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Kidney International, Vol. 57 (2000), 2308–2318

Thrombin is a novel regulator of hexokinase activity in mesangial cells

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Thrombin is a novel regulator of hexokinase activity in mesangial cells.

Background. Hexokinase (HK) activity is fundamentally important to cellular glucose uptake and metabolism. Phorbol esters increase both HK activity and glucose utilization in cultured mesangial cells via a protein kinase C (PKC)- and extracellular signal-regulated kinases 1 and 2 (ERK1/2)-dependent mechanism. In adult kidneys, increased HK activity has been reported in both glomerular injury and in diabetes, but the mechanisms responsible for these changes are unknown. Thrombin, a known activator of both PKC and ERK1/2, is increased in the settings of renal injury and diabetes. Thus, thrombin may contribute to the observed changes in HK activity in vivo.

Methods. Thrombin and thrombin receptor agonists were tested for the ability to increase HK activity and glucose metabolism in murine mesangial (SV40 MES 13) cells. ERK1/2 activation was also evaluated in parallel. Thrombin inhibition (hirudins), PKC depletion, Ser-Thr kinase inhibition (H-7), MEK1/2 inhibition (PD98059), pertussis toxin (PTX), and general inhibitors of transcription or translation were then tested for the ability to attenuate these effects.

Results. Thrombin ($\geq$0.01 U/mL) mimicked the effect of phorbol esters, increasing HK activity >50% within 12 to 24 hours ($P < 0.05$). This effect was inhibited by hirudins, mimicked by thrombin receptor agonists, and accompanied by increased Glc utilization. H-7, PD98059, and general inhibitors of transcription or translation—but not PTX—prevented thrombin-induced HK activity at 24 hours. PKC depletion and PD98059 also blocked the associated phosphorylation and activation of ERK1/2.

Conclusions. Thrombin increases mesangial cell HK activity via a PTX-insensitive mechanism involving thrombin receptor activation, PKC-dependent activation of ERK1/2, and both ongoing gene transcription and de novo protein synthesis. As such, thrombin is a novel regulator of HK activity in mesangial cells and may play a role in coupling renal injury to metabolism.

Key words: hexokinase, glucose, protein kinase C, extracellular signal-regulated kinases 1 and 2, renal injury.

Received for publication May 25, 1999
and in revised form December 17, 1999
Accepted for publication January 20, 2000

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Hexokinases (HKs) are fundamentally important to all cells. By catalyzing the phosphorylation of glucose (Glc) to yield Glc-6-P, they play a central role in both the facilitated uptake and subsequent metabolism of Glc. In the insulin-sensitive peripheral tissues that account for the bulk of systemic Glc uptake and utilization (that is, skeletal muscle and adipose), insulin is a major regulator of Glc metabolism and HK activity. In contrast, the kidney does not depend on insulin for normal Glc metabolism and is characterized by a state of Glc overutilization in the setting of diabetes, which differs markedly from the state of Glc underutilization observed in diabetic muscle and adipose tissue [1–10]. It is therefore of great interest that increased renal HK activity has been reported not only in experimental diabetes, but also in genetic obesity [8], compensatory renal hypertrophy [9], and puromycin aminonucleoside nephrosis [11]. The molecular mechanisms underlying these changes and the normal determinants of HK activity in the kidney, however, are not known. Moreover, the contributions of individual cell types to these changes have not been addressed. As the principal renal cell type affected by a variety of pathologic conditions, including diabetes, we are particularly interested in the regulation of HK activity in mesangial cells.

In preliminary testing, several known regulators of HK activity in extrarenal tissues (for example, insulin [12], adrenomimetics [13], and glucocorticoids [14]) were found to have no significant effect on HK activity in cultured mesangial cells (R.B. Robey, unpublished observations), suggesting that this cell type may exhibit novel regulatory characteristics with regard to Glc-phosphorylating capacity. Compatible with this interpretation, we recently reported the novel regulation of mesangial cell HK activity by phorbol esters [15]. The ability of phorbol esters to increase HK activity required the sequential activation of protein kinase C (PKC) and extracellular signal-regulated kinases 1 and 2 (ERK1/2). The corresponding activators of these signaling interme-

diates that are capable of altering mesangial cell HK activity in vivo, however, are not known.

The neutral serine protease α-thrombin (thrombin) is a potent mesangial cell mitogen and is a known activator of both PKC [16] and the classic mitogen-activated protein kinase (MAPK or ERK1/2) pathway [17]. Interestingly, thrombin has also been shown to stimulate Glc uptake and utilization in platelets [18, 19], which strongly express the protease-activated receptor type 1 (PAR1) thrombin receptor [20]. This receptor is proteolytically activated by thrombin via targeted cleavage of its extracellular NH_2-terminus, resulting in a new NH_2-terminal "tethered ligand" capable of receptor activation via an intramolecular mechanism [20]. PAR1 is also strongly expressed by mesangial cells and has been shown to mediate a number of thrombin's cellular effects in this cell type [21, 22], but the effects of thrombin and PAR1 activation on Glc metabolism and on HK activity in particular have not been described. We therefore tested the ability of PAR1 activation by thrombin to alter HK activity in cultured mesangial cells.

METHODS

Reagents

Bordetella pertussis toxin (PTX) was obtained from Calbiochem (La Jolla, CA, USA), as were the specific MEK1/2 inhibitor PD98059 and the recombinant hirudin variant [$Lys^{33}/Arg^{36}/Arg^{47}$]-rHV1. Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was obtained from Fluka (Milwaukee, WI, USA), and yeast Glc-6-phosphate dehydrogenase (G6PDH) was obtained from Boehringer Mannheim (Indianapolis, IN, USA). All cell culture reagents, including serum and additives, were supplied by GIBCO BRL (Gaithersburg, MD, USA). The hexapeptide PAR4 thrombin receptor agonist (GYPGKF) used in these studies corresponds to the NH_2-terminal tethered ligand of proteolytically activated murine PAR4 ($MmPAR4^{33/38}$) and was the generous gift of Dr. Trevor Walker (University of Edinburgh, Edinburgh, UK). The corresponding PAR1 hexapeptide agonist (SFLLRN) is identical to the human PAR1-tethered ligand ($HsPAR1^{50/55}$) and was obtained from Advanced ChemTech (Louisville, KY, USA). All other reagents, including high-activity bovine α-thrombin, the recombinant leech hirudin variant [Lys^{47}]-rHV2, phorbol 12-myristate 13-acetate (PMA), phorbol-12,13-didecanoate (PDD), 4α-phorbol-12,13-didecanoate (4α-PDD), H-7 (1-(5-Isoquinolinesulfonyl)-2-methylpiperazine·HCl), cycloheximide (CHX), NADP, ATP, and the 14-mer PAR1 agonist peptide $HsPAR1^{42/55}$ (SFLLRNPNDKYEPF), were obtained from Sigma (St. Louis, MO, USA), unless otherwise noted, and were of the finest quality available.

