LPA is a novel lipid regulator of mesangial cell hexokinase activity and HKII isoform expression

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Coy, Platina E., Navin Taneja, Iris Lee, Claudie Hecquet, Jane M. Bryson, and R. Brooks Robey. Lysophosphatidic acid: a novel lipid regulator of mesangial cell hexokinase activity and HKII isoform expression. Am J Physiol Renal Physiol 283:F271–F279, 2002. First published January 29, 2002; 10.1152/ajprenal.00093.2001.—The prototypical extracellular phospholipid mediator, lysophosphatidic acid (LPA), exhibits growth factor-like properties and represents an important survival factor in serum. This potent mesangial cell mitogen is increased in conditions associated with glomerular injury. It is also a known activator of the classic mitogen-activated protein kinase (MAPK) pathway, which plays an important role in the regulation of mesangial cell hexokinase (HK) activity. To better understand the mechanisms coupling metabolism to injury, we examined the ability of LPA to regulate HK activity and expression in cultured murine mesangial cells. LPA increased total HK activity in a concentration- and time-dependent manner, with maximal increases of >50% observed within 12 h of exposure to LPA concentrations ≥25 μM (apparent ED50 2 μM). These effects were associated with increased extracellular signal-regulated kinase (ERK) activity and were prevented by the pharmacological inhibition of either MAPK/ERK kinase or protein kinase C (PKC). Increased HK activity was also associated with increased glucose (Glc) utilization and lactate accumulation, as well as selectively increased HKII isoform abundance. The ability of exogenous LPA to increase HK activity was both Ca2+ independent and pertussis toxin insensitive and was mimicked by LPA-generating phospholipase A2. We conclude that LPA constitutes a novel regulator of mesangial cell HK activity and HKII isoform abundance. This regulation requires sequential activation of both Ca2+ independent and pertussis toxin insensitive pathways and is mimicked by LPA-generating phospholipase A2. We conclude that LPA constitutes a novel regulator of mesangial cell HK activity and HKII isoform expression.

Lysophosphatidic acid; mitogen-activated protein kinase; glucose; phospholipase A2; renal injury

HEXOKINASES (HKs) play a central role in glucose (Glc) uptake and utilization and are of fundamental importance to all cells. By catalyzing the phosphorylation of Glc to yield glucose-6-phosphate (Glc-6-P), HKs initiate all major pathways of Glc utilization and thereby maintain the downhill concentration gradient that permits facilitated Glc entry into cells. In the mammalian kidney, this important Glc-ATP phosphotransferase activity can be accounted for by the expression of three high-affinity HK isoforms, HKI, HKII, and HKIII (13). HKI represents the principal renal isoform and accounts for roughly two-thirds of the total HK activity of the kidney. The remainder is equally attributable to the two lower abundance renal isoforms, HKII and HKIII. Very little is presently known, however, about the normal expression, regulation, or function of individual isoforms in specific renal cell types. Increased cortical HK activity has been reported in conditions associated with alterations in kidney structure or function (2, 7, 34, 36, 37), but the specific cell types and HK isoforms responsible for these changes have not been defined. Given the central physiological and pathological importance of glomerular mesangial cells, we are particularly interested in the expression and regulation of HKs in this cell type.

We have previously reported the regulation of mesangial cell HK activity by phorbol esters (31), thrombin (33), and growth factors (32), and we have described similar regulation, in preliminary form, by proinflammatory cytokines (38). Interestingly, the common capacity of these diverse stimuli to increase HK activity appears to involve a shared requirement for classic mitogen-activated protein kinase (MAPK) pathway activation. Another common feature of these stimuli is their association with renal injury, injury-related conditions, or injury responses. These observations, coupled with the in vivo association between increased HK activity and pathological renal conditions, suggest a teleological relationship and have led us to hypothesize that HKs play an important adaptive role in mesangial cells and the kidney. The existence of common regulatory features may also suggest a general mechanism for coupling Glc metabolism to cellular injury. As an

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indirect test of this hypothesis, we have investigated other classic MAPK pathway-activating, injury-associated stimuli for the ability to regulate HK activity and expression in mesangial cells.

