Regulation of mesangial cell hexokinase activity by PKC and the classic MAPK pathway

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Regulation of mesangial cell hexokinase activity by PKC and the classic MAPK pathway. Am. J. Physiol. 277 (Renal Physiol. 46): F742–F749, 1999.—Phorbol esters increase glucose (Glc) uptake and utilization in a variety of cell types, and, in some cells, these changes have been attributed to increased Glc phosphorylation and better functional coupling of hexokinases (HKs) to facilitative Glc transporters. Phorbol esters are potent mesangial cell mitogens, but their effects on HK-catalyzed Glc phosphorylation and metabolism are unknown. When examined in murine mesangial cells, active, but not inactive, phorbol esters increased HK activity in a time- and dose-dependent manner. Maximal induction of HK activity at 12–24 h was accompanied by parallel increases in both Glc utilization and lactate production and was blocked by the specific MEK1/2 inhibitor PD-98059 (IC50 = 3 µM). This effect involved early activation of protein kinase C (PKC), MEK1/2, and ERK1/2, and the prolonged time course of subsequent HK induction was attributable, in part, to requirements for ongoing gene transcription and de novo protein synthesis. Mesangial cell HK activity thus exhibits novel regulatory behavior involving both PKC and classic MAPK pathway activation, suggesting specific mechanisms whereby PKC activation may influence Glc metabolism.

glomerular mesangial cells; hexokinase; protein kinase C; MEK1/2; ERK1/2; mitogen-activated protein kinase

Glucose (Glc) uptake and metabolism are of fundamental importance to all mammalian cells, and hexokinases (HKs) play a central role in these processes by catalyzing the phosphorylation of Glc to yield Glc 6-phosphate (Glc-6-P). By this mechanism, HKs maintain the favorable downhill concentration gradient that permits facilitated Glc entry into cells. In addition, they initiate all subsequent pathways of Glc utilization, including the glycolytic pathway, the pentose phosphate pathway, and the uronic acid pathway. Thus primary changes in total HK activity can, in principle, have profound effects on Glc uptake and utilization, as well as on the ultimate metabolic fate(s) of Glc.

Total HK activity in the kidney increases throughout development (6, 21, 29) and declines slightly after weaning to adult levels (21, 33). In the adult kidney, total HK activity increases along the axial nephron, with the highest specific activities observed distally, particularly in the thick ascending limb of Henle’s loop (23). Thus the specific activities observed in the adult renal medulla typically exceed those observed in the cortex (3, 6, 32, 33). HK activity in the adult kidney is specifically increased in genetically obese mice (27), in experimental diabetes (2, 24–28), in compensatory unilateral renal hypertrophy (28), and in puromycin aminonucleoside-induced nephrosis (10). Increases in cortical activity have been described in experimental diabetes, and increased glomerular activity has been demonstrated both during development and in the setting of experimental nephrosis. However, the contributions of individual cell types and the molecular mechanisms underlying these changes have been largely unexplored. Thus a description of the regulatory characteristics of HK activity in an individual renal cell type such as the glomerular mesangial cell should be of great interest as well as great physiological, developmental, and pathophysiological relevance.

Phorbol esters act as diacylglycerol mimetics to activate protein kinase C (PKC) and have been shown to increase Glc uptake by a variety of cell types (7, 9, 13). In the case of thymocytes, they appear to do so primarily via increased Glc phosphorylation and better functional coupling of HKs to transport (17). The mitogenic capacity of phorbol esters has also been linked to their ability to stimulate Glc uptake (7). Although these compounds are known mitogens for mesangial cells (11), their effects on Glc metabolism, and on Glc phosphorylation in particular, in this cell type have been largely unexplored. In a preliminary report published over a decade ago, Kreisberg et al. (12) reported both contraction and increased glycolysis by cultured mesangial cells treated with phorbol 12-myristate 13-acetate (PMA). These authors speculated that increased Glc metabolism could provide the requisite energy necessary for phorbol ester-stimulated contraction by these cells. Although the effect of phorbol esters on mesangial cell contraction has subsequently been characterized in greater detail (31), the molecular mechanisms underlying the associated increase in Glc metabolism have not yet been reported. Glc uptake and metabolism by these cells have been extensively investigated, but little is known about the relative contributions of HKs to these processes, and the regulation of Glc phosphorylating capacity in this cell type has not been addressed. Thus, to better understand this important family of enzymes in the kidney, we sought to characterize the general regulatory behavior of HK activity in SV40 MES 13 (murine mesangial) cells, which exhibit morphological and biochemical characteristics of normal mesangial cells in culture (16). More specifically, we sought to characterize the effects of phorbol esters on HK activity and to directly test the hypothesis that phorbol esters

