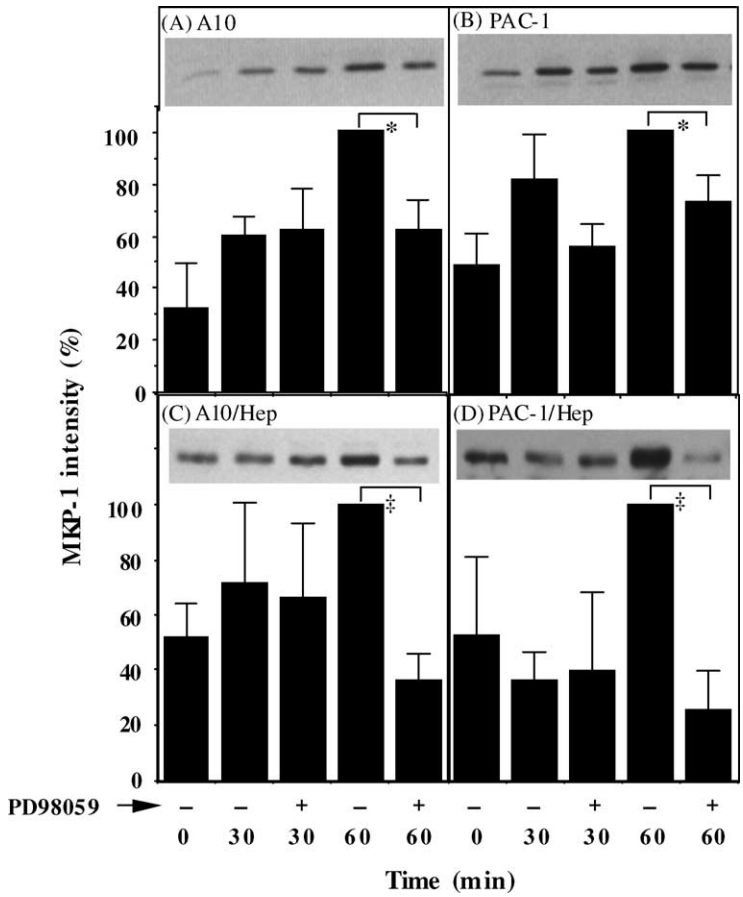

Fig. 3. Diminished serum-dependent increases in MKP-1 expression in the presence of the Erk inhibitor, PD98059. Quiescent A10 cells (A and C) or PAC-1 cells (B and D) were harvested before (0 min) or 30 or 60 min after stimulation with 5% serum, in the presence or absence of PD98059 (50 μM), as indicated. (A and B) Naïve cells; (C and D) heparin-selected cells. The photographs are of typical Western blots, and the histograms underneath show the mean ± SD of the signal intensities obtained by laser densitometry of such blots from three to five independent experiments, expressed relative to the intensity of the inhibitor-free sample at 60 min taken as 100%. Inhibition by PD98059 is significant in both cell types at 60 min ($^{*}P < 0.05$, $^{‡}P < 0.01$).

Activated Erk may increase MKP-1 either by inducing its transcription, stabilizing the protein by phosphorylation, or both. To distinguish between these possibilities, we examined levels of MKP-1 mRNA. In A10 cells, with or without heparin selection, MKP-1 mRNA levels were not increased during 2 hr of treatment with serum (Fig. 4A and C). Inclusion of heparin with the serum stimulus was likewise without effect. In PAC-1 cells, serum caused an increase of about 4-fold in MKP-1 mRNA, but only after 1 hr of exposure (Fig. 4B). Heparin did not significantly diminish the response, and levels returned to near basal values by 2 hr. Similar responses were noted in heparin-selected PAC-1 cells, except that the increase at 1 hr was about 10-fold.

4. Discussion

The present study demonstrates that heparin at 1 μg/mL does not increase phosphatase activity in VSMC, and in fact decreases the serum-stimulated levels of both Erk/MAPK and its regulatory phosphatase, MKP-1. This concentration is antiproliferative for these, as well as for primary VSMC [34]. Thus, the antiproliferative action of heparin on VSMC appears to involve kinase suppression that is independent of phosphatase activation. Previous studies with VSMC and smooth muscle-like mesangial cells showed an antiproliferative response to unfractionated bovine lung heparin down to 0.05–0.5 μg/mL, with A10 cells being somewhat less sensitive than other cell lines tested [29,34]. Therefore, we have avoided the higher concentrations of heparin frequently used in mechanistic studies of antiproliferation, but potentially complicated by enhanced growth factor interactions, displacement of matrix components, charge effects, etc. Our previous studies on suppression of mitogenic signaling [29–31,34] have focused on 1 μg/mL heparin, comparable to natural concentrations of endogenous heparan sulfates found in plasma [38]. Thus, although concentration dependence has not been studied in the present work, the conditions used are relevant to assessing the potential role of phosphatases in the heparin responsiveness of signaling events.