Cell culture

Mycoplasma-free SV40 MES 13 (murine mesangial) cells were obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 27. These cells are derived from glomerular explants of SV40 transgenic mice and exhibit both biochemical and morphologic features of normal mesangial cells in culture [15, 23]. Cells were maintained in DME:F12 (3:1) medium containing 6 mmol/L Glc and were supplemented with both 14 mmol/L HEPES and 5% fetal bovine serum. Cells were routinely grown to confluence in a humidified 37°C/5% CO_2 incubator before testing, and all experiments reported herein were performed between passages 30 and 40 to minimize the effects of phenotypic variation in continuous culture. Where appropriate, cells were serum-deprived for 24 hours prior to and during testing. When inhibitors were employed, cells were typically pretreated with inhibitor alone for 30 to 60 minutes before testing.

Hexokinase assays

Hexokinase activity was measured as the total Glc-phosphorylating capacity of whole cell extracts using a standard G6PDH-coupled assay [24]. In brief, cell lysates were prepared by a modification of the method of O'Doherty et al [25] in 900 mmol/L KCl, 20 mmol/L $MgCl_2$, 10 mmol/L ethylenediaminetetraacetic acid (EDTA), 11.1 mmol/L monothioglycerol, 0.25% Triton X-100, 10 mmol/L Glc, and 20 mmol/L Tris·HCl, pH 8.1, as described previously [15]. Freshly prepared aliquots were then assayed spectrophotometrically for the Glc- and ATP-dependent reduction of NADP in the presence of excess G6PDH (the final assay mixture composition was 1 U/mL G6PDH, 0.5 mg/mL NADP, 6.7 mmol/L ATP, 7.7 mmol/L MgCl, 4.2 mmol/L Glc, 45 mmol/L KCl, 1 mmol/L NaH_2PO_4, 10.6 mmol/L monothioglycerol, 0.01% Triton X-100, 0.5 mmol/L EDTA acid, and 42 mmol/L Tris·HCl, pH 8.5). All assays were performed at 25°C under conditions of linear HK-limited NADPH formation, and total HK activity, normalized for cellular protein content, was expressed as the Glc phosphorylation rate relative to paired unstimulated controls [15].

Extracellular signal-regulated kinases 1 and 2 phosphorylation and activity assays

Extracellular signal-regulated kinases 1 and 2 phosphorylation was evaluated by immunoblot analysis of whole cell lysates using specific monoclonal antibodies directed against a common ERK1/2 epitope (total ERK1/2) and the dual-phosphorylated tripeptide motif of ERK1/2 (phospho-ERK1/2) as described previously [15]. The corresponding ERK1/2 phosphotransferase activity was measured by a specific immunopreciptated kinase (IP/kinase) activity assay using a commercially available kit (New England BioLabs, Beverly, MA, USA) according

to the manufacturer's recommendations. In brief, activated ERK1/2 immunoprecipitates were prepared from cell lysates using immobilized monoclonal antibodies directed against dual-phosphorylated (that is, activated) ERK1/2. Immunoprecipitates were then assayed for the ability to specifically Ser phosphorylate an Elk-1 fusion protein in vitro. Total phosphotransferase activity was assessed by immunoblotting with rabbit polyclonal IgG specific for phospho-Elk-1 (Elk-1-P) followed by chemiluminescent detection using horseradish peroxidase-conjugated antirabbit secondary antibodies and the Phototope®-HRP Western Detection System (New England BioLabs). An Eagle-Eye® II still videoimaging system (Stratagene, La Jolla, CA, USA) was employed for autoradiogram image acquisition, and analysis of the resulting digital images was performed using NIH Image version 1.61 software for Macintosh computers (National Institutes of Health, Bethesda, MD, USA). Control IP/kinase assays were routinely performed in parallel using unstimulated cell lysates with and without the addition of functional MEK-activated recombinant ERK2.

Glucose utilization and lactate production assays

Glucose utilization and lactate production were assayed as the net disappearance of Glc and the net accumulation of lactate in the culture medium, respectively. Cells were routinely tested in defined growth medium containing 6 mmol/L Glc and lacking phenol red. At appropriate time points, medium aliquots were assayed spectrophotometrically for both Glc and lactate content via standard enzymatic coupled reactions as described previously [15]. Glc was assayed by spectrophotometric monitoring of chromagen formation coupled to Glc oxidase [26], and the lactate assay was based on similar chromagen formation coupled to lactate oxidase. All measures of medium Glc and lactate content were performed in the presence of nonlimiting concentrations of Glc and under conditions of linear net Glc utilization and lactate accumulation.

Statistical analysis

Data are presented as the mean ± SEM of at least three independent experiments unless otherwise noted. Statistical comparisons were performed by analysis of variance (ANOVA) or paired *t*-testing where appropriate using a significance level of 95% and StatView 5.0 software for Macintosh computers (SAS Institute, Cary, NC, USA).

