In our present studies utilizing a well characterized proximal tubule cell line, LLCPK1, we determined that all four EET regioisomers (5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET) stimulated [3H]thymidine incorporation, with 14,15-EET being the most potent. In contrast, no mitogenic effects were seen with arachidonic acid, other cP450 arachidonate metabolites (12R-hydroxyeicosatetraenoic acid (12R-HETE), 14,15-dihydroxyeicosatrienoic acid (14,15-DHET), or 20-HETE), or lipoxygenase metabolites (55-HETE, leukotriene B4, or lipoxin A4). We found that their metabolically more stable sulfonimide (SI) analogs (11,12-EET-SI and 14,15-EET-SI) were also potent mitogens. In addition, 14,15-EET-SI also increased cell proliferation as well as expression of both c-fos and egr-1 mRNA. The protein kinase C and A inhibitors, H-7 and H-8, or the cyclooxygenase inhibitor, indomethacin, had no effect upon 14,15-EET-induced [3H]thymidine incorporation, but the selective tyrosine kinase inhibitor, genistein, significantly inhibited it. Immunoprecipitation and immunoblotting demonstrated increased tyrosine phosphorylation of PI3-kinase and epidermal growth factor receptor (EGFR) within 1 min of EET administration. EETs also stimulated association of PI3-kinase with EGFR. PI3-kinase inhibitors, wortmannin and LY 294002, markedly inhibited 14,15-EET-SI-stimulated [3H]thymidine incorporation. In addition, 14,15-EET-SI administration stimulated tyrosine phosphorylation of src homologous and collagen-like protein (SCH) and association of SCH with both growth factor receptor-binding protein (GRB2) and EGFR. Mitogen-activated protein kinase was also activated within 5 min. Pretreatment of the cells with the mitogen-activated protein kinase inhibitor, PD98059, inhibited the 14,15-EET-SI-stimulated [3H]thymidine incorporation. Moreover, immunoblotting indicated that 14,15-EET stimulated tyrosine phosphorylation of the specific pp60src substrate p120 and e-Src association with EGFR. 14,15-EET increased src kinase activity within 1 min. Our data indicate that EETs are potent mitogens for renal epithelial cells, and the mitogenic effects of the EETs are mediated, at least in part, by the activation of Src kinase and initiation of a tyrosine kinase phosphorylation cascade.

Cytochrome P450 epoxidease catalyzes the NADPH-dependent epoxidation of arachidonic acid to 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acids (EETs)1 in a regio- and stereo-selective manner. The EETs are produced predominantly by epoxygenases of the 2C family of cP450s, which have been localized in the kidney to the mammalian proximal tubule. The proximal tubule contains the highest concentration of cP450 within the mammalian kidney (1) and expresses minimal cyclooxygenase or lipoxygenase activity (2). The EETs or their hydration products have been implicated in modulation of vascular tone and renal glomerular hemodynamics (3), renal proximal tubule function, and regulation of mitogenesis (4, 5). Recently, EETs have been suggested to be an endothelial-derived hyperpolarizing factor (6), although there is controversy about this issue (7, 8). Direct administration of EETs inhibits amiloride-sensitive sodium transport (9) and 86Rb uptake in LLC-PK1, a nontransformed, immortalized cell line from pig kidney with certain proximal tubule characteristics (10).

EETs have also been proposed as second messengers for hormones and growth factors in the proximal tubule. We have shown that epidermal growth factor (EGF) stimulates EET production in rat proximal tubule suspensions and primary cultured rabbit proximal tubule cells, and EETs may mediate both EGF-stimulated calcium influx and mitogenesis in proximal tubules (11). Omata et al. demonstrated EET production upon stimulation with angiotensin II (12). EETs may mediate the effect of high (>10^-7 M) angiotensin II to increase cytosolic calcium ([Ca^2+]_i) and decrease Na^-/H^+ exchange activity in cultured rabbit proximal tubule cells and isolated rat proximal tubules (13–15). In the present studies, we examined mitogenic signaling mechanisms of EETs in renal epithelial cells. We determined the mitogenic effects of all four EET regioisomers and utilized metabolically more stable sulfonimide analogs to determine potential intracellular signaling mechanisms mediating mitogenic effects. We demonstrate here that EETs activate pp60src and initiate a tyrosine kinase cascade that mediates their mitogenic effects. This is the first evidence that cP450-mediated arachidonic acid metabolites can signal through such pathways.