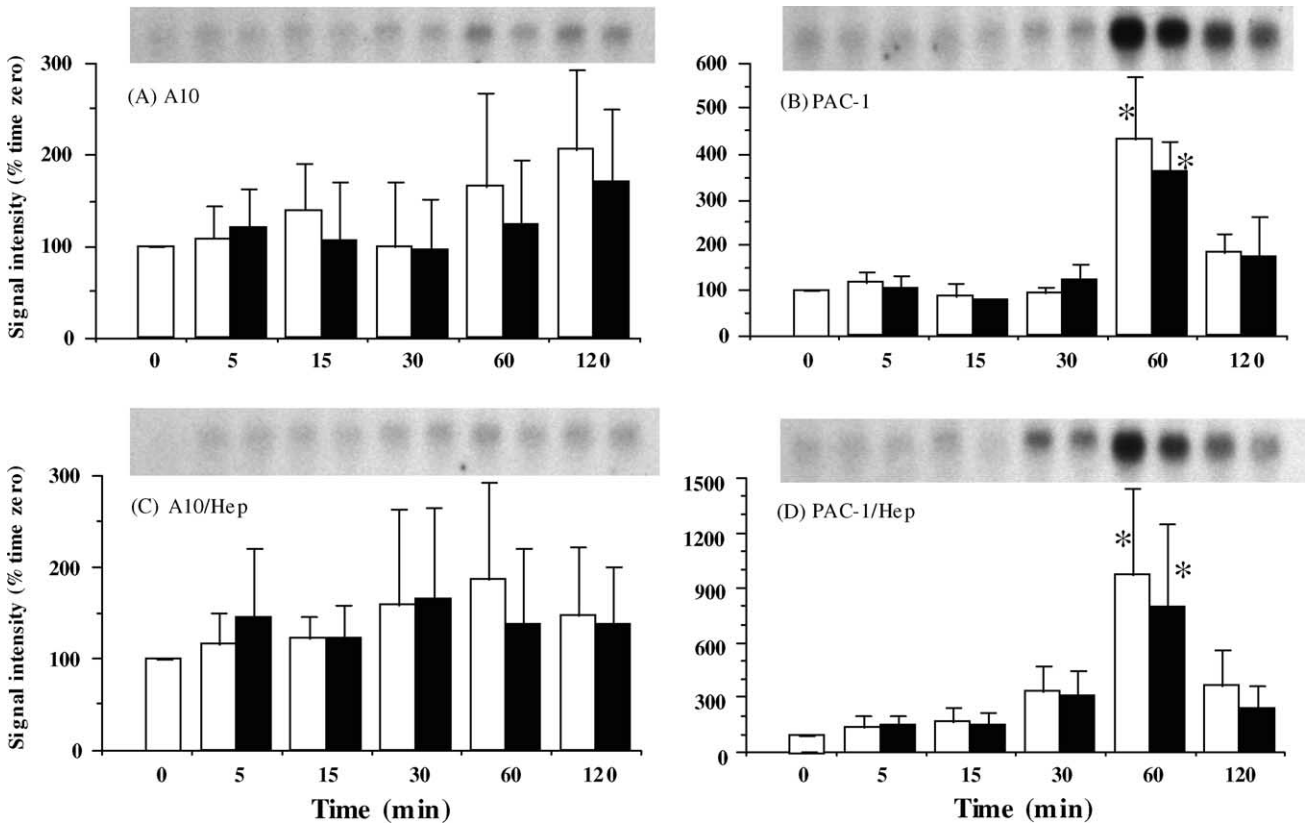

Fig. 4. Northern blotting of MKP-1 mRNA following serum stimulation. Quiescent cultures of A10 cells (A), PAC-1 cells (B), heparin-selected A10 cells (C), or heparin-selected PAC-1 cells (D) were treated with 5% serum for the indicated times, in the absence (open bars) or presence (filled bars) of 1 μg/mL heparin. The photographs show representative Northern blots. Histograms show the signal intensity from densitometry, corrected to 18S rRNA hybridized on the same blot and normalized to the basal level (time 0) as 100%. Values are mean ± SD from three and four independent experiments with A10 cells and PAC-1 cells, respectively. ∗ ($P < 0.01$) significant difference from time zero.