Lysophosphatidic acid (LPA; 1- or 2-acyl-sn-glycerol-3-phosphate) is a ubiquitous glycerophospholipid with a diverse array of biological actions. In addition to its well-defined role as a natural precursor for the formation of more complex phospholipids, LPA has recently been recognized as an important extracellular signaling molecule with a number of growth factor-like properties (27). LPA is a normal constituent of serum, where it is present at 1–5 μM tightly bound to albumin (8). Circulating LPA represents both a major vasoactive lipid (40) and an important serum survival factor (20). Platelet-derived LPA probably accounts for the bulk of circulating LPA (8) and has been best characterized (27). However, extracellular LPA is also generated by a variety of noncirculating cell types, including glomerular mesangial cells, through the actions of extracellular phospholipases (26), such as inflammatory group II phospholipase A₂ and phospholipase D-like phosphodiesterases (27, 40, 41). The cellular actions of LPA are mediated by specific G protein-coupled receptors (GPCRs) that couple to multiple G protein effectors. Activation of these GPCRs is associated with a wide range of biological responses (25–27) that include mesangial cell proliferation (17) and contraction (16). In addition to being a potent mesangial cell mitogen, LPA specifically activates the classic MAPK pathway in this cell type (10, 17) and has been implicated in the pathogenesis of renal injury (15). However, the precise physiological and pathophysiological roles of LPA are poorly defined (27), and the metabolic consequences of LPA stimulation have been largely unexamined. We therefore investigated the ability of LPA to alter both HK activity and individual HK isoform abundance in cultured murine mesangial cells.

MATERIALS AND METHODS

Reagents. LPA was obtained from Cayman Chemical (Ann Arbor, MI). Fatty acid-free BSA and grade I yeast Glc-6-P dehydrogenase (G6PDH) were both obtained from Roche Molecular Biochemicals (Indianapolis, IN). The Ca²⁺ chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) was obtained as the cell-permeable tetraacetoxymethyl ester from Calbiochem (La Jolla, CA), as were the (BAPTA) was obtained as the cell-permeable tetraacetoxy-amin-3-avone (PD-98059), the Ca²⁺-methoxy ionophore calcimycin (A-23187) and ionomycin, Borrelia burgdorferi pertussis toxin (PTX), the MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK)-selective inhibitor 2-(2-amino-3’-methoxyflavone (PD-98059), the Ca²⁺-dependent, or classic, PKC (cPKC) isoform-selective inhibitor Go-6976, and the alternative MEK-selective inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U-0126) was procured from Promega (Madison, WI), and the fluorescent Ca²⁺ indicator fura 2 was obtained as its caged acetoxymethyl ester (fura 2-AM) from Molecular Probes (Eugene, OR). All cell culture reagents, including media and additives, were obtained from Gibco BRL (Grand Island, NY). Electrophoretic and immunoblotting reagents, including nitrocellulose membranes, were obtained from Bio-Rad (Hercules, CA). All other reagents, including ATP, β-NADP, and porcine pancreatic phospholipase A₂, were obtained from Sigma (St. Louis, MO) unless otherwise noted.

Cell culture. Mycoplasma-free SV40 MES 13 murine mesangial cells (22) were obtained from the American Type Culture Collection (Rockville, MD) at passage 27 and were maintained in DMEM-F12 (3:1) containing 6 mM Glc and supplemented with 14 mM HEPES and 5% fetal bovine serum as described previously (31, 33). Cells were uniformly tested between passages 30 and 40 to minimize the effects of phenotypic variation in continuous culture. To induce quiescence and remove the confounding influence of serum constituents, confluent cell monolayers were routinely serum deprived for 12–24 h before and during testing. When inhibitors were employed, cells were pretreated for at least 30 min before stimulation with LPA.

LPA preparation and use. Aqueous 10 mM stock solutions of LPA were prepared in physiological Ca²⁺-, Mg²⁺-, and H₂O-free PBS containing 0.1% (wt/vol) fatty acid-free BSA. Aliquots were stored at −30°C until immediately before use. LPA stock was then added directly to the culture medium with fatty acid-free BSA supplementation as indicated to maintain the final BSA concentration at 0.001% (wt/vol).