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1 The abbreviations used are: EET, epoxyeicosatrienoic acids; EGF, epidermal growth factor; EGFR, EGF receptor; HETE, hydroxyeicosatetraenoic acid; ERK, extracellular signal-regulated kinase; SCH, Src homologous and collagen-like protein; SI, sulfonimide; TBS, Tris-buffered saline; DHET, dihydroxyeicosatrienoic acid; PY, phosphotyrosine; PI3-kinase, phosphatidylinositol 3-kinase; GDPβS, guanyl-5'-yl thiophosphate; GRB2, growth factor receptor binding protein-2; LTBP, leukotriene B4.
EXPERIMENTAL PROCEDURES

Materials—A 1:1 bicarbonate-buffered mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 nutrient medium was purchased from Sigma. (±)-11,12-EET, (±)-14,15-EET (16), and their sulfonamide analogs (see Fig. 1) were synthesized as follows: EET (94 mg, 0.29 mmol) and N-hydroxysuccinimide (37 mg, 0.32 mmol) were azetropically dried with benzene (3 × 20 ml), then dissolved in dry tetrahydrofuran (5 ml) and cooled to 0 °C. To this was added dichlorocarbodi imide (66 mg) all at once with vigorous stirring. After 12 h at room temperature, the solvent was removed in vacuo, and the crude product was purified by SiO2 column chromatography using ethyl acetate (30:70) to give the corresponding N-hydroxysuccinimide ester as a gum (92 mg, 75%). TLC (SiO2): 30% ethyl acetate in hexane, Rf 0.2; 1H NMR (250 MHz, CDCl3): δ 0.92 (t, J = 7.0, 6H, 2H), 1.32–1.40 (m, 6H, 1.82–1.85 (m, 6H), 2.2–2.25 (m, 6H), 5.34–5.52 (m, 6H). The active ester (92 mg), methanesulfonimidate (209 mg), and 4-methylaminopyridine (29.5 mg) were dried in vacuo (0.1 mm Hz) for 2 h, mixed with anhydrous hexamethyphosphoramide (0.25 ml), and heated at 90 °C under argon atmosphere. After 1.5 h, the reaction mixture was cooled and partitioned between water and ethyl acetate. The aqueous layer was extracted twice more with ethyl acetate. The combined organic extracts were dried over MgSO4 and evaporated in vacuo, and the residue was purified preparative TLC using ethyl acetate/hexane (1:1) to afford (±) EET-SI (51 mg, 58%) or a colorless oil. TLC (SiO2): ethyl acetate/hexane (1:1), Rf 0.29; 1H NMR (250 MHz, CDCl3): δ 0.93 (t, J = 6.6 Hz, 3H), 1.23–1.60 (m, 6H), 1.73–1.82 (m, 2H), 2.05–2.19 (m, 2H, 2.20–2.52 (m, 4H), 2.72–2.89 (m, 3H), 3.02–3.30 (m, 2H), 3.29 (m, 3H), 3.50–3.61 (m, 6H, 3.61–3.80 (m, 3H), 4.03–4.10 (t, J = 5.5 Hz, 2H), 4.25–4.35 (m, 2H), 4.50–4.80 (m, 2H), 5.15–5.35 (m, 2H), 5.64–5.90 (m, 2H), 6.10–6.30 (m, 2H). 13C NMR (63 MHz, CDCl3): δ 14.05, 22.53, 24.18, 25.64, 25.82, 26.14, 26.45, 27.55, 31.56, 31.64, 35.78, 41.36, 57.02, 58.01, 124.09, 124.93, 125.73, 128.31, 128.55, 129.11, 130.72, 187.20. 5H-HETE, 12R-HETE, LTB4, and lipoxin A4 were from Biomol (Plymouth Meeting, PA). EGF (receptor grade) was purchased from Collaborative Research (Bedford, MA). [3H]Thymidine, [α-32P]ATP, and [γ-32P]ATP (specific activity: 3000 Ci/mmol) were from NEN Life Science Products, Genestin, H-7, PD-98059, H-8, and SHC were obtained from Calbiochem. Rabbit muscle enolase was from Boehringer Mannheim. All other chemicals were from Sigma. Polyclonal and monoclonal PY-20 anti-phosphotyrosine antibodies were obtained from Zymed Laboratories Inc. (San Francisco, CA). Polyclonal and monoclonal anti-phosphotyrosine-kinase-3 (PI3)-kinase antibodies and polyclonal anti-EGF receptor antibody were from Upstate Biotechnology Inc. (UBI, Lake Placid, NY). Polyclonal anti-Src antibody 327, a mouse mAb that specifically recognizes mammalian pp60c-src, was obtained from Oncogene Research Products (Cambridge, MA). Polyclonal anti-Src (sc-018), which recognizes pp60c-src, pp62c-src, and pp69c-src, polyclonal anti-ERK antibodies, and polyclonal anti-A-garose beads were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-SHC, monoclonal anti-EGRF, and monoclonal anti-GRB2 antibodies were from Transduction Laboratories (Lexington, KY).

