Reactive Lipid Species from Cyclooxygenase-2 Inactivate Tumor Suppressor LKB1/STK11

CYCLOPENTENONE PROSTAGLANDINS AND 4-HYDROXY-2-NONENAL COVALENTLY MODIFY AND INHIBIT THE AMP-KINASE KINASE THAT MODULATES CELLULAR ENERGY HOMEOSTASIS AND PROTEIN TRANSLATION*

Received for publication, September 2, 2005, and in revised form, November 14, 2005 Published, JBC Papers in Press, November 25, 2005, DOI 10.1074/jbc.M509723200

Tracy M. Wagner1, James E. Mullally, and F. A. Fitzpatrick2
From the Department of Medicinal Chemistry, University of Utah, Huntsman Cancer Institute, Salt Lake City, Utah 84112

LKB1, a unique serine/threonine kinase tumor suppressor, modulates anabolic and catabolic homeostasis, cell proliferation, and organ polarity. Chemically reactive lipids, e.g. cyclopentenone prostaglandins, formed a covalent adduct with LKB1 in MCF-7 and RKO cells. Site-directed mutagenesis implicated Cys210 in the LKB1 activation loop as the residue modified. Notably, ERK, JNK, and AKT serine/threonine kinases with leucine or methionine, instead of cysteine, in their activation loop did not form a covalent lipid adduct. 4-Hydroxy-2-nonenal, 4-oxo-2-nonenal, and cyclopentenone prostaglandin A and J, which all contain α,β-unsaturated carbonyls, inhibited the AMP-kinase kinase activity of cellular LKB1. In turn, this attenuated signals throughout the LKB1 → AMP kinase pathway and disrupted its restraint of ribosomal S6 kinases. The electrophilic β-carbon in these lipids appears to be critical for inhibition because unreactive lipids, e.g. PGB1, PGE2, PGF2α, and TxB2, did not inhibit LKB1 activity (p > 0.05). Ectopic expression of cyclooxygenase-2 and endogenous biosynthesis of eicosanoids also inhibited LKB1 activity in MCF-7 cells. Our results suggested a molecular mechanism whereby chronic inflammation or oxidative stress may confer risk for hypertrophic or neoplastic diseases. Moreover, chemical inactivation of LKB1 may interfere with its physiological antagonism of signals from growth factors, insulin, and oncogenes.

The classic model of tumor suppressors as recessive genes stipulates that biallelic inactivation is necessary for tumorigenesis (1–3). This model fits Rb1, adenomatous polyposis coli, and p53 in many familial and sporadic cancers (1, 4). Paradoxically, tumors often retain one functional allele of some tumor suppressor genes, e.g. 27Kip1 (5), phosphatase tensin homolog (6), LKB1, an AMP-kinase kinase and tumor suppressor, is a unique link to the AMP kinase pathway (Fig. 1). LKB1, an AMP-kinase kinase and tumor suppressor, is a unique link to the AMP kinase pathway (Fig. 1).

2 Holds the Dee Glenn and Ida W. Smith Chair for Cancer Research. To whom correspondence should be addressed: Dee Glenn and Ida W. Smith Chair of Cancer Research, Huntsman Cancer Institute, 2000 Circle of Hope, University of Utah, Salt Lake City, UT 84112-5550. Tel.: 801-581-6204; Fax: 801-585-0011; E-mail: frank.fitzpatrick@hci.utah.edu.