Intracellular Ca²⁺ assays. Changes in free intracellular Ca²⁺ were monitored spectrofluorometrically using the Ca²⁺-sensitive fluorophore fura 2 as described previously (14). In these experiments, cell monolayers were grown to confluence on untreated glass coverslips and serum deprived for 24 h before analysis. Fura 2 loading was accomplished by preincubation in normal serum-free growth medium lacking phenol red and supplemented with both 14 mM HEPES, pH 7.4, and 2.5 μg/ml fura 2-AM for 1 h at 37°C. After being thoroughly washed to remove excluded fura 2-AM, individual coverslips were transferred to a Sykes-Moore chamber (Bellco, Vineland, NJ) mounted on an inverted microscope/PTI Deltascan (Princeton, NJ) spectrofluorometer system. The Ca²⁺ concentration of the assay medium was uniformly identical to that of normal growth medium (~1.4 mM). At appropriate times, LPA was introduced into the system, and Ca²⁺-sensitive changes in intracellular fura 2 fluorescence were monitored at 510 nm after excitation at either 340 (F₃₄₀) or 380 nm (F₃₈₀). Intracellular free Ca²⁺ content ([Ca²⁺]i) was routinely analyzed by the ratiometric method (F₃₄₀/F₃₈₀). Ca²⁺ ionophore addition was employed at the completion of each run to evaluate cell integrity and validate each assay.

Cell lysate preparation. Whole cell lysates suitable for both enzyme activity assays and immunoblot analysis were routinely prepared in ice-cold 45 mM Tris-HCl, 50 mM K₂HPO₄, 10 mM Glc, 11.1 mM monothioglycerol, 0.5 mM EDTA, and 0.2% (vol/vol) Triton X-100, pH 8.2. Supplementation with protease inhibitors was found to be unnecessary as long as 0.2% (vol/vol) Triton X-100 was added. LPA was added directly to the culture medium with fatty acid-free BSA supplementation as indicated to maintain the final BSA concentration at 0.001% (wt/vol).

HK assays. HK activity was assayed as the total Glc phosphorylating capacity of fresh whole cell lysates using a standard G6PDH-coupled assay as reported previously (31, 33), with minor modifications. The final assay mixture con-
sisted of 1 U/ml G6PDH, 0.5 mg/ml β-NADP, 6.7 mM ATP, 7 mM MgCl2, 4 mM Glc, 2.5 mM KH2PO4, 1 mM Na2HPO4, 11.1 mM monothioglycerol, 0.01% Triton X-100, 25 μM EDTA, and 45 mM Tris-HCl, pH 8.5. Total protein content was measured by the Bradford method (4), using bovine γ-globulin (Bio-Rad) as a standard. All data were expressed as specific HK activity in units (U) per gram protein, where 1 U is defined as that amount of enzyme activity that results in the coupled formation of 1 μmol NADPH/min at 25°C. To facilitate comparisons, specific activities are reported as percent activity relative to unstimulated time-paired control cells.

**Glc utilization and lactate production assays.** Net rates of Glc utilization and lactate production were measured as net Glc disappearance and net lactate accumulation in culture supernatants, respectively. After 18 h of absence of LPA, cells were rinsed with Ca2+- and Mg2+-free PBS and incubated for 6 h in phenol red-deficient medium containing 6 mM Glc. Aliquots of medium were subsequently analyzed for both Glc and lactate content as described previously (31, 33).

**ERK phosphorylation and activity assays.** The combined activities of ERK1/2 were analyzed using an in vitro immunoprecipitated kinase activity assay from New England Biolabs (Beverly, MA) as described previously (31, 33). In brief, activated ERK1/2 immunoprecipitates were prepared using a phospho-specific monoclonal antibody directed against the dual-phosphorylated pTyr motif in activated ERK1/2. These immunoprecipitates were then assayed for their ability to specifically serine-phosphorylate (pS383) an Elk-1 chimeric fusion protein in vitro by immunoblotting using a rabbit polyclonal phospho-Elk-1-specific antibody. Alternatively, ERK1/2 phosphorylation was analyzed as immunodetectable phospho-ERK content in whole cell lysates as described previously (31, 33). The Phototope chemiluminescent detection system from New England Biolabs was used to analyze all immunoblots.