Cell Culture—LLCPKc4, an established proximal tubule epithelial cell line derived from pig kidney (17), was routinely cultured in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (Hyclone Laboratories, Logan, UT) at 37 °C in a 5% CO2 cell culture incubator. The medium was changed every 2–3 days. For studies of [3H]thymidine incorporation, cells were grown in 24-well plates. For cellular RNA isolation and immunoprecipitation, cells were grown in 100- and 60-mm plates, respectively.

[3H]Thymidine Incorporation Assay and Cell Growth Curves—Confluent cells in 100-mm dishes were detached by trypsinization, resuspended in growth medium, seeded at a density of 3 × 105/well in 24-well plates, and incubated for 2–3 days until they were 80% confluent. The medium was then changed to serum-free medium and incubated for an additional 3 days. EETs and/or the indicated drugs in the corresponding medium was then changed to serum-free medium and incubated for an additional 3 days. EETs and/or the indicated drugs in the corresponding medium was then changed to serum-free medium and incubated for an additional 3 days. EETs and/or the indicated drugs in the corresponding medium was then changed to serum-free medium and incubated for an additional 3 days. EETs and/or the indicated drugs in the corresponding medium was then changed to serum-free medium and incubated for an additional 3 days. EETs and/or the indicated drugs in the corresponding medium was then changed to serum-free medium and incubated for an additional 3 days. EETs and/or the indicated drugs in the corresponding medium was then changed to serum-free medium and incubated for an additional 3 days. EETs and/or the indicated drugs in the corresponding medium was then changed to serum-free medium and incubated for an additional 3 days.
with EC_{max} \sim 256 \text{ nM} and an EC_{50} of \sim 8 \text{ nM} (Fig. 2A). Arachidonic acid in concentrations from 1 to 40 \text{ \mu M} failed to stimulate \[^{3}H\]thymidine incorporation in LLCPKc14 (n = 14). Administration of the product of P450 arachidonic acid \(\alpha\)-hydroxylation, 20-HETE, did not stimulate \[^{3}H\]thymidine incorporation in LLCPKc14 (n = 7). Similarly, no mitogenic effects were seen with the cP450 arachidonate metabolite, 12R-HETE (n = 6). The lipoxigenase metabolites, 5S-HETE, LTB4, and lipoxin A4, also failed to stimulate \[^{3}H\]thymidine incorporation in LLCPKc14 (n = 4–6). However, as indicated in Fig. 2B, all four EET regioisomers (5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET) increased \[^{3}H\]thymidine incorporation in LLCPKc14 cells in a concentration-dependent manner, with 14,15-EET being the most potent. In contrast, the hydration product of 14,15-

EET, 14,15-dihydroxyeicosatrienoic acid (14,15-DHET), produced only minimal increases in \[^{3}H\]thymidine incorporation (not shown).

EETs are susceptible to oxidative degradation; to block acetyl CoA-dependent activation and lysophospholipid acylation, the EET carboxylate was blocked by condensation with methanesulfonimide to generate the corresponding sulfonimide derivatives (11,12-EET-SI and 14,15-EET-SI). Shown in Fig. 1 are structures of 14,15-EET and its sulfonimide derivative, 14,15-EET-SI. These sulfonimide analogs were also found to be potent mitogens for LLCPKc14 cells, as determined by increased \[^{3}H\]thymidine incorporation (Fig. 2C). As with the parent compound, 14,15-EET-SI was found to be relatively more potent than 11,12-EET-SI. Therefore, 14,15-EET-SI was utilized for further investigation of the mitogenic signaling pathways. In addition to \[^{3}H\]thymidine incorporation, 14,15-EET-SI increased cell proliferation in the absence of fetal calf serum (Fig. 2D).