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could affect mesangial cell Glc metabolism via an effect on total Glc phosphorylating capacity.

MATERIALS AND METHODS

Materials. Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was obtained from Fluka (Milwaukee, WI), PD-98059 was from Calbiochem (La Jolla, CA), and Leuconostoc mesenteroides Glc-6-P dehydrogenase (G6PDH) was from Boehringer-Mannheim (Indianapolis, IN). Electrophoretic and immunoblotting reagents were routinely obtained from Bio-Rad (Hercules, CA), and all cell culture reagents were supplied by Life Technologies (Gaithersburg, MD). All antibodies, substrates, and the chemiluminescent detection system employed for both immunoblot analysis and immunoprecipitation/kinase (IP/kinase) activity assays were obtained from New England Biolabs (Beverly, MA). All other chemicals, including PMA, phorbol-12,13-diacetate, and 1% (vol/vol) Triton X-100, cell extracts were routinely prepared by a modification of the methods of O'Doherty et al. (19) and Braithwaite et al. (5). In brief, cells were routinely preincubated with inhibitor alone for 30 min prior to the addition of phorbol esters. Cell lysate preparation. Cell lysates for HK activity assays were routinely prepared by a modification of the methods of O'Doherty et al. (19) and Braithwaite et al. (5). In brief, cells were routinely preincubated with inhibitor alone for 30 min prior to the addition of phorbol esters. Cell lysate preparation. Cell lysates for HK activity assays were routinely prepared by a modification of the methods of O'Doherty et al. (19) and Braithwaite et al. (5). In brief, cells were routinely preincubated with inhibitor alone for 30 min prior to the addition of phorbol esters. Cell lysate preparation. Cell lysates for HK activity assays were routinely prepared by a modification of the methods of O'Doherty et al. (19) and Braithwaite et al. (5). In brief, cells were routinely preincubated with inhibitor alone for 30 min prior to the addition of phorbol esters. Cell lysate preparation. Cell lysates for HK activity assays were routinely prepared by a modification of the methods of O'Doherty et al. (19) and Braithwaite et al. (5). In brief, cells were routinely preincubated with inhibitor alone for 30 min prior to the addition of phorbol esters. Cell lysate preparation. Cell lysates for HK activity assays were routinely prepared by a modification of the methods of O'Doherty et al. (19) and Braithwaite et al. (5). In brief, cells were routinely preincubated with inhibitor alone for 30 min prior to the addition of phorbol esters.
vated ERK1/2 were used to assess phospho-ERK1/2 content. Following electrophoretic separation by SDS-PAGE and transfer to nitrocellulose, cell lysates were probed with primary antibodies at a 1:1,000 dilution. Specific protein bands were then visualized by chemiluminescent detection of horseradish peroxidase-conjugated anti-rabbit secondary antibodies using the Phototope-HRP Western Detection System (New England BioLabs) per the manufacturer’s recommendations. Specific ERK1/2 phosphotransferase activity was also assayed by an IP/kinase activity assay using a commercially available kit (New England Biolabs) according to the manufacturer’s recommendations. In brief, activated ERK1/2 immunoprecipitates were prepared from cell lysates using immobilized phospho-specific ERK1/2 monovalent antibodies directed against dual-phosphorylated (i.e., activated) ERK1/2. These immunoprecipitates were then assayed for the ability to specifically Ser-phosphorylate an Elk-1 fusion protein in vitro. Quantitative immunoblotting was performed using rabbit polyclonal IgGs specific for phospho-Elk-1 and chemiluminescent detection as described above. Control IP/kinase assays were routinely performed in parallel using unstimulated cell lysates with and without the addition of functional MEK-activated recombinant ERK2. Quantitative comparisons were made by scanning transmission densitometry of the resulting autoradiograms using an Eagle-Eye II still videomaging system (Stratagene, La Jolla, CA). Analysis of digital images was routinely performed using NIH Image v1.61 software for Macintosh computers (NIH, Bethesda, MD).