MKP-1 participates in a negative feedback loop to regulate Erk activity. MKP-1 phosphorylation is MEK1 dependent and MKP-1 is a substrate for Erk1 and Erk2 [15,39]. Consistent with these results, the MEK1 inhibitor, PD98059, blocks induction of MKP-1 in mesangial cells [40]. This could possibly be due to a non-specific effect on protein synthesis. However, that even at 100 μM the inhibitor was without effect on the mitogenic responsiveness of cells stimulated through the stress-activated protein kinase pathway suggests otherwise [41]. The phosphorylation of MKP-1 by Erk does not modify the phosphatase activity of MKP-1, but rather increases net activity by stabilizing the protein [15]. Inhibition of MKP-1 mRNA expression leads to prolonged activation of Erk [7]. Therefore, Erk increases MKP-1 which in turn decreases Erk activity, completing the feedback loop. In this scenario, suppression of Erk by heparin can be viewed as preceding an effect on MPK-1, and can account for the observed decrease in MKP-1. Consistent with this interpretation, heparin decreased serum- and phorbol ester-stimulated Erk activity after 2 min of exposure [30], before changes in MKP-1 are observed (e.g. Figs. 1 and 4).

Decreased MKP-1 activity has been shown to contribute to VSMC proliferation [11]. Presumably, this effect is predominantly through increased kinase activity, and the suppression of multiple kinase activities by heparin determines an antiproliferative response despite a decrease in MKP-1. However, additional functions of MKP-1 may be affected in heparin-treated cells. One effect of MKP-1 over-expression is to activate Raf-1 and MEK [42]. This somewhat paradoxical positive feedback regulation of upstream components of the Erk pathway appears to be mediated by Ras, and not to reflect removal of signal repression by inactivation of Erk. Thus, although MKP-1 decreases activity of the MEK target, Erk, it may activate Raf-1/MEK signaling through pathways other than those using Erk. The down-regulation of MKP-1 by heparin might enhance heparin's antimitogenic effects *via* such pathways. Thus, a caveat to the conclusion that the effects of heparin on VSMC predominantly involve kinase suppression is that decreases in both Erk and MKP-1 could act together to diminish mitogenic signaling.

The Erk-dependent effects of heparin on MKP-1 are not due to changes in mRNA levels; these levels are not affected by serum in A10 cells, and are increased by serum in PAC-1 cells only after 60 min, when changes in protein level have already nearly plateaued. This suggests that the major effect of Erk on the early regulation of MKP-1 in

these VSMC lines is on protein expression, perhaps due to increased translational efficiency or decreased degradation. Although the question of phosphorylation has not been addressed in the present study, Erk-dependent phosphorylation of MKP-1 is a well-established mechanism of achieving protein stabilization [15], and this suggests an explanation for the increased protein levels observed here. The late increase in MKP-1 mRNA in PAC-1 cells is insensitive to heparin and may represent an unknown, Erk-independent pathway.

VSMC preparations are generally heterogeneous in their growth characteristics [43]. Their sensitivity to heparin can also be variable [34,44,45], and this suggests that exposure to heparin may select cells that are resistant to heparin's antiproliferative effects. Several previous studies have documented changes in the antiproliferative and/or signaling effects of heparin after selection for growth in the presence of heparin, typically at concentrations of 100–200 μg/mL [45–50]. Because we are examining effects on mitogenic stimulation of VSMC that are observed at a heparin concentration of 1 μg/mL, we selected cells for growth in 10 μg/mL heparin. This concentration is much lower than used in previous studies, but higher than required to elicit acute effects during stimulation of quiescent VSMC. Thus, it is not clear whether the heparin-selected cells studied here are altered in the same way as those in previous studies. Nevertheless, two observations potentially relevant to heparin resistance are presented here. First, heparin-selected cells display decreased levels of MKP-1 in comparison to their unselected counterparts, and fail to respond to acute heparin treatment with decreased MKP-1. Secondly, the ability of heparin to suppress Erk activation is lost in heparin-selected A10 cells. Because MKP-1 expression is associated with decreased VSMC proliferation, as discussed above, the diminished expression of MKP-1 after heparin selection is consistent with a role for decreased MKP-1 levels in the heparin-resistant phenotype. We suggest that decreased MKP-1 levels in unselected VSMC stimulated with serum in the presence of heparin are due to a decrease in Erk activity and Erk-dependent MKP-1 induction. However, serum stimulation of heparin-selected cells results in Erk activation comparable to that seen in unselected cells, but still produces a decreased induction of MKP-1. This suggests that, at least in heparin-selected cells, additional mechanisms must be regulating MKP-1 expression. Finally, while the mechanism of the loss of an acute response of Erk to heparin in heparin-selected A10 cells is not obvious, it does indicate that signaling pathways involved in the mitogenic response may alter adaptively under heparin selection.

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