14,15-EET-SI Activated Immediate Early Gene Transcription—Quiescent LLCPKc14 cells were exposed to 20 \text{ \mu M} 14,15-EET-SI for the indicated times, the cells were lysed, total cellular RNA was isolated, and levels of mRNA expression were determined by Northern blot hybridization. 14,15-EET-SI increased steady state levels of both \(c\)-fos and \(egr\)-1 mRNA within
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Fig. 3. 14,15-EET-SI-induced immediate early gene activation. Quiescent LLCPKc4 cells were exposed to vehicle (Me2SO) alone (lane 1), 20 μM 14,15-EET-SI (lanes 2–6), 10−8 M phorbol 12-myristate 13-acetate (lane 7), or 100 nM EGF (lane 8) for 15 min (lane 2), 30 min (lanes 1, 3, 7, and 8), 1 h (lane 4), 3 h (lane 5), or 6 h (lane 6), then the cells were lysed, total cellular RNA was isolated, and mRNA expression of c-Fos or Egr-1 was determined by Northern blot analysis as described under "Experimental Procedures." To assess the uniformity of RNA loaded into each gel well, the blots were stripped and reprobed with a cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

15 min, with maximal increases at 30 min. The message levels rapidly declined in the subsequent 3 h (Fig. 3). In contrast, 14,15-EET-SI did not induce any significant increases in COX-2 mRNA levels, nor did it induce expression of acyl CoA oxidase mRNA, a marker of peroxisomal proliferator activator receptor activation (not shown).

14,15-EET-SI Induced Activation of Tyrosine Phosphorylation Cascade—To investigate signaling mechanisms of the mitogenic effects of 14,15-EET, we tested a range of inhibitors. The isoquinoline sulfonamides, H-7 and H-8, are potent inhibitors of serine/threonine kinases and of cyclic nucleotide-dependent protein kinases. Although neither is specific, H-7 has relative specificity for PKC, whereas H-8 is most effective for protein kinase A and cGMP-dependent protein kinase (19, 20). No inhibition of 14,15-EET-SI-induced [3H]thymidine incorporation into DNA of LLCPKc4 cells was seen with either H-7 or H-8. In addition, the cyclooxygenase inhibitor, indomethacin, did not alter 14,15-EET-SI-induced [3H]thymidine incorporation. However, the tyrosine kinase inhibitor, genistein, blocked [3H]thymidine incorporation in a concentration-dependent manner, with an IC50 of ~10 μM (Fig. 4A). Similar inhibitory effects were seen for 11,12-EET-SI-stimulated [3H]thymidine incorporation (not shown). After treatment with 20 μM 14,15-EET-SI, the cells were lysed and immunoprecipitated with polyclonal anti-phosphotyrosine (PY), then immunoblotted with monoclonal anti-phosphotyrosine (PY-20). We found that administration of 14,15-EET-SI led to increases in tyrosine phosphorylation of several LLCPKc4 proteins, including bands of molecular mass ~175, ~120, ~85, and ~60 kDa (Fig. 4B).

To characterize the 85-kDa tyrosine-phosphorylated protein, cell lysates were subjected to either immunoprecipitation with anti-phosphotyrosine and immunoblotting with an anti-P13-kinase antibody or immunoprecipitation with anti-P13-kinase and immunoblotting with anti-phosphotyrosine. The results indicated in Fig. 5A confirmed that 14,15-EET-SI administration induced tyrosine phosphorylation of the 85-kDa regulatory subunit of P13-kinase. Increased tyrosine phosphorylation of this protein was detectable within 1 min of 14,15-EET-SI addition and remained elevated for up to 30 min (the longest time period tested). The P13-kinase inhibitor, wortmannin, was found to inhibit 14,15-EET-SI-stimulated DNA synthesis in a concentration-dependent manner. Pretreatment of the cells with 1 nM wortmannin for 30 min inhibited 21% of the stimulation, and 10 nM wortmannin almost totally (95%) abolished the stimulatory effect of 20 μM 14,15-EET-SI, with IC50 ~ 5 nM (Fig. 5B). At the concentrations used in the present studies, wortmannin has been shown to inhibit P13-kinase specifically and to have no inhibitory effect upon phospholipase C, phospholipase D, phospholipase A2, or adenylate cyclase (21). Furthermore, similar effects were shown by another structurally and mechanistically distinct, highly specific P13-kinase inhibitor, LY294002 (22), with an IC50 value of ~5 μM (Fig. 5C). Wortmannin and LY294002 administration resulted in similar inhibition of 11,12-EET-SI-stimulated [3H]thymidine incorporation (not shown).