MEK1/2 activity assays. Mitogen/extracellular signal-regulated kinase kinase 1 and 2 (MEK1/2) phosphotransferase activity was assayed by an IP/kinase activity assay similar to that employed to measure ERK1/2 kinase activity. In brief, rabbit polyclonal IgGs specific for phospho-MEK1/2 (New England Biolabs) and protein A-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were used to selectively immunoprecipitate activated MEK1/2 from cell lysates. The resulting immunoprecipitates were then assayed for the ability to specifically phosphorylate inactive recombinant murine ERK2 (New England Biolabs) in vitro. Phospho-ERK2 detection and quantitation was accomplished by immunoblotting with mouse monoclonal IgGs specific for dual-phosphorylated ERK1/2 followed by chemiluminescent detection and analysis as described above.

Statistical analysis. Unless otherwise noted, data are presented as experimental means ± SE for at least three independent measures. Statistical comparisons were performed by paired t-testing where appropriate using a significance level of 95% and StatView 5.0 software for Macintosh computers (SAS Institute, Cary, NC).

RESULTS

SV40 MES 13 cells exhibited morphological and biochemical characteristics of normal mesangial cells in culture. SV40 MES 13 cells, originally derived from glomerular explants of mice transgenic for the SV40 early region, have been shown to exhibit morphological and biochemical characteristics of normal mesangial cells in culture (16). As previously reported, we found that these cells stained for SV40 large T antigen, exhibited the typical stellate morphology of mesangial cells in culture, and, unlike fibroblasts which lack D-amino acid oxidase, were capable of normal growth following equimolar substitution of D-valine for its natural L-stereoisomer in normal growth medium. In addition, they uniformly expressed mesangial cell markers such as the intermediate filaments vimentin and desmin, but failed to express detectable levels of epithelial cell markers such as cytokeratins by immunoblot analysis (data not shown). In preliminary experiments, we observed similar levels of basal HK activity in both SV40 MES 13 cells and cultured rat mesangial cells maintained in normal growth medium (27 ± 3 vs. 26 ± 1 U/g protein; not significant). Serum deprivation of SV40 MES 13 cells for 48 h decreased basal HK activity slightly but significantly by ~20% (28 ± 3 vs. 22 ± 3 U/g protein; P < 0.001), and similar results were observed for cultured rat mesangial cells. Since, in preliminary studies, phorbol esters increased total HK activity similarly in both cell types (22), we sought to further characterize this response in SV40 MES 13 cells.