1 Supported by a predoctoral fellowship from the American Foundation for Pharmaceutical Education.

* This work was supported in part by United States Public Health Services Grants R01 AI26730 and PO1 CA73992 and the Huntsman Cancer Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

EXPERIMENTAL PROCEDURES

Materials—Supplies used were Dulbecco’s modified Eagle’s medium and supplements (Invitrogen); bovine insulin and gentamicin (Invitrogen); PG (Cayman Chemicals); 4-HNE and 4-ONE (Cayman Chemicals); 5-aminomimidazole-4-carboxamide ribonucleoside (AICAR) (Toronto Research Chemicals Inc); Complete™ protease inhibitor mixture and FuGENE 6 transfection reagent (Roche Applied Science); polyclonal antibodies directed against LKB1, phospho-Thr172-AMPKα, AMPKα, phospho-Scr79-ACC, ACC, phospho-Thr389-S6K, S6K, ERK, JNK, AKT, IKKα, IKKγ and COX-2 (Cell Signaling Technologies); rapamycin (Cell Signaling Technologies); horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology); PVDF membranes and Western Lighting™ chemiluminescence reagents (PerkinElmer Life Sciences); neutradvin-conjugated beads (Pierce); hemagglutinin epitope-tagged constructs for LKB1 (gift from Dr. Tomi Makela, University of Helsinki, Finland); and a QuikChange™ site-directed mutagenesis kit (Stratagene).

Cell Culture—MCF-7 breast cancer cells (ATCC) were maintained in minimal essential medium at 37 °C in a humidified incubator with 5% CO2. The medium was supplemented with 2 mM-l-glutamine, 1.5 g/liter sodium bicarbonate, 0.1 mM nonessential amino acids, 1 mM sodium bicarbonate.
pyruvate, 0.01 mg/ml bovine insulin, 0.01 mg/ml gentamicin, and 10% fetal bovine serum. RKO colon cancer cells were maintained in minimal essential medium supplemented with 1.5 g/liter sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 0.01 mg/ml gentamicin, and 10% fetal bovine serum. In certain experiments, cells were incubated in serum-depleted medium containing 1% fetal bovine serum for 6 h prior to treatments described below.

**Isolation of Kinases Covalently Labeled by PG-Amidopentylbiotin**—MCF-7 and RKO cells were treated with 10–60 μM PG-A1-amidopentylbiotin or H926212-PGJ2-amidopentylbiotin for 4 h. The cells were lysed in 250 mM sucrose, 50 mM Tris, pH 7.4, 5 mM MgCl₂, 1 mM EGTA, 1× Complete™ protease inhibitor, 2 mM sodium fluoride, and 2 mM sodium orthovanadate. The lysates were sonicated 10 for 1 s at 4 °C. After centrifugation at 10,000 g for 10 min, samples containing 100 μg of protein from total cell lysates were incubated with 100 μl of neutravidin beads in 1 ml of phosphate-buffered saline with 0.4% Tween 20 for 16 h at 4 °C. The samples were then centrifuged at 500 g for 5 min to isolate neutravidin-biotin complexes (NA pulldown). The beads were washed three times with 1 ml of phosphate-buffered saline, 0.4% Tween 20. The samples were dissolved in 50 μl of Laemmli loading buffer, 0.5% β-mercaptoethanol and heated at 95 °C for 10 min. Protein samples (15 μg) were fractionated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% w/v nonfat dry milk in TBS-T, then incubated for 12 h at 4 °C with primary antibodies directed against LKB1 (1:1000), IKKα (1:1000), IKKγ (1:1000), JNK (1:1000), ERK (1:1000), and AKT (1:1000), followed by horseradish peroxidase-conjugated, goat anti-rabbit secondary antibody (1:4000). Antigen-antibody complexes were detected with Western Lighting™ ECL reagents.

**Transfection**—LKB1-HA or LKB1 (C210S)-HA was transfected into RKO cells using 1 μg/ml DNA, 3 μl Lipofectamine2000™ for 20 h following the manufacturer’s protocol. COX-2 was transfected into MCF-7 cells using 1 μg/ml DNA, 3 μl Lipofectamine2000™ for 24 h following the manufacturer’s protocol. Transfection efficiency was measured by immunochemical determination of COX-2 protein in cell lysates. Samples were lysed and fractionated, as above, and membranes were incubated for 12 h at 4 °C with primary antibodies directed against COX-2 (1:1000).