Immunoblotting with an anti-EGF receptor antibody identified the ~175-kDa protein that was tyrosine-phosphorylated in response to 14,15-EET-SI (Fig. 6A). Increases in EGF receptor tyrosine phosphorylation were noted within the first 5 min of 14,15-EET-SI addition. Moreover, immunoprecipitation with a P13-kinase antibody and immunoblotting with an anti-EGFR antibody indicated that 14,15-EET-SI induced the association of P13-kinase with EGF receptors (Fig. 6B).

Tyrosine phosphorylation of the adaptor protein SHC has been reported to occur in response to activation of both tyrosine kinase receptors and nontyrosine kinase receptors. To determine if 14,15-EET-SI stimulates SHC tyrosine phosphorylation, we probed anti-PY immunoprecipitates with SHC antibody and determined that 14,15-EET-SI-stimulated tyrosine phosphorylation of the three SHC isoforms: 66 kDa, 52 kDa, and 46 kDa. Initial increases in tyrosine phosphorylation were detected in 52-kDa and 66-kDa SHC, with subsequent tyrosine phosphorylation of 46-kDa SHC (Fig. 7A). Phosphorylated SHC is known to bind to SH2 domains of tyrosine kinase receptors and to recruit the adaptor protein, GRB2, to the plasma membrane. By immunoprecipitating with anti-SHC and then immunoblotting with antibodies against GRB2 or EGF receptors, we further corroborated that 14,15-EET-SI-activated SHC simultaneously co-immunoprecipitated both GRB2 and EGF receptors (Fig. 7, B and C), i.e. 14,15-EET-SI-induced association of SHC not only with GRB2 but also with EGF receptors.

GRB2 has been shown to bind to the guanine nucleotide exchanging factor, SOS, leading to Ras activation and Ras-dependent activation of the mitogen-activated protein kinase cascade. In this regard, we determined that 14,15-EET-SI induced tyrosine phosphorylation of both ERK1 and ERK2. As shown in Fig. 8A, both ERK1 and ERK2 were phosphorylated on tyrosine residues after treatment with 20 μM 14,15-EET-SI. To determine whether there was a role for the mitogen-activated protein kinase signaling pathway in EET-stimulated [3H]thymidine incorporation, we utilized the mitogen-activated protein kinase kinase inhibitor, PD98059 (23, 24). Pretreatment of the cells with PD98059 dose-dependently inhibited the 14,15-EET-SI-stimulated [3H]thymidine incorporation, with IC50 ~ 5 μM (Fig. 8B). In other experiments, PD98059 also inhibited 11,12-EET-SI-stimulated [3H]thymidine incorporation (not shown).

14,15-EET-SI Activated c-Src Kinase—Further studies were undertaken to determine the mechanism by which 14,15-EET-SI increased tyrosine phosphorylation in LLCPKc4 cells. Immunoblotting with antibodies against the specific pp60c-src substrate, p120, indicated increased tyrosine phosphorylation of...
this protein within 1 min after 14,15-EET-SI addition (Fig. 9A).
In additional experiments, cell lysates were immunoprecipitated with anti-EGFR antibody and immunoblotted with a polyclonal antibody that recognizes the kinase catalytic region highly conserved in c-Src, c-Fyn, and c-Yes members of the Src kinase family. Immunoreactive c-Src, or Src-like kinases, were found to be associated with the EGF receptor within 1 min of 14,15-EET-SI administration to LLCPK14 cells (Fig. 9B). In contrast, there was minimal, if any, association of Src-like kinases with the EGF receptor in the first 5 min after EGF addition (Fig. 9B).