RESULTS

Thrombin increases mesangial cell HK activity in a time- and dose-dependent manner

Thrombin increased total HK activity in SV40 MES 13 cells in both a time- and dose-dependent manner. As

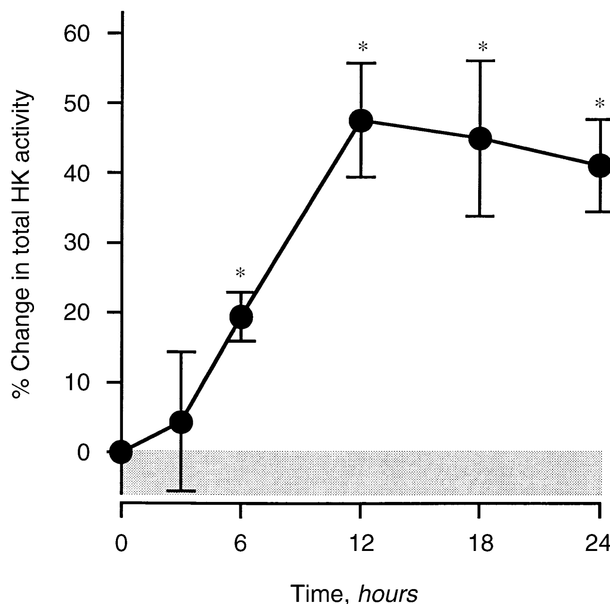

Fig. 1. Thrombin increases mesangial cell hexokinase (HK) activity in a time-dependent fashion. Thrombin (1 U/mL) increased HK activity approximately 20% within six hours and as much as approximately 50% above basal levels during maximal induction observed between 12 and 24 hours (*$P < 0.03$). All data are presented as the mean ± SEM for at least four independent measures.

depicted in Figure 1, the maximal HK induction was observed within 12 to 24 hours of exposure to 1 U/mL thrombin. Although HK activity began to decline thereafter, the total activity of stimulated cells was still at least 10 to 15% higher than unstimulated controls at 36 hours (data not shown). As shown in Figure 2, the effect of thrombin at 24 hours was concentration dependent, with significant increases in HK activity uniformly observed at thrombin activities >0.001 U/mL ($P < 0.05$) and a plateau effect, with increases of as much as 40 to 50% observed between 0.01 and 10.0 U/mL. Preliminary testing in cultured rat mesangial cells and an SV40-transformed human mesangial (C2M15) cell line that also exhibits biochemical and morphologic features of normal mesangial cells in culture [27] yielded similar results (data not shown). To better understand the molecular mechanisms underlying these responses, we further characterized the response in SV40 MES 13 cells exposed to 1 U/mL thrombin for 24 hours.

Hirudins inhibit thrombin-induced mesangial cell HK activity

To evaluate the specificity of thrombin's effect on mesangial cell HK activity, we tested recombinant leech hirudins for the ability to inhibit this effect. These proteins specifically bind to the active site of thrombin and thereby inhibit its proteolytic activity. As shown in Figure 3, the recombinant type 2 hirudin variant [Lys^{47}]-

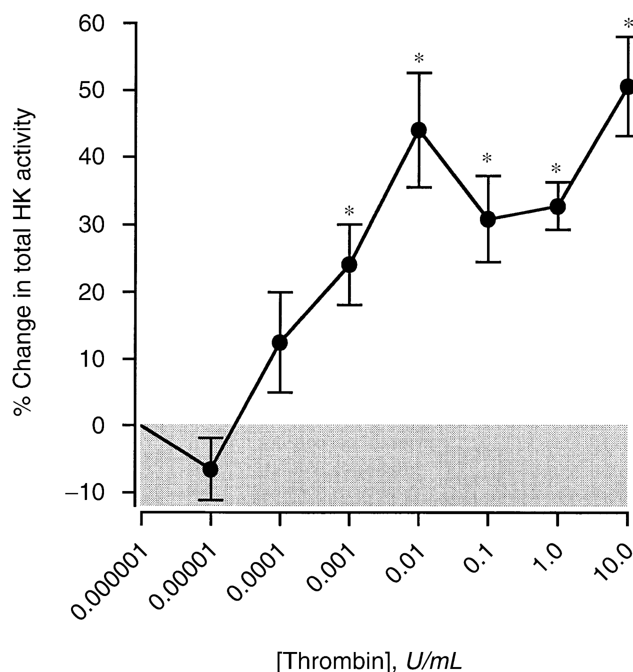

Fig. 2. Thrombin exhibits a dose-dependent effect on mesangial cell HK activity. Thrombin significantly increased total HK activity in cultured mesangial cells exposed to thrombin activities > 0.001 U/mL for 24 hours (*$P < 0.05$ vs. unstimulated controls). Data are presented as the mean ± SEM for at least four independent measures, and all thrombin activities are reported in NIH units defined as specific proteolytic activity in a standardized assay relative to a thrombin reference standard.

rHV2 inhibited thrombin-induced (Fig. 3A), but not basal (Fig. 3B) HK activity. Another recombinant hirudin variant, [$Lys^{33}/Arg^{36}/Arg^{47}$]-rHV1, was also tested with similar results (data not shown), further suggesting specificity for thrombin.

PAR1 thrombin receptor agonists mimic thrombin's effect on HK activity

The PAR1 thrombin receptor activator peptides $HsPAR1^{50/55}$ (SFLLRN) and $HsPAR1^{42/55}$ (SFLLRNPND KYEPF) each correspond to the NH_2-terminus of proteolytically activated human PAR1 and have been shown to specifically mimic the effect of proteolytic PAR1 activation. As shown in Figure 4, $HsPAR1^{42/55}$ mimicked the effect of thrombin on mesangial cell HK activity. Similar results were obtained with the hexapeptide agonist $HsPAR1^{50/55}$ (data not shown). In contrast, the PAR4-activating hexapeptide $MmPAR4^{60/65}$, corresponding to the murine PAR4-tethered ligand sequence (GYPGKF), had no significant effect on HK activity. Exposure to 1, 10, 100, 200, or 500 μmol/L $MmPAR4^{60/65}$ for 24 hours resulted in total HK activities that were 93 ± 3% (N = 3, P = NS), 101 ± 8% (N = 3, P = NS), 102 ± 8% (N = 3, P = NS), 108 ± 5% (N = 4, P = NS), and 111 ± 9% (N = 3, P = NS) of unstimulated control cells, respectively. In contrast, cells exposed to 1 U/mL thrombin and tested in parallel increased total HK activity by approximately 30% ($P < 0.05$).