**HK immunoblot analysis.** Whole cell lysates were electrophoretically resolved and transferred to nitrocellulose membranes for immunoblotting as described previously (32). Blots were routinely stained with 0.1% (wt/vol) Ponceau S in 5% acetic acid to confirm both the uniformity of gel loading and the efficiency of membrane transfer. To minimize nonspecific binding, blots were routinely washed with Tween 20/Tris-buffered saline (TTBS; 100 mM Tris-HCl, 0.9% (wt/vol) NaCl, 0.1% (vol/vol) Tween 20, pH 7.5) and preincubated in TTBS supplemented with 5% (wt/vol) nonfat dry milk for 1 h at 25°C. Blots were then incubated with primary antibodies in TTBS containing 5% BSA overnight at 4°C or for 4 h at 25°C, and secondary antibody was probed in TTBS with 5% nonfat milk for 1 h at 25°C. Specific rabbit polyclonal antipeptide antisera directed against the COOH-terminal 18 residues of rat/human HKII were used for all HKII immunoblots and were either generated commercially (Research Genetics) or were generously provided by Dr. Daryl K. Granner (Vanderbilt University). Rabbit polyclonal antipeptide antisera directed against the COOH-terminal 11 residues of human HKI and against the COOH-terminal 9 residues of human HKIII were also generated commercially (Alpha Diagnostic, San Antonio, TX). Rat brain lysates, recombinant human HKII (a gift of Drs. Richard L. Printz and Daryl K. Granner, Vanderbilt University), and mouse lung lysates were used as positive controls in HKI, HKII, and HKIII immunoblot analysis, respectively. Specific protein bands were visualized using a commercially available chemiluminescent detection system (New England Biolabs). Specificity was assessed by parallel analysis using preimmune serum or immunoglobulin controls where appropriate. The ability of these antibody preparations to specifically identify their target isoforms has been previously validated by the parallel use of independent isoform-specific antibody preparations either obtained commercially (Santa Cruz Biotechnology or Chemicon) or supplied by Drs. Daryl K. Granner or John E. Wilson (Michigan State University). Quantitative densitometric assessment of the resulting autoradiograms was performed on a Macintosh G3 computer using public domain National Institutes of Health Image 1.62 software (http://rsb.info.nih.gov/nih-image/).

**Statistics.** All data were expressed as means ± SE for at least three independent measurements, unless otherwise indicated. Statistical comparisons were routinely made by two-tailed paired t-testing or ANOVA with Scheffe’s post hoc F-comparisons where appropriate, using a significance level of 95% and StatView 5.0.1 statistical software for Macintosh computers (SAS Institute, Cary, NC).

**RESULTS**

**Exogenous LPA increases mesangial cell HK activity in a concentration- and time-dependent manner.** As shown in Fig. 1A, LPA increased total mesangial cell HK activity at 24 h in a concentration-dependent fashion, with maximal activation observed at concentrations ≥25 μM (apparent ED50 2 μM). As depicted in Fig. 1B, a maximally effective concentration of LPA (50 μM) increased total HK activity within 4 h, and maximal increases of >50% were observed within 12 h.

Fig. 1. Lysophosphaticid acid (LPA) increases mesangial cell hexokinase (HK) activity in a concentration- and time-dependent manner. A: LPA increased total HK activity in cultured mesangial cells at 24 h at concentrations of ≥1 μM, and maximal stimulation was observed at concentrations of ≥25 μM (apparent ED50 = 2 μM). Total HK activity in unstimulated control cells was 24 ± 2 U/g protein and corresponds closely to that reported previously for both this cell line and for primary cultures of rat mesangial cells (31). LPA maximally increased HK activity to 33 ± 4 U/g protein in these experiments. B: exposure to a maximally effective concentration of LPA (50 μM) increased total HK activity within 4 h, an effect that peaked within 12 h and persisted for an additional 12 h. Values are means ± SE for at least 6 independent measurements. *P < 0.05. **P < 0.01.
These increases persisted for at least an additional 12 h and were similar to those reported previously for other inducing stimuli in this cell type (31, 33). In contrast, the BSA vehicle alone had no independent effect on HK activity in these cells (data not shown).