To demonstrate 14,15-EET stimulation of Src-like kinase activity and to further define the identity of the kinase involved, we utilized a specific monoclonal antibody to immunoprecipitate pp60src for an in vitro Src kinase activity assay. Within 1 min, 14,15-EET-SI increased Src kinase activity (Fig. 9C). The stimulated enzymatic activity peaked at 45 min and was elevated for up to 1.5 h, confirming that pp60src kinase was activated in LLCPK14 cells by 14,15-EET-SI.

**DISCUSSION**

Previous studies suggested that cP450 arachidonic acid metabolites were mitogens for both mesenchymal and epithelial cells (4, 5, 11, 25, 26). The mechanisms by which cP450 AA metabolites induced mitogenesis were not explored in these earlier studies. In the present study, we determined that all four regioisomers of EETs induced [³H]thymidine incorporation in LLC-PK14, a nontransformed proximal tubule-like cell line, and that the signaling pathway involved activation of a tyrosine kinase cascade. These mitogenic effects appeared to be relatively specific for EETs in these cells, since no increases in [³H]thymidine incorporation were seen with the parent compound, arachidonic acid, with the hydration product of 14,15-EET, 14,15-DHET, with nonepoxysenase cP450 arachidonate metabolites (12R-HETE, 20-HETE), or with lipoxygenase metabolites (5S-HETE, LTB4, lipoxin A4).

It has been established that prostaglandins and leukotrienes mediate their biologic actions at least in part through specific cell surface G-protein-coupled receptors. On the other hand, little is known about the signaling mechanisms responsible for most of the biological activities, including mitogenic effects, attributed to EETs. There have been reports of a specific binding site for 14,15-EET in monocytes (27, 28) and for 12(R)-HETE in microvessel endothelial cells (29). Studies have also indicated that EETs activate calcium-activated K⁺ channels in vascular smooth muscle cells but have no effect when added directly to the cytoplasmic surface of excised inside-out patches (30). More recent studies have suggested that channel activation requires intermediate signaling steps involving G-proteins (31). The addition of GTPγS to excised patches restored 11,12-EET activation, which was blocked by GDPβS. These studies implicated Gαs in this response (30). Whether EETs interact through a G-protein-coupled receptor or directly activate het-
erotrimeric G-proteins remains to be determined. There is evidence that unsaturated fatty acids, including arachidonic acid, inhibit Gz activation by binding directly to G za; similar effects have been reported for arachidonic acid on the small G-proteins, Ras and Rac (32–34). 14,15-EET has also been reported to stimulate ADP-ribosylation of a yet-to-be characterized 52-kDa protein in rat liver cytosol (35), but to date, no studies have directly investigated the effects of EETs on G-protein activation.

In LLCPKc4 cells, the EET-stimulated tyrosine kinase-signaling cascade activated mitogen-activated protein kinase. Our data suggest that c-Src (pp60c-src) is the cytosolic tyrosine kinase that initiated the tyrosine phosphorylation cascade. pp60c-src and the structurally related members of the Src family

![Fig. 5. A, 14,15–EET-SI induced activation of PI3-kinase in LLCPKc4 cells. Quiescent LLCPKc4 cells were treated with vehicle (Me2SO) alone (lanes 1–2), 20 μM 14,15-EET-SI (lanes 3–6), or 100 nM EGF (lanes 7 and 8) for 1 min (lanes 1, 3, and 7), 5 min (lanes 2, 4, and 8), 15 min (lane 5), or 30 min (lane 6), and tyrosine-phosphorylated proteins were immunoprecipitated with polyclonal anti-phosphotyrosine antibodies and immunoblotted with a monoclonal anti-PI3-kinase antibody. B and C, effect of PI3-kinase inhibition on DNA synthesis. PI3-kinase inhibitors, wortmannin (B) or LY294002 (C), inhibited 14,15-EET-SI-stimulated [3H]thymidine incorporation in LLCPKc4 cells. I.P., immunoprecipitation; I.B., immunoblotting.](image)

![Fig. 6. A, 14,15-EET-SI induced tyrosine phosphorylation of EGF receptors in LLCPKc4 cells. Lanes and timing are as in Fig. 5A. Cell lysates were immunoprecipitated with a polyclonal anti-PY antibody and immunoblotted with a monoclonal anti-EGFR antibody. B, 14,15-EET-SI induced association of PI3-kinase with EGF receptors. The lanes and timing are identical to A. Cell lysates were immunoprecipitated with a polyclonal anti-PI3-kinase antibody and immunoblotted with monoclonal anti-EGFR antibody. I.P., immunoprecipitation; I.B., immunoblotting.](image)