**Site-directed Mutagenesis**—A C210S LKB1 mutant was constructed using a QuikChange™ site-directed mutagenesis kit following the manufacturer’s protocol. Residue 210 was converted from a Cys to a Ser by a TGC to a TCC substitution. The identity of the product was confirmed by DNA sequencing.

**AMPK, ACC, and S6K Phosphorylation Assays by Western Blot**—MCF-7 cells were treated with 0–60 μM of the designated PGs, Tx, 4-HNE, or 4-ONE for 4 h unless otherwise stated. Following this incubation, 2 mM AICAR (27), an AMP mimetic, was added to cells for 30 min. In certain experiments cells were treated with 60 μM PG for 4 h, and 50 nM rapamycin for 2 h prior to AICAR treatment. The cells were lysed as described above; 15 μg of protein was fractionated by SDS-PAGE, and proteins were transferred to PVDF membranes. The membranes were probed with primary antibodies directed against phospho-Thr172-AMPKα, phospho-Ser79-ACC FIGURE 1. LKB1 signaling pathway. LKB1 (STK11) functions as an AMP-kinase kinase in cells. Activity of LKB1 is limited in cells with adequate ATP. Accumulation of AMP, or addition of its mimetic AICAR, activates the AMP-kinase kinase activity of LKB1, which converts AMPKα → phospho-Thr172 AMPKα. In turn, phospho-Thr172 AMPKα converts ACC → phospho-Ser79 acetyl-CoA carboxylase. Phospho-Thr172 AMPKα also phosphorylates TSC1/2, which inhibits mTOR-mediated conversion of S6K → phospho-Thr389 S6K. Cells sense changes in their AMP/ATP ratio and use the LKB1→AMPKα kinase cascade to maintain proper anabolic ↔ catabolic homeostasis. LKB1 has a dual role as a tumor suppressor and metabolic regulator by antagonizing the phosphatidylinositol 3-kinase-AKT pathway, which propagates anabolic signals from insulin and proliferation signals from growth factors/oncogenes.
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**RESULTS**

Covalent Modification of Cellular LKB1 by Electrophilic Lipids—Cyclopentenone PG and 4-HNE react covalently with IKK (16, 17). Thus, these lipids may modify other serine/threonine kinases with homology to the Cys-residue in IKK. LKB1, a unique serine/threonine kinase tumor suppressor, has a nucleophilic Cys that aligns with the Cys-residue in the activation loop of IKKα and IKKβ. In contrast, ERK, JNK, p38, and AKT kinases have Met or Leu residues in the corresponding position (Fig. 2A). To determine whether electrophilic PGs could react directly with LKB1 and other kinases, we used PGA1 amidopentylbiotin (PGA1-APB). This PG analog has the characteristic.

Site-directed mutagenesis demonstrated that Cys210 in the activation loop of LKB1 is required for lipid adduct formation. RKO cells express negligible amounts of LKB1; therefore, we transfected them with plasmids encoding LKB1 or the corresponding C210S mutant. PGA1-amidopentylbiotin formed a covalent adduct with wild type LKB1 protein but not with the LKB1-C210S mutant protein (Fig. 3A). Adduct formation with LKB1 was not dependent on ectopic overexpression of the LKB1 protein. PGA1-amidopentylbiotin formed adducts with wild type LKB1 expressed physiologically in MCF-7 cells (Fig. 3B). Additionally, adduct formation is not unique to PGA1, as both PGA1-amidopentylbiotin and Δ12-PGJ2-amidopentylbiotin formed adducts with LKB1 in MCF7 cells (Fig. 3B).