Increased HK activity is associated with increased net Glc utilization and lactate production. We have previously demonstrated that increased mesangial cell HK activity is associated with increased Glc metabolism (31, 33). To examine the ability of LPA to increase Glc utilization, we monitored both Glc disappearance and lactate accumulation in the culture medium as described before (31, 33). As shown in Fig. 2, LPA (50 μM) increased both net Glc utilization and net lactate production. These changes temporally corresponded with increased HK activity in these cells.

LPA-stimulated HK activity requires classic MAPK pathway activation. We have previously demonstrated the involvement of the classic MAPK pathway in the regulation of mesangial cell HK activity by phorbol esters, thrombin, and growth factors (31–33), and similar regulation has been suggested for proinflammatory cytokines (38). Because this pathway constitutes a known effector pathway for LPA signaling, we examined MEK-selective inhibitors for the ability to prevent LPA-increased HK activity in cultured mesangial cells. Both U-0126 (Fig. 3A) and PD-98059 (Fig. 3B) prevented LPA-increased HK activity at 24 h in a concentration-dependent manner, suggesting a requirement for classic MAPK pathway activation. The apparent IC50 for U-0126 (0.4 μM) was much lower than that for PD-98059 (5.3 μM), in agreement with the reported relative potencies of these inhibitors (9). To further investigate the involvement of the classic MAPK pathway, we also tested the ability of LPA to directly activate the distal pathway components ERK1/2. Concentrations of LPA capable of increasing HK activity (≥1 μM) were uniformly capable of increasing the dual phosphorylation of the tripeptide activation motif of ERK1/2 (pTEpY) within 10 min (Fig. 4). LPA also increased ERK1/2 activity within 1 min, an effect that was maximal within 5 min and persisted for at least 15–30 min (Fig. 5A). The ability of PD-98059 to prevent the activation of ERK1/2 by LPA is compatible with a role for LPA-stimulated classic MAPK pathway activation in these responses (Fig. 5B). In agreement with our previous findings (31), MEK inhibition abro-

**Fig. 2.** LPA stimulates mesangial cell glucose (Glc) metabolism. Cultured mesangial cells exposed to 50 μM LPA (+LPA; •) uniformly exhibited higher net Glc utilization and net lactate accumulation rates than paired unstimulated control cells (−LPA; ○). These changes were observed between 18 and 24 h after LPA treatment and corresponded temporally to a 24 ± 4% increase in total HK activity (P < 0.003). Values are means ± SE for 6 independent experiments. *P < 0.01.

**Fig. 3.** Mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibition prevents LPA stimulation of HK activity. MEK-selective inhibition by both U-0126 (apparent IC50 = 0.4 μM; A) and PD-98059 (apparent IC50 = 5.3 μM; B) prevented LPA-stimulated HK activity in a concentration-dependent manner. Values are means ± SE for at least 9 independent experiments. *P < 0.001 vs. unstimulated control cells. †P < 0.03 vs. paired LPA-stimulated cells in the absence of inhibitor.
gated basal ERK1/2 activity (Fig. 5B) and decreased basal HK activity by as much as 10–20% (Fig. 3, A and B), suggesting a minor requirement for classic MAPK pathway activation in the maintenance of basal mesangial HK activity.

**LPA increases HK activity via a Ca^{2+}-independent mechanism.** LPA signaling has been characterized as both Ca^{2+} dependent and Ca^{2+} independent (27, 28).

To investigate the general involvement of Ca^{2+} in LPA induction of HK activity, we first examined the ability of the Ca^{2+} chelator BAPTA to attenuate the effects of LPA. In a series of three independent experiments, 50 μM LPA increased total HK activity by >60% (Fig. 6A). Pretreatment with BAPTA (1–10 μM) did not prevent this increase and had no independent effect on basal HK activity (Fig. 6A). Consistent with these observations, the Ca^{2+} ionophores A-23187 (Fig. 6B) and ionomycin (Fig. 6C) both failed to mimic the effect of LPA in normal growth medium containing ~1.4 mM Ca^{2+}. Continuous exposure to A-23187 or ionomycin at concentrations >2.5 and ≥500 μM, respectively, were associated with increased cell detachment and toxic cell morphology, precluding routine testing at higher concentrations (data not shown). To validate the Ca^{2+} independence of these effects, we also directly evaluated the ability of LPA to increase [Ca^{2+}]_{i} in these cells. Interestingly, LPA concentrations shown to be maximally effective at increasing classic MAPK pathway activation and HK activity in our model (25–50 μM) were not associated with demonstrable increases in [Ca^{2+}]_{i} (Fig. 6D). In contrast, Ca^{2+} ionophores were uniformly capable of increasing [Ca^{2+}]_{i} under conditions that were not associated with increased HK activity (Fig. 6D), supporting the contention that the observed increases in HK activity were not coupled to [Ca^{2+}]_{i}.