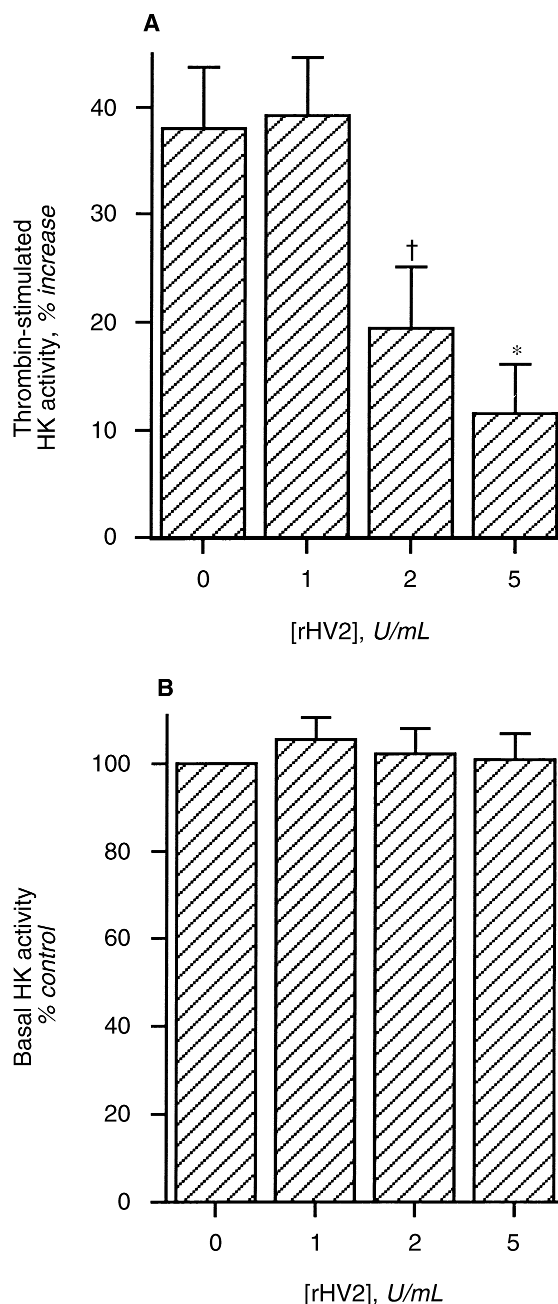

Fig. 3. Hirudins inhibit thrombin-induced (*A*), but not basal (*B*), mesangial cell HK activity. To assess the specificity of our observations, we tested the ability of hirudins to inhibit HK induction by 1 U/mL bovine thrombin. The recombinant type 2 hirudin variant [Lys^{47}]-rHV2 (rHV2) inhibited thrombin-induced HK activity in a dose-dependent fashion (A), whereas basal HK activity remained unaffected (B). The depicted data represent the mean ± SEM of at least four independent experiments (†$P < 0.02$ and *$P < 0.04$ vs. thrombin-stimulated controls).

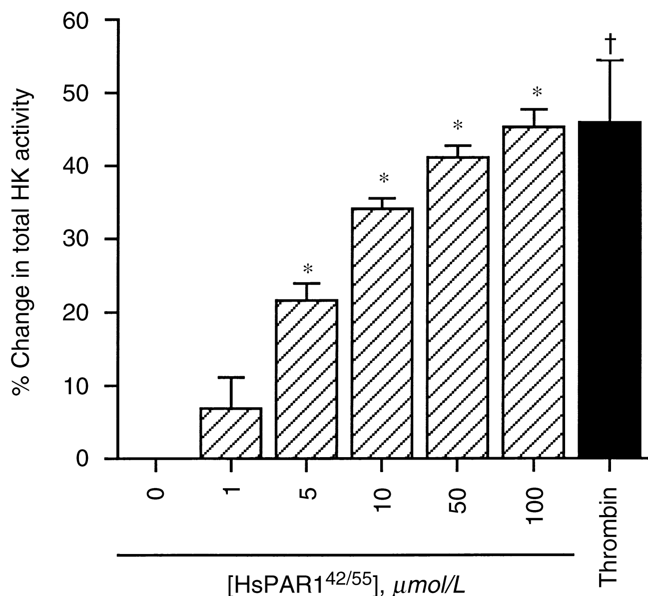

Fig. 4. PAR1 thrombin receptor peptide agonists mimic the effect of thrombin on HK activity. The specific PAR1 peptide agonist HsPAR1$^{42/55}$ (SFLLRNPNDKYEPF) increased HK activity in a dose-dependent manner (▨) and fully mimicked the effect of 1 U/mL thrombin (■) at 24 hours (*$P < 0.003$ and †$P < 0.04$ vs. unstimulated controls). Data are presented as the mean ± SEM for at least three independent measures. Importantly, the hexapeptide PAR1 agonist HsPAR1$^{50/55}$ (SFLLRN) also mimicked these effects at 24 hours (data not shown).

Thrombin's effect on total HK activity is PTX-insensitive

G-protein–coupled receptors typically activate a single type of G protein. PAR1, however, is known to couple with both PTX-sensitive G_i and PTX-insensitive G_q proteins [22, 28, 29]. We therefore attempted to functionally characterize the type of G-protein interaction responsible for mediating activated PAR1's effect on HK activity. To this end, we tested the ability of 100 ng/mL PTX to influence total HK activity at 24 hours. As shown in Figure 5, continuous PTX exposure had no significant effect on either basal or stimulated HK activities.

Ser-Thr kinase inhibition attenuates thrombin-induced mesangial cell HK activity

Thrombin is also a well-known activator of PKC in cultured mesangial cells, and general Ser-Thr kinase inhibition by H-7 has been shown to inhibit some of the cellular effects of thrombin in this cell type [30]. We therefore tested H-7 for the ability to inhibit thrombin-induced HK activity in cultured mesangial cells. To this end, we employed H-7 concentrations (25 to 50 μmol/L) previously shown to be effective in blocking PKC activation and thrombin action in this cell type without adversely affecting cell viability with prolonged 24-hour exposure [30, 31]. As shown in Figure 6, 50 μmol/L H-7 prevented the increase in HK activity by approximately 70% ($P < 0.05$), consistent with a postreceptor role for Ser-Thr kinases such as PKC in mediating this effect. Basal HK activity was also slightly, albeit significantly, affected by H-7 at this concentration, suggesting that Ser-Thr kinase signaling intermediates such as PKC may also play a small role in the maintenance of basal activity. These changes were not accompanied by alterations in the cellular exclusion of trypan blue (data not shown), suggesting that they are not primarily attributable to changes in cell viability.

