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Interleukin-1β Activates PI 3-Kinase in Renal Mesangial Cells

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Interleukin-1β rapidly stimulates the activation of phosphatidylinositol 3-hydroxyl kinase in renal mesangial cells. This interleukin-1β dependent activation is associated with a rapid increase in phosphatidyl inositol 3,4,5 phosphate in renal mesangial cells. The increase in PI 3-kinase activity is inhibited by wortmannin. In addition wortmannin partially inhibited IL-1β induced PGE_2 production and potentiated IL-1β induced nitric oxide production. These experiments suggest that IL-1β can activate PI 3-kinase in renal mesangial cells and that the enzyme plays a role in IL-1β induced PGE_2 and NO formation in the renal mesangial cell.

Interleukin-1 (IL-1) is a pleotropic cytrokine capable of triggering a variety of signal transduction pathways in many mammalian cells. These postreceptor events include activation of a GTP-binding protein with no associated activation of adenyl cyclase (1), activation of adenyl cyclase (2,3), hydrolysis of phospholipids by non-phosphtidylinositol phospholipase C (4,5), release of ceramide from sphingomyelin after activation of sphingomyelinase (6), and release of arachidonic acid from phospholipids via cytosolic phospholipase A_2 ($cPLA_2$) after the activation of a PLA_2 activating protein (PLAP) (7,8). Several investigators have reported phosphorylation of cellular proteins within the first 15 minutes after IL-1 receptor binding. Most consistently IL-1 activates protein kinases which phosphorylate either a serine or threonine residue. In addition IL-1 is capable of activating several members of the MAP kinase family of dual specificity kinases including ERK's 1 and 2. JNK 1 and 2 and p38 MAP kinase. It has been assumed that the functional IL-1 type 1 receptor is a single chain receptor based primarily on the ability of Chinese hamster ovary cells expressing recombinant murine Type I IL-1R to transduce an IL-1 signal (9). This concept has been challenged by the molecular cloning and characterization of a second subunit of the interleukin-1 receptor complex (10). This recombinant accessory protein increases the binding affinity of the recombinant Type I IL-1R for IL-1 when the two receptor proteins are co-expressed. Neither the Type I IL-1R or its accessory protein are known to contain SH2 or SH3 domains and suggests that the receptor is incapable of directly interacting with cellular non-receptor tyrosine kinases. In the renal mesangial cell we previously demonstrated that tyrosine kinase activation was necessary for the IL-1 induced expression of cyclooxygenase 2 and the inducible nitric oxide synthase. We therefore designed experiments to ask the question whether IL-1β could activate PI 3-kinase in renal mesangial cells.

MATERIALS AND METHODS

Rat mesangial cell culture. Primary mesangial cell cultures were prepared from male Sprague-Dawley rats as previously described. Cells were grown in RPMI-1640 medium supplemented with 15% (v/v) heat-inactivated fetal

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calf serum 0.6% (v/v) insulin, 100 U/ml penicillin, 100 μg/ml streptomycin, 250 μg/ml amphotericin B, and 15 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES). All experiments were performed with confluent cells grown in 175 cm^2 flasks (about 1.5 - 2.0 $\times$ 10^7 cells) and used at passages 3-6.

Measurement of PGE_2 by gas chromatography mass spectrometry. PGE_2 in the media was determined by stable isotope GC-MS. At the end of predetermined times, media was removed and spiked with 10-20 ng tetradeuterated (d_4-PGE_2). The medium was then acidified to pH 3.5 and PGE_2 is extracted by 1 ml octadecyl columns (Baker). Extracts were derivatized for GC-mass spectrometric analysis. The samples were analyzed as the pentafluorobenzyl methoxime trimethylsilyl ether by negative ion chemical ionization using methane as the reagent gas. Ions monitored were *m/z* 524 (d_o PGE_2) and *m/z* 528 (d_4 PGE_2). Mass spectrometry was performed on a Hewlett-Packard 5985^B using a 25-m Ultra 1 (Hewlett Packard) capillary column, and data analysis performed using Vector 2 (Teknivent, St. Louis, MO) software.