Active, but not inactive, phorbol esters induced total HK activity in a time- and dose-dependent manner. As shown in Fig. 1A, exposure to 1 µM PMA increased total HK activity in SV40 MES 13 cells by over 20% within 5 h (P = 0.07), and a significant increase of almost 40% was observed within 12 h (P < 0.002).
Maximal activity was observed within 24 h and was over 50% greater than in unstimulated controls (P < 0.02). Thereafter, total HK activity began to decrease but was still over 40% higher than controls at 36 h. In Fig. 1B, the dose dependence of PMA-induced HK activity at 24 h in these cells can also be appreciated. PMA significantly increased total HK activity at concentrations as low as 10 nM (P < 0.05), and maximal activation was observed within 24 h of exposure to PMA concentrations ≥100 nM. To ensure that induction of HK activity by phorbol esters was specific for PKC activation, we also tested both PDD and its inactive 4α-analog, 4α-PDD, for the ability to induce total HK activity. As shown in Fig. 2, the active phorbol ester PDD increased HK activity at 24 h in a dose-dependent fashion and mimicked the effects of PMA. At concentrations ≥10 nM, PDD significantly increased HK activity by ~60% (P < 0.05 vs. unstimulated control cells). In contrast, 4α-PDD, which is incapable of activating PKC (1), or vehicle alone (DMSO) failed to alter total HK activity when tested in parallel. Consistent with these observations, the ability of particulate cell extracts to specifically phosphorylate a PKC pseudosubstrate increased over 10-fold within 1 min of 1 µM PMA stimulation, an effect that persisted for at least 30 min. In contrast, the phosphotransferase activities of corresponding soluble (cytosolic) extracts decreased as much as ~86% within this time period, suggesting activation and membrane translocation of PKC. At 24 h, both particulate and cytosolic activities were decreased by ~72% and ~97%, respectively, consistent with down-regulation of PKC activity in response to prolonged phorbol ester exposure.

Sustained exposure to phorbol esters was not required for maximal induction of HK activity at 24 h. Although the findings above suggested short-term activation and long-term depletion of PKC activity, long-acting phorbol esters (e.g., PMA) are capable of eliciting cellular responses attributable to sustained PKC activation (18). We therefore also tested the ability of brief PMA exposure (≤1 h) to mimic the effect of continuous 24-h exposure. To this end, we exposed cells to 1 µM PMA for varying time periods before thorough washing and replacement with phorbol ester-free medium. Both 0.5-h and 1.0-h exposures resulted in over 60% increases in total HK activity at 24 h (P < 0.02 vs. unstimulated controls) that were indistinguishable from that observed in cells continuously exposed to PMA over the same time period.

Increased total HK activity was accompanied by parallel increases in both Glc utilization and lactate production. As demonstrated in Fig. 3, net Glc utilization and net lactate production were both increased within 18–24 h of phorbol ester stimulation, and these changes temporally corresponded to maximal induction of HK activity. Regression analysis revealed a linear relationship between net Glc utilization and net lactate accumulation that was the same for both stimulated and unstimulated cells. Moreover, the slope of the line generated using all data (m = 1.7 lactate/Glc; r² = 0.86) was not different from that generated from data obtained from unstimulated or PMA-stimulated cells alone or from that defined by the individual means (see inset).
HK induction by phorbol esters was PD-98059-inhibitable and was associated with the early activation of MEK1/2. Since the downstream effects of PKC activation in mesangial cells may be mediated, in part, by classic mitogen-activated protein kinase (MAPK) pathway activation (15), we tested the ability of the specific MEK1/2 inhibitor PD-98059 to block induction of HK activity by phorbol esters. As shown in Fig. 4, this compound inhibited PMA-stimulated HK activity in a dose-dependent manner with an apparent IC<sub>50</sub> of ~3 µM and at concentrations considered specific for the inhibition of MEK1/2 activity. Basal HK activity was not similarly affected, and vehicle alone (DMSO) failed to mimic this effect, suggesting that the effect was specific for PD-98059 and was not attributable to direct inhibition of HK activity by this compound. Consistent with this interpretation, 1 µM PMA markedly increased MEK1/2 activity within 1 min of PMA exposure, and activity rapidly declined thereafter toward normal levels (see Fig. 5A). As expected, PD-98059 also inhibited the activation of MEK1/2 by PMA (data not shown).