Thrombin increases HK activity via a classic MAPK pathway-dependent mechanism

We have previously shown that the specific MEK1/2 inhibitor PD98059 is capable of blocking the induction of HK activity by phorbol esters [15]. We therefore tested the ability of MEK1/2 inhibition to similarly block HK induction by thrombin. As demonstrated previously for phorbol esters, 50 μmol/L PD98059 completely prevented the induction of HK activity by 1 U/mL thrombin at 24 hours: Total HK activities in the presence or absence of PD98059 were 102 ± 7% and 134 ± 8% of unstimulated control values ($P < 0.02$), respectively. To further evaluate the involvement of the classic MAPK pathway, we also directly tested the ability of thrombin to activate ERK1/2. Both thrombin and PAR1 peptide agonists increased ERK1/2 phosphorylation (Fig. 7A) and activity (Fig. 7B) within five minutes. Cellular PKC depletion by prolonged (24 hours) exposure to 1 μmol/L PMA completely inhibited the increases in ERK1/2 phosphorylation (Fig. 7A) and activity (Fig. 7B) observed following thrombin or PAR1 peptide agonist treatment. As expected, thrombin's ability to activate ERK1/2 was also blocked by PD98059 inhibition of MEK1/2 (data not shown).

Thrombin-stimulated HK activity requires both ongoing gene transcription and de novo protein synthesis

We have previously shown that phorbol esters increase mesangial cell HK activity within 12 to 24 hours via a mechanism that requires both ongoing gene transcription and de novo protein synthesis [15]. To test whether thrombin-stimulated HK activity exhibits similar requirements, we tested both the general transcriptional inhibitor DRB and the general translational inhibitor CHX for the ability to block HsPAR1$^{42/55}$-induced HK activity. As with phorbol esters, both agents completely prevented the increase in HK activity normally seen following PAR1 activation (Table 1). A small, but significant, inhibition of basal HK activity was also observed following a 24-hour exposure to 20 μmol/L DRB, consistent with our previously reported observations [15].

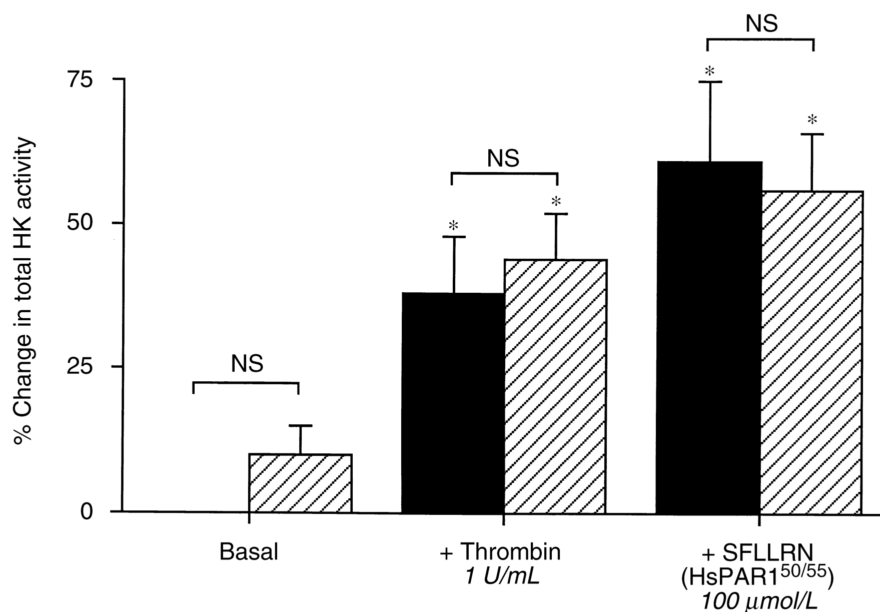

Fig. 5. PAR1-mediated HK induction by thrombin is not pertussis toxin (PTX) inhibitable. Total HK activity was increased in mesangial cells stimulated by either thrombin (1 U/mL) or the hexapeptide PAR1 agonist $HsPAR1^{50/55}$ (SFLLRN; 100 μmol/L) for 24 hours (*$P < 0.05$ vs. unstimulated controls). The presence of 100 ng/mL PTX had no significant effect (P = NS) on either stimulated or unstimulated HK activities. The depicted data represent the mean ± SEM of at least four independent experiments. Symbols are: (■) –PTX; (▨) + 100 ng/mL PTX.

Increases in total HK activity are accompanied by increased Glc metabolism

We have also previously shown that the phorbol ester PMA increases Glc metabolism by cultured mesangial cells, as measured by net Glc disappearance and lactate accumulation in the culture medium, and that these changes temporally correspond to PMA-induced increases in total HK activity [15]. To confirm that thrombin-induced changes in total HK activity were also associated with changes in mesangial cell Glc metabolism, we evaluated the effect of thrombin on these parameters. As shown in Figure 8, both thrombin and the active phorbol ester PDD mimicked the effect of PMA on Glc metabolism, whereas the inactive 4α-stereoisomer of PDD (4α-PDD), which is incapable of activating PKC [32], did not.