**LPA increases HK activity via a PTX-insensitive mechanism.** The cellular effects of LPA are mediated by specific G protein-coupled receptors that are known to couple with multiple G protein effectors, including...
both PTX-sensitive Gi and PTX-insensitive Gq or G11 proteins (3). We therefore investigated the ability of PTX to block LPA-stimulated HK activity in mesangial cells. In cells pretreated with PTX (0.1 μg/ml), LPA (50 μM) increased total HK activity by 45 ± 14% within 24 h (P = 0.02 vs. paired unstimulated control cells; n = 6), and this increase was not significantly different (P = 0.55) from that observed in paired LPA-stimulated cells in the absence of PTX (41 ± 10%; P = 0.01 vs. paired unstimulated control cells).

LPA-stimulated HK activity is prevented by PKC inhibition. LPA has been shown to signal via both PKC-dependent and -independent pathways (24). Because the classic MAPK pathway can also be activated by both PKC-dependent and -independent mechanisms in mesangial cells (Refs. 31, 33; Robey RB, unpublished observations), we examined the ability of the general PKC-selective inhibitor bisindolylmaleimide I to attenuate LPA-stimulated HK activity. At 5 μM, bisindolylmaleimide I had no significant effect on basal HK activity (91 ± 15% of paired control values) but completely prevented both PMA (80 ± 8 vs. 159 ± 5%; P < 0.0001) and LPA-stimulated (89 ± 12 vs. 135 ± 5%; P < 0.0001) HK activity at 24 h, consistent with a common requirement for PKC activation in these responses. In contrast, an inactive analog, bisindolylmaleimide V, had no significant effect on either stimulated or unstimulated HK activity (data not shown), suggesting specificity for PKC.

LPA mimics the effects of phorbol esters on mesangial cell HK activity. The effects of LPA on classic MAPK pathway activation and total HK activity are indistinguishable from those reported previously for direct stimulation of PKC by phorbol esters (31). The inability of phorbol esters to augment LPA-increased HK activity (data not shown) is consistent with a common mechanism of action, presumably via phorbol ester-activatable, Ca2+-independent, or “novel” (nPKC) PKC isoforms. As an indirect test of this hypothesis, we also examined the Ca2+-independent or “classic” PKC (εPKC) isoform-selective inhibitor Gö-6976 for the ability to prevent LPA-stimulated HK activity. In a series of four independent experiments, Gö-6976 did not affect LPA-stimulated HK activity (150 ± 6 vs. 147 ± 10% of paired, unstimulated control values), suggesting the general involvement of Ca2+-independent nPKC activity in these effects. The inability of Ca2+ ionophores to directly increase mesangial cell HK activity (Fig. 6, B and C) is compatible with this contention, as is the general inability of Ca2+ chelation by BAPTA to prevent increased HK activity by both phorbol esters and thrombin (data not shown).

Phospholipase A2 mimics the effect of exogenous LPA on HK activity. Extracellular LPA can be generated via deacylation of cell surface phosphatidic acid by inflammatory type II phospholipase A2 (PLA2), which is released during pathological conditions such as inflammation (25, 27). We therefore examined exogenous porcine secretory PLA2 addition to the culture medium for the ability to mimic the effects of LPA on mesangial cell HK activity. Consistent with a role for PLA2-generated endogenous LPA production, total HK activity was increased at 24 h by the addition of as little as 0.1 U/ml PLA2 (127 ± 9% vs. controls; P < 0.03), an effect that was maximal at 2 U/ml PLA2 (138 ± 7% vs. controls; P < 0.01).