Nitrite determination. The conditioned incubation medium was collected and nitrite content in the supernatant measured by the addition of Griess regent (1% sulfanilamide-0.1% naphthylethyl-enediamine dihydrochloride in 2% phosphoric acid). The absorbance at 550 nm was measured and the amount of nitrite obtained by extrapolation from a standard curve using sodium nitrite as a standard. The nitrite production was corrected for protein determined by the micro bicinchoninic acid assay.

Measurement of PI 3-kinase in vitro. For this assay mesangial cells were stimulated for 0,2,5,10 and 15 mins with IL-1 (50 U/ml). At the end of the appropriate incubation time the media was quickly aspirated and replaced with 1 ml of ice-cold lysis buffer. Lysis buffer consisted of 1% Triton X-100; 20 mM Tris-HCl, pH 8.0; 137 mM NaCl; 10% glycerol; 2 mM EDTA; 1mM sodium orthovanadate; 1mM PMSF; 10 μg/ml leupeptin and 1μg/ml aprotinin. Cells were then scraped and the suspension centrifuged for 15 mins at 14,000 $\times$ g in the cold. Cell lysates (200 μg protein) were then immunoprecipated for 3 hr with agarose-conjugated anti-phosphotyrosine antibody. Precipitates were then washed with lysis buffer containing 50 μM vanadate and three times with 10 mM Tris-HCl. PI 3-kinase activity was measured by addition of 10 μg sonicated PI (Avanti Polar Lipids) and 10 μCi of γ-[^{32}P]ATP. Reactions were carried out for 15 mins in the presence of 200 μM adenosine at room temperature and stopped by the addition of 0.1 ml 1 N HCl and 0.2 ml chloroform:methanol (1:1 v/v). Lipids are separated on oxalate-impregnated TLC plates using a solvent system of chloroform:methanol:water:28% ammonia (45:35:7.5:2.5, v/v/v/v). TLC plates are then exposed to X-ray film at −80°C. Quantitation of the radioactivity incorporated into lipids was measured by quantitative densitomery following autoradiography.

Measurement of PIP_3 levels in intact cells. To determine whether PIP_3 levels increase in cells stimulated by IL-1β a further series of experiments were carried out. Mesangial cells in 175 cm^2 flasks were labeled with $H_3[^{32}P]O_4$, 0.5 mCi/ml in phosphate-free RPMI containing 20 mM HEPES pH 7.4, for 2 hrs at 37°C. IL-1β was then added to the appropriate flasks for 0, 2, 5, 10 and 15 mins. The reaction was then terminated by the addition of 3.5 ml methanol:chloroform (2:1, v/v). Phases were then separated by addition of 1.25 ml each of 2.4 N HCl and chloroform and the lower organic phase recovered after centrifugation. The upper phase was re-extracted with 1 ml of chloroform:methanol (2:1, v/v). The combined lower phases were washed twice with 1 ml methanol, 0.1 M EDTA in water (1:0.9, v/v) and dried under nitrogen. Dried cellular lipids were then deacylated by adding 1.8 ml of methylamine reagent (methanol: 25% methylamine: n-butyl alcohol, 45.7:42.8:11.4, v/v/v). The deacylated lipids were dried in vacuo, resuspended in 2 ml water extracted three times with 2 ml n-butyl alcohol:light petroleum ether:ethyl formate (20:4:1, v/v/v), dried under nitrogen and suspended in 160 μl water for HPLC. For HPLC ammonium phosphate pH 3.8 was added to the sample to a final concentration of 10 mM and samples chromatographed on a Partisil 5 SAX column. Cellular lipids were separated using a 60 min linear gradient of 0-0.25 M ammonium phosphate, pH 3.8, followed by a 50 min linear gradient from 0.25 to 1 M ammonium phosphate, pH 3.8. 1 ml fractions were collected for liquid scintillation counting (11).