![Fig. 7. A, 14,15-EET-SI induced tyrosine phosphorylation of SHC in LLCPKc4 cells. Lanes and timing are as in Fig. 5A. Cell lysates were immunoprecipitated with a polyclonal anti-PY antibody and immunoblotted with a monoclonal anti-SHC antibody. B, 14,15-EET-SI induced association of GRB2 with SHC. Lanes and timing are as in A except that lane 9 is PC12 lysate as a positive control. Cell lysates were immunoprecipitated with a polyclonal anti-SHC antibody and immunoblotted with a monoclonal anti-GRB2 antibody. C, 14,15–EET-SI induced association of SHC with EGF receptor. Lanes and timing are as in A. Cell lysates were immunoprecipitated with a polyclonal anti-SHC antibody and immunoblotted with a monoclonal anti-EGFR antibody. I.P., immunoprecipitation; I.B., immunoblotting.](image)
An increasing number of intracellular signaling pathways and cellular responses have been shown to be dependent upon Src activation, including morphological changes and cell proliferation (36). Recent studies have found that seven-transmembrane receptors, which signal through G-protein activation, mediate Ras-dependent activation of mitogen-activated protein kinase by activation of c-Src or Src-like kinases, phosphorylation of the SHC adapter protein, SHC-GRB2 complex formation, and recruitment of Ras guanine nucleotide exchange factor activity (SOS) (37–39), which then leads to activation of mitogen-activated protein kinase signaling pathways. Agonists known to utilize this pathway include, among others, angiotensin II, catecholamines, thrombin, and endothelin (40–43). The mechanism of c-Src activation has still not been clarified; for Gi-activating receptors, it appears that G
\[\alpha_{\text{bg}}\] subunits are involved (38), whereas for other receptors, G
\[\alpha_{\text{on}}/G_{\text{b,11}}\] may be involved (44). These G-protein receptor-mediated tyrosine-phosphorylated proteins utilize growth factor receptors such as EGFRs and platelet-derived growth factor receptors as “scaffolds” (38, 45); unlike the sequence of events occurring when EGFRs and platelet-derived growth factor receptors bind their ligands, these receptors are not activated by autophosphorylation in this process but are tyrosine-phosphorylated by Src-like kinases. Src, GRB2, and SOS may be associated via interactions with SHC, which interacts with SH2 domains of the receptor. It is therefore of interest that the present studies indicate that 14,15-EET-SI also activated Src kinase and initiated a tyrosine phosphorylation cascade that utilized the EGF receptor as a scaffold and resulted in mitogen-activated protein kinase activation.
Although the role of this tyrosine kinase cascade in stimulation of mitogenesis is based in large part on the use of inhibitors, at the concentrations we employed genistein is a potent and highly tyrosine-specific protein kinase inhibitor, with minimal inhibition of serine/threonine-specific protein kinases (57). Similarly, at the concentrations utilized in the present studies, wortmannin and LY294002 have been shown to be highly specific for PI3-kinase (21, 22), and PD98059 is highly specific for mitogen-activated protein kinase kinase (23, 24).

In summary, these results demonstrate that epoxycyclostearic acid are potent mitogens for renal epithelial cells and indicate that the mitogenic effects are mediated by activation of Src kinase and initiation of a tyrosine kinase phosphorylation cascade. This is the first evidence that these lipid mediators can signal through tyrosine kinase activation; further studies will be required to elucidate the role of the EETs in agonist-mediated activation of tyrosine phosphorylation cascades.