Exogenous LPA selectively increases HKII isoform abundance. We have previously reported that cultured mesangial cells express all three renal HK isoforms (32). To better understand the relative contributions of individual HK isoforms to LPA-stimulated increases in total HK activity, we examined the corresponding effects on individual isoform abundance. As shown in Fig. 7A, immunoreactive HKI, HKII, and HKIII isoforms were uniformly detectable in both unstimulated and LPA-stimulated mesangial cells. LPA treatment increased HKII protein abundance approximately three-fold within 24 h (Fig. 7B), whereas no corresponding changes were observed for either HKI or HKIII. The ability of MEK inhibition by PD-98059 to prevent the increase in HKII abundance by LPA (Fig. 7C) is compatible with a requirement for classic MAPK pathway activation in this effect.

![Fig. 7. LPA selectively increases HKII isoform abundance. Whole cell lysates (40 μg total protein) from both unstimulated and LPA-stimulated (50 μM × 24 h) cells were analyzed by quantitative immunoblot analysis using HK isoform-specific antibodies.](http://ajprenal.physiology.org/)
DISCUSSION

The present work details previously unreported cellular actions of LPA, as well as a novel mechanism of HK regulation in mesangial cells. We have demonstrated that this simplest of glycerophospholipids increases mesangial cell HK activity in a concentration- and time-dependent manner, and several lines of evidence suggest that these changes are biologically relevant. First, LPA-stimulated HK activity was associated with increases in both net Glc utilization and net lactate production. Second, increased HK activity was observed in the 1–5 μM LPA concentration range found in normal serum (8). Last, maximal induction was observed at LPA concentrations that have been associated with pathological conditions (≥25 μM). In addition to its well-characterized role as an injury-generated platelet-derived factor (8), LPA is formed during the oxidation of low-density lipoproteins (LDL) and constitutes a major active component of atherogenic oxidized LDL (23, 35). Increased LPA content (>50 μM) has also been reported in both plasma (44) and malignant effusions (43) in the setting of neoplastic disease. Similar increases (>65 μM) have been observed in plasma as a consequence of in vitro phospholipase D-like phosphodiesterase activity (40), which has been implicated in a broad spectrum of physiological and pathophysiological processes. Finally, mesangial cells are known to generate extracellular LPA via the action of inflammatory group II PLA2 (30), so local concentrations may be even higher in pathological conditions associated with increased glomerular phospholipase activity. Taken together, our findings clearly have both physiological and pathophysiological implications.

We have demonstrated that LPA-stimulated HK activity requires both PKC and classic MAPK pathway activation. We previously reported the ability of phorbol esters to increase HK activity by a similar mechanism, although the corresponding endogenous activators of PKC were not identified (31). We subsequently suggested that thrombin constitutes one such endogenous stimulus and reported that not all activators of PKC are capable of increasing HK activity in this cell type (Refs. 31, 33; Robey RB, unpublished observations). The ability of LPA to mimic the effects of phorbol esters suggests that LPA may constitute another endogenous inducer of HK activity via this mechanism. Like thrombin, LPA is known to signal via specific GPCRs that are capable of coupling to multiple G protein effectors, including Gq, Gi, and G12/13 (25). Thus the inability of PTX to prevent LPA-stimulated HK activity suggests a role for PTX-insensitive G protein effectors (e.g., Gq or G12/13) in this process. LPA did not appreciably alter [Ca2+]i at concentrations associated with maximally increased HK activity in our model. These findings strongly suggest a Ca2+-independent mechanism of action. They do not, however, exclude the ability of LPA to modulate intracellular Ca2+ in this cell type, as biphasic functional responses to LPA have previously been reported in mesangial cells (10).

Both the inability of BAPTA to inhibit LPA-increased HK activity and the failure of Ca2+ ionophores to mimic these effects are compatible with this interpretation. Indirect support for the Ca2+ independence of these effects may also be found in the inability of Gö-6976 (a Ca2+-dependent or εPKC isomser-selective inhibitor) to mimic the effects of bisindolylmaleimide I (a general PKC-selective inhibitor) in preventing phorbol ester-stimulated HK activity. Taken together, these findings suggest the involvement of nPKC isoforms in these responses, and this may explain, in part, why not all activators of PKC are uniformly capable of increasing HK activity in these cells (31, 33). However, PKC signaling exhibits great diversity in mesangial cells, and specific nPKC isoform involvement has not yet been established.