**Inhibition of Cellular LKB1 Serine/Threonine Kinase Activity by Reactive Lipid Species**—The AMP-kinase kinase activity of LKB1 can be determined by measuring the cellular conversion of AMPKα into phospho-Thr172-AMPK. Basal levels of phospho-Thr172-AMPKα were low but detectable in MCF-7 cells grown in 10% FCS (Fig. 4A, lane 1). Phospho-Thr172-AMPKα levels rose in cells incubated with the AMP mimetic, AICAR (Fig. 4A, lane 3), and in cells placed in 1% FCS for 6 h (Fig. 4A, lane 5). PGA1 inhibited the formation of phospho-Thr172-AMPKα by these stimuli (Fig. 4A, lanes 2, 4, and 6). Several different reactive lipid species that have electrophilic β-carbons, including 4-HNE, 4-ONE, 15-deoxy-Δ12, Δ14-PGJ2, Δ12-PGJ2, and PGA1, inhibited the AMP-kinase kinase activity of LKB1 in MCF-7 cells stimulated with AICAR (Fig. 4B). Inhibition was concentration-dependent (Fig. 4C) with IC50 (half-maximal inhibition, mean ± S.E.) of 26.8 ± 4.3 μM PGA1 (n = 10), 12 ± 2.0 μM Δ12-PGJ2 (n = 8), 3.0 ± 1.4 μM 4-HNE (n = 4), and 2.5 ± 1.0 μM 4-ONE (n = 3). The electrophilic β-carbon appeared to be critical for inhibition, because unreactive lipids, including cyclopentenone PGB1, PGE2, PGF2α, 15-keto-PGF2α, and TbX4, at concentrations of 60–100 μM did not inhibit LKB1 activity (Fig. 4B).

**Inhibition of the LKB1-AMPKα-mTOR-S6 Kinase Signaling Pathway by Reactive Lipid Species**—Inhibition of LKB1 by reactive lipids affected proximal and distal components in its signaling pathway downstream from AMPKα. For instance, PGA1, Δ12-PGJ2, and 4-HNE inhibited the phosphorylation of ACC, a substrate for phospho-Thr172-AMPKα, whereas PGB1 did not (Fig. 5A). Likewise, signaling through the S6 kinase pathway was affected. The basal level of phospho-Thr429-S6 kinase was readily detectable in MCF-7 cells, which must be grown in

(1:1000), phospho-Thr389-p70 S6K (1:1000), and total p70 S6K (1:1000), followed by horseradish peroxidase-conjugated, goat anti-rabbit secondary antibody (1:5000). Protein bands were detected with Western Blotting. The bands were analyzed using a Kodak Image Station 440TM, and the net band intensity was converted to a percentage of the control. Experiments were repeated 3–10 times, and data depict the mean ± S.E.

Covalent modification of cellular kinases by PGA1-amidopentylbiotin; comparison of LKB1 with other serine/threonine kinases. A shows the amino acid sequence alignment of the activation loop regions of JNK, ERK, p38, AKT, IKK, and LKB1 kinases. Cys179 in IKK corresponds with Cys210 in LKB1 (box and arrow). B shows immunoblots of total ERK, JNK, AKT, IKK, and LKB1 kinases in whole cell lysates (WC) from MCF-7 cells treated 4 h at 37°C with 20 μM PGA1, (WC, lane 1), 20 μM PGA1-amidopentylbiotin (WC, lane 2), or 20 μM aminopentylbiotin (WC, lane 3). Whole cell lysates were incubated with neutravidin immobilized on agarose to sequester kinases with a biotin epitope, introduced de novo from their covalent reaction with PGA1-amidopentylbiotin. The immunoblots of kinases sequenced on neutralin-agarose beads (NA pulldown) shows that only IKK and LKB1 contained a biotin epitope after incubation with PGA1-APB (NA pulldown, lane 2). None of the kinases contained a biotin epitope after incubation with PGA1 (NA pulldown, lane 1) or aminopentylbiotin (NA pulldown, lane 3), as controls.