RESULTS

Activation of PI 3-kinase by IL-1β. Exposure of rat mesangial cells to IL-1β produced a rapid phosphorylation of PI to PIP (PI 3-phosphate). Fig. 1 shows such an experiment with lane 1 (time 0) as control, lane 2 (IL-1 for 1 min) and lane 2 (IL-1 for 2 mins) showing significant increases in formation of PIP. For comparison a known activator of PI 3-kinase is shown as a positive control. Lanes 4 and 5 show increased activation of PI 3-kinase by WEHI-3 supernatant (which contains IL-3), at 1 and 2 mins of stimulation. Fig. 2 shows a time course of activation with peak responses seen at 2 mins and return to basal levels 10 mins post stimulation. Fig. 2 also demonstrates that wortmannin a fungal toxin completely inhibits the ability of IL-1β to increase PIP in renal mesangial cells, thus confirming the formation of PIP is due to the activation of PI 3-kinase.

IL-1β increases PIP_3 in intact mesangial cells. To determine if IL-1β by activating PI 3-

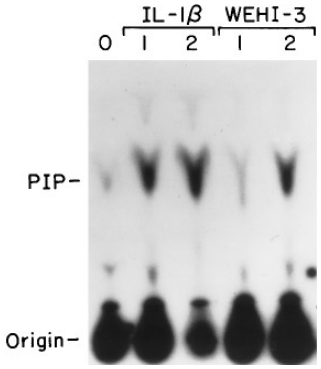

FIG. 1. IL-1β-induced increases in PI 3-kinase activity immunoprecipated with anti-phosphotyrosine antibody from mesangial cells. Supernatants were removed at 1 min and 2 mins after stimulation with IL-1β or WEHI-3 supernatant as positive control. The conversion of PI to PIP in the presence of γ-[^{32}P]ATP was analysed by TLC.

kinase can lead to increases in PIP_3 (phosphatidyl inositol 3,4,5 trisphosphate) in the mesangial cell, labelled cells were stimulated with IL-1β and lipid extracts subjected to HPLC as described in methods. Fig. 3 shows the results of these experiments. It demonstrates PIP_3 formation and confirms the rapid formation of PIP_3 in intact mesangial cells when stimulated by IL-1β.

Effects of wortmannin on IL-1β induced PGE_2 and NO formation. Fig. 4 demonstrates that IL-1β induced PGE2 formation is partially inhibited by wortmannin with maximal inhibition occuring at 300 nM concentrations of wortmannin. In contrast wortmannin dose dependently potentiated IL-1β induced NO formation.

DISCUSSION

Several members of the cytokine family have been demonstrated to activate PI 3-kinase in a variety of biological systems. In contrast there are no reports demonstrating activation of PI 3-kinase by IL-1. Many of the extracellular ligands which activate PI 3-kinase interact with

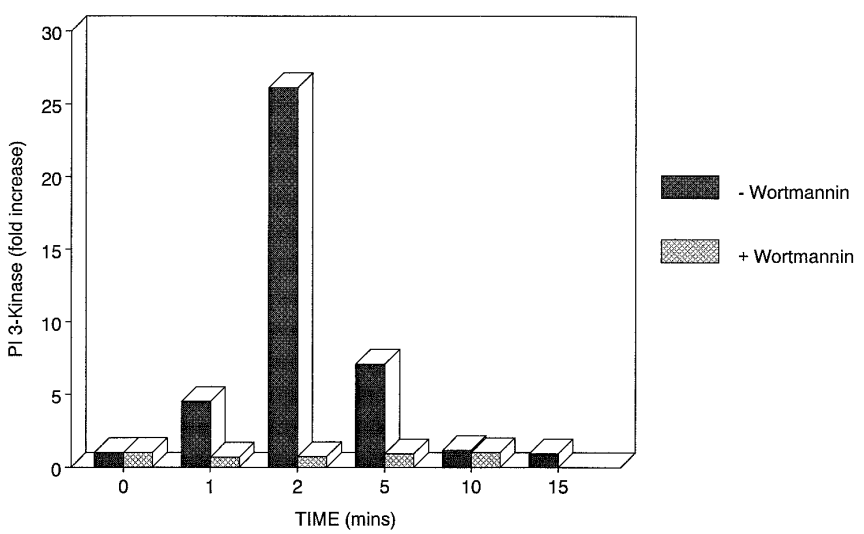

FIG. 2. Time course of activation of PI 3-kinase in renal mesangial cell by IL-1β. Effect of wortmannin is shown.