The observed increases in mesangial cell HK activity are specific for thrombin

Finally, we tested the ability of other activators of PKC to mimic the effects of phorbol esters and thrombin on mesangial cell HK activity. The vasoactive peptides angiotensin II (Ang II) and endothelin-1 (ET-1) are potent mesangial cell mitogens that share several common signaling intermediates with thrombin in this cell type, including PKC [33]. We therefore tested the ability of both Ang II and ET-1 to influence total HK activity in cultured mesangial cells. Interestingly, both Ang II and ET-1 failed to mimic the effects of thrombin or PAR1 peptide agonists on HK activity. In fact, 24-hour exposure to 0.1, 1, 10, 100, 200, 500, or 1000 nmol/L ET-1 resulted in total HK activities 96 ± 5% (N = 4, P = NS), 103 ± 10% (N = 4, P = NS), 99 ± 10% (N = 4, P = NS), 110 ± 6% (N = 5, P = NS), 104 ± 16% (N = 3, P = NS), 98% (N = 1), and 93% (N = 1) of control values, respectively. Similarly, cells exposed to 1 nmol/L or 1 μmol/L Ang II for 24 hours resulted in HK activities that were 104 ± 3% (N = 3, P = NS) and 117 ± 3% (N = 4, P = NS) of unstimulated controls, respectively. Control cells exposed to 1 μmol/L PMA and evaluated in parallel uniformly increased HK activity as described previously [15].

DISCUSSION

In addition to its central role in hemostasis, thrombin regulates a number of important cellular responses to vascular injury, including cell growth, cell migration, and matrix accumulation. Although best characterized in platelets [34, 35] and in vascular endothelial cells [36], thrombin's cellular actions have been demonstrated in a variety of other cell types [37–40], including glomerular mesangial cells [30, 33, 41–43]. These diverse cellular effects require thrombin's proteolytic activity and are largely mediated by specific interaction with one or more recently identified members of the protease-activated receptor family (PARs). In addition to being a potent mesangial cell mitogen [30, 41, 44], thrombin has also been shown to alter a number of important mesangial cell functions such as contraction [44], prostaglandin E_2 production [44], and expression of both tissue-type plasminogen activator and plasminogen activator inhibitor 1 [30]. The G-protein–coupled heptaspanning PAR1 thrombin receptor is strongly expressed in mesangial cells [21, 22] and mediates a number of these effects [30, 33, 41, 44–46].

The present demonstration of thrombin-induced HK

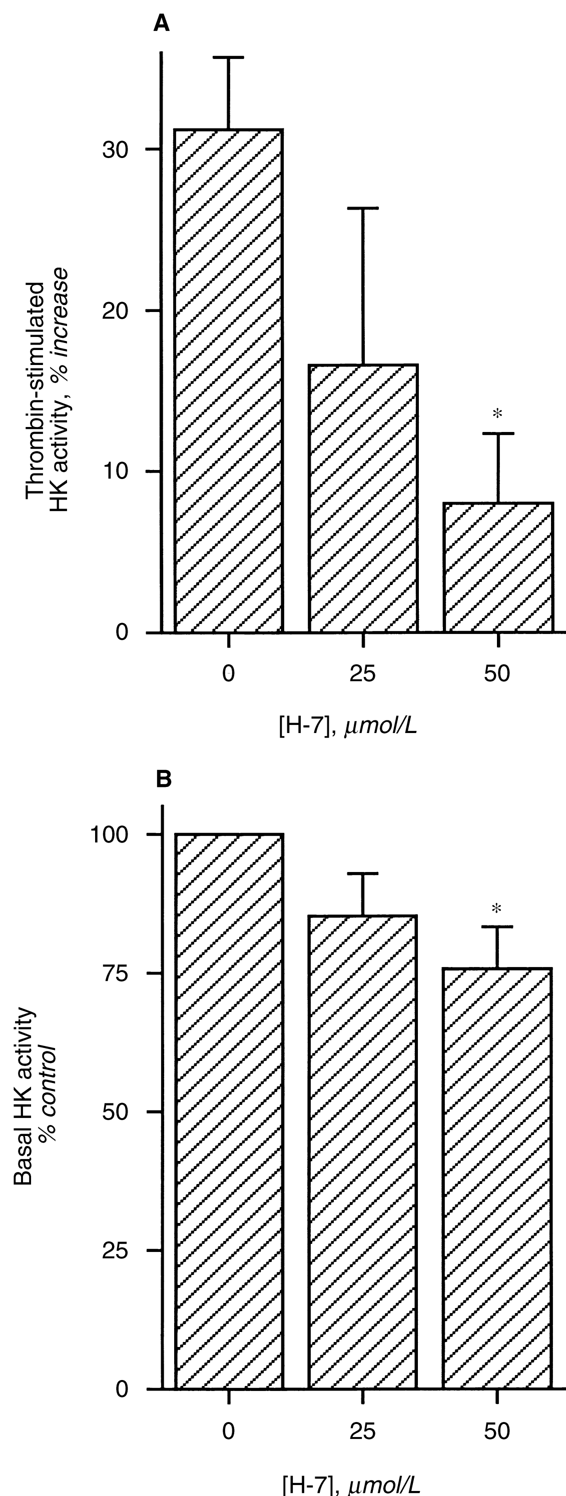

Fig. 6. H-7 inhibits thrombin-induced mesangial cell HK activity. (*A*) H-7 inhibited thrombin-induced HK activity at 24 hours in a dose-dependent fashion and at concentrations known to inhibit PKC activity (*$P < 0.05$). (*B*) A smaller, albeit statistically significant, effect was also observed on basal activity at higher concentrations of H-7. All data are presented as the mean ± SEM of at least four independent determinations.