Phorbol esters increased the phosphorylation and activity of ERK1/2 in a time and dose-dependent manner, and these effects were also blocked by PD-98059. To further evaluate the involvement of the classic MAPK pathway, we tested the ability of PMA to induce the specific phosphorylation and activation of ERK1/2. As depicted in Fig. 5B, specific dual-phosphorylated ERK1/2 content increased markedly within 1 min of PMA exposure, was maximal within 5 min, and declined thereafter, albeit never to basal levels over the 60-min period monitored. As shown in Fig. 6A, ERK1/2 activity increased approximately 10-fold within 1 min of PMA stimulation, and the time course of activation paralleled increases in dual-phosphorylated ERK1/2 content (see Fig. 5B). The effect of PMA was dose dependent, and only those concentrations capable of inducing total HK activity at 24 h were found to be capable of ERK1/2 activation at 5 min (see Fig. 6B). As shown in Fig. 6C, pretreatment of cells with 1 µM PMA for 24 h to deplete active PKC completely blocked the subsequent ability of phorbol esters to induce ERK1/2 activity. In Fig. 6D, we similarly confirmed the ability of 50 µM PD-98059 to completely block the downstream activation of ERK1/2 by 1 µM PMA at 5 min.

PMA induction of HK activity required both ongoing gene transcription and de novo protein synthesis. The prolonged time course of HK induction following PMA treatment also prompted us to examine the effects of general inhibitors of gene transcription and protein translation on this effect. As depicted in Fig. 7, the general transcriptional inhibitor DRB also inhibited PMA-stimulated HK activity at 24 h in a dose-dependent manner with an apparent IC<sub>50</sub> of ~5-10 µM. At this concentration, DRB significantly inhibited PMA-stimulated activity (P = 0.0005) without a corresponding effect on basal activity. At 20 µM, however, DRB inhibited PMA-stimulated activity by 81 ± 5% (P < 0.0005) while decreasing basal activity by 19 ± 5% (P < 0.0005). These effects were mimicked by both 1 µg/ml actinomycin D and 10 µg/ml cycloheximide, which completely blocked the induction of HK activity by PMA at 24 h (=100%; P = 0.05 and P < 0.02, respectively) and resulted in corresponding decreases in basal activity of 16 ± 14% (not significant) and 22 ± 6% (P < 0.02), respectively.
phorbol esters to increase mesangial cell HK activity is relevant not only to the understanding of mesangial cell Glc metabolism but also to HK regulation in general. To better understand the role and regulation of HK activity in mesangial cells, we have attempted to characterize this regulatory response and some of its underlying molecular mechanisms.

We have shown that phorbol esters increase total HK activity in both a time- and dose-dependent manner. Maximal induction of HK activity occurred within 12–24 h of phorbol ester stimulation, and activity remained persistently elevated for at least an additional 12–24 h. The prolonged time course of HK induction, coupled with the observation that 30-min PMA exposure was sufficient for maximal HK induction at 24 h, suggests 1) that PKC activation initiates an early chain of signaling events culminating 12–24 h later in increased HK activity and 2) that sustained PKC activation is not required for this effect. Our findings of early activation and subsequent depletion of PKC with continued exposure to PMA are consistent with these interpretations. Since PKC is the only known intracellular target for diacylglycerol-mimetic phorbol esters, the demonstration that active, but not inactive, phorbol esters were capable of increasing HK activity supports a causal relationship between PKC activation and subsequent HK induction.

We have also shown that increases in HK activity were accompanied by corresponding increases in both net Glc utilization and net lactate accumulation. Regression analysis of the data suggested that both stimulated and unstimulated cells generated at least five molecules of lactate for every three molecules of Glc consumed (~1.7 lactate/Glc) and did not suggest metabolic uncoupling by phorbol esters. Thus glycolysis

DISCUSSION

The specific HK activities reported herein for cultured mesangial cells were comparable in magnitude to those previously reported for both isolated rat glomeruli (6, 10, 23) and whole rat renal cortex (3, 32, 33), typically in the 10–30 U/g protein range. Although much lower than the specific activities observed in the distal nephron and the whole medulla (23), they are comparable in magnitude to those reported for normal murine muscle and adipose (5), tissues responsible for mediating the bulk of peripheral Glc utilization. Thus mesangial cells clearly exhibit a substantial capacity for Glc phosphorylation.