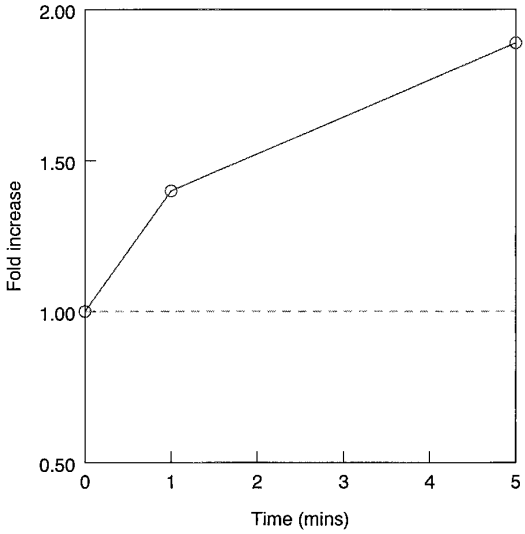

FIG. 3. Time course of PIP_3 levels in mesangial cell after stimulation with IL-1β. Levels are expressed as fold stimulation over baseline to normalize for variability of incorporation of ^{32}P label into PIP_3.

their homologous receptors which have intrinsic tyrosine kinase activity or have SH2 and/or SH3 motifs in their intracellular domains. The IL-1R1 receptor does not appear to have these motifs in its intracellular domain. Furthermore the recently cloned second subunit of the IL-1 receptor complex does not appear to have SH2 and/or SH3 motifs. It does have however a potential protein kinase C acceptor site (10). This raises the interesting possibility that PI 3-kinase can be activated by PKC or a stress activated protein kinase as has been suggested (12). Our experiments do however indicate that IL-1β can activate PI 3-kinase in the renal mesangial cell and this leads to the rapid formation of PIP_3 intracellularly. In published studies

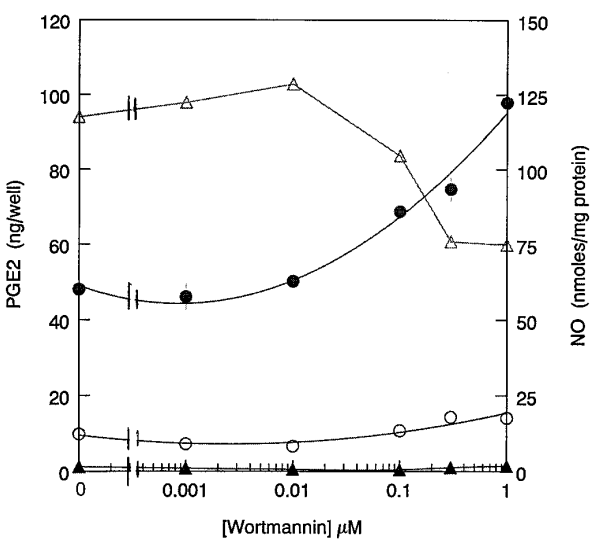

FIG. 4. Effect of wortmannin on IL-1β induced PGE_2 and NO production by mesangial cells. (▲-▲) PGE_2 control; (○-○) nitric oxide control; (△-△) PGE2 IL-1β stimulated; (●-●) nitric oxide, IL-1β stimulated.

it has been suggested that PIP_3 can activate PKCζ (13) which in turn can phosphorylate IκB (14). This phosphorylation of IκB signals its ubiquitin dependent degradation and releases the inhibitory protein from the nuclear factor κB, which then allows translocation to the nucleeus (15-17). We have previously demonstrated that antisense oligonucleotide to PKCζ inhibited IL-1β induced PGE_2 production and COX-2 protein expression in renal mesangial cells by 40-50% (18). It is therefore intriguing that wortmannin inhibited IL-1β induced PGE_2 production by 40%. These experiments suggest that PI 3-kinase is partially involved in IL-1β signalling in the renal mesangial cell and can influence the PGE2 response to IL-1β stimulation.

ACKNOWLEDGMENT

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