Although both PD-98059 and U-0126 effectively and selectively inhibit MEK signaling, they do so in a fundamentally different manner. PD-98059 binds to inactive MEK at a site distinct from its ATP- and ERK-binding sites, thereby preventing its activation by upstream effectors (1). U-0126 binds to the same site, but, in contrast to PD-98059, is capable of both binding and inhibiting the activated form of MEK (9). In principle, therefore, PD-98059 may be less effective than U-0126 in cells with significant levels of basal classic MAPK pathway activation. Our findings are in agreement with the reported potencies of these individual inhibitors (9), although a modest ongoing requirement for basal MEK activation could also contribute to observed differences in the effectiveness of these individual inhibitors in our model. The ability of both inhibited activation (PD-98059) and inhibited catalytic activity (U-0126) to prevent LPA-increased mesangial cell HK activity at concentrations deemed specific for MEK validates the specific involvement of MEK in this process.

Phorbol esters, thrombin, and growth factors increase mesangial cell HK activity via classic MAPK pathway activation (31–33). A uniform requirement for both ongoing gene transcription and de novo protein synthesis has suggested a role for increased HK expression in these effects (31–33). Associated increases in HKII isoform abundance (Refs. 32, 38; Robey RB, unpublished observations) suggests a specific role for HKII expression. In the present work, we have demonstrated that LPA-increased HK activity requires classic MAPK pathway activation and is associated with increased HKII abundance. These findings, coupled with similar demonstrations for other stimuli (32, 38), suggests that HKII may constitute the principal inducible isoform in this cell type and account for the observed increases in total HK activity. Interestingly, HKII represents the principal regulated HK isoform in the insulin-sensitive peripheral tissues that are responsible for the bulk of systemic Glc utilization (e.g., muscle and adipose), and the expression of this isoform has been widely regarded as a marker of insulin sensitivity with respect to Glc metabolism. It is therefore of considerable interest that neither insulin nor IGF-I increases HK activity in cultured mesangial cells at
physiological concentrations (Ref. 32; Robey RB, unpublished observations). It is also interesting to note that the corresponding regulation of HKII expression in skeletal myotubes does not require classic MAPK pathway activation (29). Presently speculative is the possibility that these cell-specific differences in HKII regulation may contribute to the contrasting tissue-specific patterns of HK activity and Glc utilization observed in the kidney and tissues such as skeletal muscle, myocardium, and adipose tissue in the setting of diabetes (2, 7, 34, 36, 37).

Finally, LPA is a known serum survival factor for a variety of cell types (11, 20, 21, 42) and has recently been reported to prevent apoptosis in mesangial cells (18). The specific mechanisms whereby this important bioactive phospholipid promotes cell survival are poorly understood, but this effect appears to require de novo protein synthesis in mesangial cells (18). We have previously suggested that increased HK activity may represent an important general adaptive response to cellular injury (31–33), and the present findings are compatible with such a hypothesis and with the contention that HKs may contribute to the salutary effects of LPA. Although not directly addressed in the present work, indirect support for such a role may be found in the ability of increased HK activity to mimic the antiapoptotic effects of growth factors in fibroblasts (12) and to decrease the susceptibility of renal epithelial cells to acute oxidant-induced cell death (5).

In summary, we have confirmed the expression of all three renal HK isoforms in cultured mesangial cells. We have also demonstrated that LPA-stimulated HK activity in this cell type requires classic MAPK pathway activation and is associated with selective increases in HKII isoform abundance. Given similar observations for phorbol ester-, thrombin-, growth factor-, and cytokine-stimulated HK activity in these cells, the present findings are compatible with the hypothesis that these diverse stimuli share a common mechanism of action. They are also in agreement with previously noted similarities between the biological actions of LPA and thrombin (19). Thus the present work both confirms and extends our previous findings and suggests another novel mechanism for coupling metabolism to cellular injury.

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