The present demonstration of regulated HK activity in cultured mesangial cells is, to our knowledge, the first such description in this cell type. In addition, the specific regulation of total Glc phosphorylating capacity by phorbol esters has not heretofore been described for any cell type that we are aware of. Thus the ability of
probably accounted for at least 85% of the Glc utilization by these cells, with the difference reflecting, in large part, the contributions of nonglycolytic Glc utilization (e.g., via the pentose phosphate or uronic acid pathways) and lactate reutilization. These findings are consistent with those reported previously by Kreisberg et al. (12) and suggest that changes in the total Glc phosphorylating capacity of cultured mesangial cells are associated with parallel changes in Glc metabolism.

In an effort to identify downstream effectors of PKC activation that contribute to the induction of mesangial cell HK activity, we also investigated the classic MAPK pathway (Raf → MEK → ERK). This pathway has been shown to mediate a number of downstream effects of PKC activation in a variety of cell types. Although PKC activation by phorbol esters has already been shown to result in ERK1/2 activation in glomerular mesangial cells (15), the physiological consequences of such activation have not been described. The ability of the specific MEK1/2 inhibitor PD-98059 to completely block PMA induction of HK activity at 24 h suggests the involvement of classic MAPK pathway signaling downstream of PKC. The demonstration of rapid PMA-stimulated ERK1/2 phosphorylation and activation is consistent with such a hypothesis, and the corresponding time course of MEK1/2 activation is temporally consistent with a role for these dual-specificity kinases in activating ERK1/2 and mediating this effect. Taken together, these findings suggest that the signaling events initiated by phorbol esters, and ultimately contributing to increased HK activity, in mesangial cells include the immediate activation of PKC, MEK1/2, and ERK1/2.

The ability of general inhibitors of both gene transcription and protein translation to block the effect of phorbol esters on HK activity also suggests that maximal induction at 24 h requires both ongoing gene transcription and de novo protein synthesis. The much smaller, albeit statistically significant, effects on basal activity suggest a more limited dependence upon continuing gene expression for maintenance of basal HK activity and may be suggestive of limited enzyme turnover. The persistence of elevated HK activity following induction provides indirect support for such a hypothesis. In addition, requirements for both ongoing gene transcription and de novo protein synthesis may explain, at least in part, the prolonged time course of HK induction following PMA stimulation.

We have previously reported in preliminary form that 24- to 48-h exposure to elevated ambient Glc at concentrations capable of PKC activation in mesangial cells failed to mimic the effect of phorbol esters on HK activity (22). It is therefore of additional interest that other known activators of PKC exhibit a variable capacity to induce HK activity in this cell type. For example, other preliminary work in our laboratory indicates that the effect of PMA can be mimicked by thrombin (20), but not by angiotensin II or endothelin-1 (R. B. Robey, unpublished observations). The reasons underlying these discrepant observations are not presently clear, but they may reflect, in part, the diversity and complexity of PKC signaling in this cell type. The inability of some known activators of PKC to increase mesangial cell HK activity also suggests that this is a specific, rather than a general, response to PKC activation.

In summary, phorbol esters are clearly capable of initiating an early chain of signaling events in mesangial cells that ultimately leads to increased HK activity. This effect involves the immediate activation of PKC and the classic MAPK pathway, and the prolonged lag time between these early signaling events and subsequent increases in Glc phosphorylating capacity may reflect specific requirements for ongoing gene expression. Although the specific gene expression requirements for induction have not yet been identified and the possibility of altered regulatory gene product expression cannot be excluded, it would be attractive to speculate that phorbol esters ultimately exert their effect on HK activity by increasing HK gene expression. We conclude that mesangial cell HK activity exhibits novel regulatory behavior that requires both PKC and classic MAPK pathway activation, as well as ongoing gene expression. In addition to suggesting specific mechanisms whereby PKC activation may influence Glc metabolism, these findings may have physiological or pathophysiological implications for certain conditions associated with altered mesangial cell PKC activation.

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