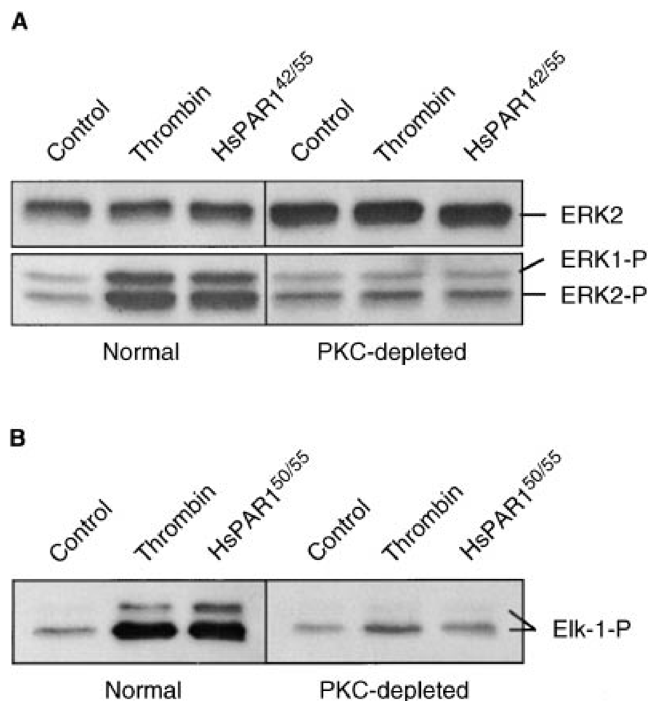

Fig. 7. Thrombin and PAR1 peptide agonists activate ERK1/2 via a PKC-dependent mechanism. (*A*) Both total ERK2 and phospho-ERK1/2 were detected and quantitated by parallel immunoblot analyses of lysates prepared from cells exposed to thrombin (1 U/mL) or the 14-mer PAR1 peptide agonist $HsPAR1^{42/55}$ (SFLLRNPNDKYEPF; 100 μmol/L) for five minutes. Both thrombin and $HsPAR1^{42/55}$ were capable of increasing the specific phosphorylation of ERK1/2, and these effects were inhibited by antecedent PKC depletion resulting from prior 24-hour exposure to 1 μmol/L PMA. (*B*) Total ERK1/2 activity was also assayed in whole cell lysates as the ability of dual-phosphorylated ERK1/2 immunoprecipitates to phosphorylate an Elk-1 fusion protein in vitro (Elk-1-P). The duplicate, slower migrating band observed in stimulated cell lysates represents a hyperphosphorylated form of the Elk-1-P fusion protein. Both thrombin (1 U/mL) and the hexapeptide PAR1 agonist $HsPAR1^{50/55}$ (SFLLRN; 100 μmol/L) were capable of increasing ERK1/2 activity within five minutes. Increases in ERK1/2 activity uniformly paralleled increases in ERK1/2 phosphorylation and were similarly blocked by antecedent PKC depletion. Representative experiments, repeated at least four times with identical results, are depicted.

activity represents a previously unreported cellular effect of this important serine protease on mesangial cells, as well as a novel mechanism of HK regulation. Thrombin increased mesangial cell HK activity in both a dose- and time-dependent manner, and these changes were accompanied by corresponding increases in net Glc utilization and net lactate accumulation. The ability of recombinant leech hirudins—specific inhibitors of thrombin's proteolytic activity—to prevent this increase in HK activity suggests specificity for thrombin and is compatible with the observation that thrombin's cellular effects are largely mediated by proteolytic activation of cell surface thrombin receptors [22]. The ability of oligopeptide PAR1 agonists (for example, $HsPAR1^{42/55}$ and

Table 1. Dependence of increased hexokinase (HK) activity following PAR1 activation on both ongoing gene transcription and de novo protein synthesis

	No inhibitors	+CHX 10 μg/mL	+DRB 20 μmol/L
Unstimulated cells	100%	97 ± 2%	84 ± 4%[a]
HsPAR1[42/55]-simulated cells	160 ± 14%[a]	81 ± 5%[b]	105 ± 8%[b]

Total HK activity was measured at 24 hours in cells treated with the PAR1 agonist HsPAR1[42/55] in the presence or absence of the general transcriptional inhibitor dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) or the general inhibitor of translation, cycloheximide (CHX). Paired data from four independent experiments are presented as mean activities relative to controls ± SEM. The inhibitory effects of both CHX and DRB were dose dependent and maximal at the concentrations depicted above (data not shown).
[a] $P < 0.03$ vs. unstimulated controls
[b] $P < 0.04$ vs. HsPAR1[42/55]-stimulated cells

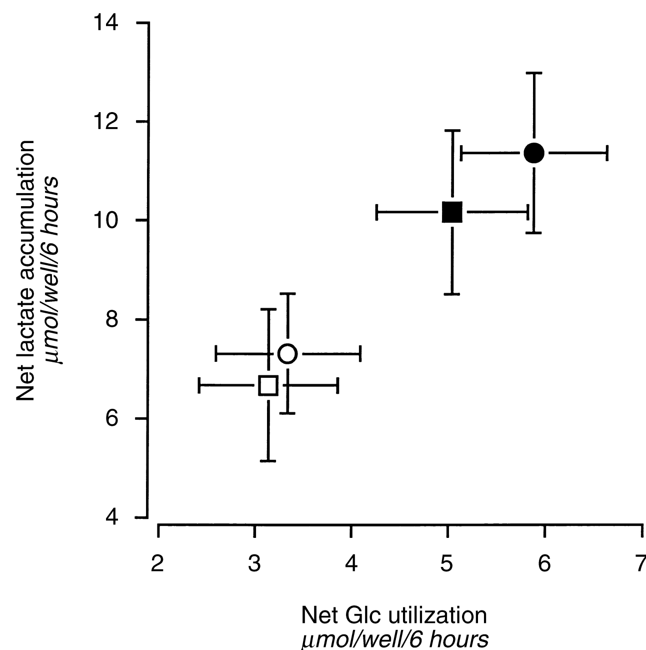

Fig. 8. Thrombin stimulates mesangial cell glucose (Glc) metabolism. Thrombin (●; 1 U/mL) increased both net Glc utilization and net lactate accumulation by cultured mesangial cells (○; control). These changes were observed between 18 and 24 hours following thrombin treatment and temporally corresponded to maximal thrombin-induced changes in total HK activity. Active (■; PDD 100 nmol/L), but not inactive (□; 4α-PDD 100 nmol/L), phorbol esters were found to mimic these effects of thrombin, consistent with a role for PKC activation in these changes. All data are presented as the mean ± SEM for at least four independent experiments.

HsPAR1[50/55]) [43, 45] to mimic this effect suggests specificity for the PAR1 thrombin receptor.