REFERENCES

1. Endou, H. C. P. (1983) Jpn. J. Pharmacol. 33, 423–433
2. Bonvelet, J.-P., Pradelles, P., and Farman, N. (1987) Am. J. Physiol. 253, F377–F387
3. Takahashi, K., Capdevila, J., Karara, A., Falck, J. R., Jacobson, H. R., and Shiina, K. (1990) J. Biol. Chem. 265, 10207–10211
4. Harris, E. C., Homma, T., Jacobson, H. R., and Capdevila, J. (1990) J. Cell. Physiol. 144, 429–443
5. Sellamyer, A., Uedelhoven, W. M., Weber, P. C., and Bonventre, J. V. (1991) J. Biol. Chem. 266, 3860–3870
6. Campbell, W. B., Gebremedhin, D., Pratt, P. F., and Harder, D. R. (1996) Circ. Res. 78, 415–423
7. Fukao, M., Hatatori, Y., Kanoe, M., Sakuma, I., and Kitabatake, A. (1997) Br. J. Pharmac. 120, 439–446
8. Van de Voorde, J., and Vanheyl, B. (1997) J. Cardiovasc. Pharmacol. 29, 827–832
9. Escalante, B. A., Staupfinder, R., Schwartzman, M., and Abraham, N. (1995) Adv. Prostaglandin, Thromboxane, Leukotriene Res. 23, 207–209
10. Staupfinder, R., Escalante, B., Schwartzman, M., and Abraham, N. G. (1994) J. Cell. Physiol. 160, 69–74
11. Burns, K. D., Capdevila, J., Wei, S., Breyer, M. D., Homma, T., and Harris, R. C. (1995) Am. J. Physiol. 269, C815–C840
12. Omata, K., Abraham, N. G., and Schwartzman, M. L. (1992) Am. J. Physiol. 262, F951–F959
13. Madhun, Z. T., Goldthwait, D. A., McKay, D., Hopfer, U., and Douglas, J. G. (1993) J. Clin. Invest. 88, 456–461
14. Romero, M. F., Madhun, Z. T., Hopfer, U., and Douglas, J. G. (1991) Adv. Prostaglandin, Thromboxane, Leukotriene Res. 21, 205–208
15. Houldier, P., Chambery, R., Achard, J. M., Freissart, M., Poggioli, J., and Paillard, M. (1996) Kidney Int. 50, 1496–1505
16. Falck, J. R., Yadagiri, P., and Capdevila, J. (1990) Methods Enzymol. 187, 385–394
17. Amsler, K., and Cook, J. S. (1982) Am. J. Physiol. 242, C94–C101
18. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
19. Hidaka, H., Inagake, M., Kawamoto, S., and Sasaki, Y. (1984) Biochemistry 23, 5036–5041
20. Hidaka, H., and Hagiwara M. (1997) Trends Pharmacol. Sci. 8, 162–164
21. Straub, S. G., and Sharp, G. W. G. (1996) J. Biol. Chem. 271, 1660–1668
22. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, F. B. (1994) J. Biol. Chem. 269, 5241–5248
23. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7686–7688
24. Waters, S. B., Holt, K. H., Ross, S. E., Suy, L. J., Guan, K. L., Saltiel, A. R., Koretzky, G. A., and Pessin, J. E. (1995) J. Biol. Chem. 270, 26883–26886
25. Sheu, H. L., Omata, K., Utsumi, Y., Tsurumi, E., Sato, T., Shimizu, T., and Abe, K. (1995) Adv. Prostaglandin, Thromboxane, Leukotriene Res. 23, 211–213
26. Lin, F., Rios, A., Falck, J. R., Belosludtsev, Y., and Schwartzman, M. L. (1995) Am. J. Physiol. 269, F806–F816
27. Wong, P. Y., Lin, K. T., Yan, Y. T., Ahern, D., Hee, J., Shen, S. Y., Bhatt, R. K., and Falck, J. R. (1995) J. Lipid Mediators 6, 199–208
28. Wong, P. Y. K., Lai, P. S., Shen, S. Y., Belosludtsev, Y. Y., and Falck, J. R. (1997) J. Lipid Med. Cell Signal. 16, 155–169
29. Stoltz, B. A., and Schwartzman, M. L. (1997) J. Ocul. Pharmacol. Ther. 13, 191–199
30. Hu, S., and Kim, H. S. (1993) Eur. J. Pharmacol. 230, 215–221
31. Li, P. L., and Campbell, W. B. (1997) Circ. Res. 80, 871–884
32. Chuang, T. H., Bohl, B. P., and Bokoch, G. M. (1993) J. Biol. Chem. 268, 26206–26211
33. Sawai, T., Asada, M., Numoi, H., Matsuda, I., Sano, S., Tsukahira, K., Takai, Y., and Katayama, K. (1995) Biochem. Biophys. Res. Commun. 195, 264–269
34. Sermon, B. A., Eccleston, J. F., Skinner, R. H., and Lowe, P. N. (1996) J. Biol. Chem. 271, 1566–1572