Three distinct PARs—PAR1, PAR3, and PAR4—that are specifically activated by thrombin have recently been cloned and characterized [20, 47, 48]. Of these receptors, renal expression has been demonstrated only for PAR1. The mechanism of thrombin receptor activation has been reviewed elsewhere [49]. In brief, targeted cleavage of each receptor's extracellular NH_2-terminus by thrombin generates a new NH_2-terminal "tethered ligand" that is capable of specific intramolecular interaction with, and activation of the receptor. Consistent with this model, synthetic oligopeptides corresponding to the putative NH_2-terminal tethered ligands of PAR1 [20, 43, 50] and PAR4 [48] have been shown to mimic thrombin's effects in cells expressing these receptors. Thus, the ability of PAR1, but not PAR4, peptide agonists to mimic the effect of thrombin on HK activity suggests that PAR1 activation is sufficient for thrombin's effect on HK activity and that the PAR4 thrombin receptor does not contribute substantially to this effect.

Unlike most G-protein–coupled receptors, which typically activate a single type of G protein, PAR1 has been shown to couple with both PTX-sensitive G_i and PTX-insensitive G_q effectors [28]. Consistent with these observations, both PTX-sensitive and PTX-insensitive cellular effects of thrombin have been demonstrated in mesangial cells and other cell types [22, 29]. The inability of coadministered 0.1 μg/mL PTX to block the effects of thrombin or PAR1 agonist peptides in our study suggests that PTX-insensitive effectors such as G_q, G_{12}, or G_s are responsible for mediating the effects of PAR1 activation on HK activity.

We have previously shown that phorbol esters initiate an immediate chain of signaling events in murine mesangial cells that culminates in increased Glc-phosphorylating capacity within 12 to 24 hours [15]. This effect requires both PKC and classic MAPK pathway activation, as well as ongoing gene transcription and de novo protein synthesis. The corresponding endogenous activators of the involved signaling pathway(s) in vivo, however, are unknown. As a known activator of both PKC [16] and the classic MAPK pathway [17], thrombin represents an important candidate effector of these changes. In the present studies, MEK1/2 inhibition by PD98059 abolished both thrombin-induced and PAR1 peptide agonist-induced HK activity, suggesting the involvement of the classic MAP kinase pathway. The ability of both thrombin and PAR1 peptide agonists to increase ERK1/2 phosphorylation and activity is consistent with this interpretation. The ability of antecedent PKC depletion to inhibit ERK1/2 phosphorylation and activity also suggests a role for PKC activation in this process. The demonstration that general inhibitors of gene transcription and translation (that is, DRB and CHX, respectively) are similarly

capable of preventing increased HK activity following PAR1 activation further suggests a related dependence on ongoing gene transcription and de novo protein synthesis.

Taken together, these findings are consistent with the hypothesis that phorbol esters and thrombin induce mesangial HK activity via a common mechanism involving the sequential activation of PKC and the classic MAPK pathway (Raf→MEK→ERK). Given the apparent common requirement for ongoing gene transcription, it would also be attractive to speculate that activation of PKC and ERK1/2 by phorbol esters or thrombin ultimately results in increased HK gene expression. Three high-affinity HK isoforms (types I, II, and III) are expressed in the kidney [51], but their intrarenal expression patterns and cellular regulation are poorly understood. Preliminary studies in our laboratory have suggested that mesangial cells express all three isoforms and HKII appears to constitute the principal regulated isoform in this cell type (abstract; Robey et al, *J Am Soc Nephrol* 10:57A, 1999). Both HKII and HKIII are low-abundance renal isoforms, together accounting for only one third of the total Glc-phosphorylating capacity of the normal kidney [51]. Their relative abundance in individual renal cell types (for example, mesangial cells), however, has not yet been reported. It is therefore of additional interest that selective increases in these lower abundance isoforms may also largely account for the increases in cortical HK activity observed in experimental diabetes (abstract; Robey et al, *J Am Soc Nephrol* 10:689A, 1999).

Interestingly, thrombin has been shown to mimic some of the cellular effects of vasoactive peptides such as ET-1 and Ang II in cultured mesangial cells [33]. SV40 MES 13 cells remain responsive to Ang II [23], and both thrombin and these vasoactive peptides are known activators of PKC in this cell type [33]. Thus, the inability of either 1 μmol/L ET-1 or superphysiologic doses of Ang II to mimic the effect of thrombin on HK activity suggests that this effect is specific for thrombin receptor activation and argues against a general mitogenic response.

In conclusion, we have demonstrated that thrombin is capable of specifically regulating HK activity in cultured mesangial cells via a PTX-insensitive PAR1-dependent mechanism that requires both ongoing gene transcription and de novo protein synthesis. This represents a novel and previously unreported cellular response to thrombin receptor activation. The specific mechanisms and the physiologic (or pathophysiologic) significance of our observations in vivo are not presently known, but they do have implications for mesangial cell Glc metabolism in the setting of renal injury. It is therefore of great interest that reports of altered HK activity in the adult kidney have been largely limited to pathological conditions associated with renal functional and/or structural abnormalities. As one of the principal cell types affected in glomerular injury of various etiologies, it would be attractive to speculate that thrombin plays an important role not only in mesangial cell Glc metabolism, but also in the functional and structural changes that accompany injury. The changes demonstrated herein are comparable in magnitude to those reported previously in the cortex of animals with experimental diabetes and are compatible with such a hypothesized role. Physiologic control of thrombin's actions is largely mediated at the level of active thrombin generation [52], which is markedly increased in the settings of vascular injury [52] and diabetes [53]. Thus, thrombin's actions are particularly relevant to the understanding of metabolic responses to renal or vascular injury. Our findings also suggest a specific mechanism whereby cellular energy metabolism may be coupled to thrombin receptor activation and cellular injury responses. We therefore speculate that HKs may play a role in either the mediation of or the response to renal injury and that thrombin may contribute to this role.

ACKNOWLEDGMENTS

This work was supported, in part, by grants-in-aid from the National Kidney Foundation of Illinois (R.B.R.) and the American Heart Association of Metropolitan Chicago (R.B.R.), as well as by a VA Merit Review Award (R.B.R.). Portions of this work were presented in preliminary form on October 26, 1998, at the 31st Annual Meeting of the American Society of Nephrology in Philadelphia, PA, USA. The authors thank Drs. Jose A.L. Arruda and Ashok K. Singh for helpful discussions and for critical reading of the manuscript. We also gratefully acknowledge the technical assistance of Ms. Katie Tinich.

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