**Prostaglandin E2 and D3 Formation**—Enzyme immunoassays were performed to assess PGF2α and PGD2 metabolite formation. COX-2 was transfected into MCF-7 cells using 1 μg/μl DNA, 3 μl of Lipofectamine 2000TM for 24 h following the manufacturer’s protocol. Cells were treated with 10 μM COX-2 inhibitor NS-398 (Cayman Chemical) or vehicle control for 1 h followed by treatment with 100 μM of arachidonic acid for 4 h. PGE2 Express EIA kit and PGD2-MOX Express kits (Cayman Chemical) were used following the manufacturer’s protocol. Medium from cell culture was sampled and analyzed at 30 min. Experiments were repeated three times, and results depict the mean ± S.E.

**Statistics**—Statistical significance was assessed by analysis of variance with Bonferroni’s or Newman-Keul’s post-hoc test for comparisons among groups.

**FIGURE 2. Covalent modification of cellular kinases by PGA1-amidopentylbiotin; comparison of LKB1 with other serine/threonine kinases.** A shows the amino acid sequence alignment of the activation loop regions of JNK, ERK, p38, AKT, IKK, and LKB1 kinases. Cys179 in IKK corresponds with Cys210 by LKB1 (box and arrow). B shows immunoblots of total ERK, JNK, AKT, IKK, and LKB1 kinases in whole cell lysates (WC) from MCF-7 cells treated 4 h at 37°C with 20 μM PGA1, (WC, lane 1), 20 μM PGA1-amidopentylbiotin (WC, lane 2), or 20 μM aminopentylbiotin (WC, lane 3). Whole cell lysates were incubated with neutravidin immobilized on agarose to sequester kinases with a biotin epitope, introduced de novo from their covalent reaction with PGA1-amidopentylbiotin. The immunoblots of kinases sequenced on neutralin-agarose beads (NA pulldown) shows that only IKK and LKB1 contained a biotin epitope after incubation with PGA1-APB (NA pulldown, lane 2). None of the kinases contained a biotin epitope after incubation with PGA1 (NA pulldown, lane 1) or aminopentylbiotin (NA pulldown, lane 3), as controls.
media containing 0.01 mg/ml insulin (Fig. 5, B and C, lane 1). Activation of the cellular LKB1-AMPKα pathway with 2 mM AICAR reduced the level of phospho-Thr<sup>389</sup>-S6K, the active form of S6K (Fig. 5, B and C, lane 3). Δ12-PGJ<sub>2</sub>-I<sub>b</sub>, alone, enhanced S6K activity in MCF-7 cells (Fig. 5, B and C, lane 2 versus lane 1). Δ12-PGJ<sub>2</sub> antagonized the effect of AICAR on cellular LKB1 activity and restored the content of phospho-Thr<sup>389</sup>-S6K to the control, tonic level (Fig. 5, B and C, lane 4 versus lane 3 and lane 1). Finally, rapamycin, which interacts directly with mTOR, overrode the inactivation of LKB1 by Δ12-PGJ<sub>2</sub> and blocked phosphorylation of S6K (Fig. 5, B and C, lanes 5 and 6). Thus, by inactivating cellular LKB1 at the apex of the AMPKα pathway, Δ12-PGJ<sub>2</sub> enhanced the distal formation of anabolically active phospho-Thr<sup>389</sup>-S6K, consistent with the scheme shown in Fig. 1.

**Inhibition of LKB1 Activity by Ectopic Expression of Cyclooxygenase-2—**

COX-2 expression and activity are increased in inflammation and in tumors (25, 26). This exposes cells to autocoid mediators and related, reactive lipid species. To extend our investigation, we tested whether or not transfection of MCF7 cells with a plasmid encoding COX-2 would inhibit LKB1 kinase activity by enabling biosynthesis of endogenous eicosanoids. Ectopic expression of COX-2 in the presence of its substrate, arachidonic acid, inhibited the kinase activity of LKB1. LKB1 activity, measured as phospho-Thr<sup>172</sup>-AMPKα formation, was designated 100% in mock-transfected MCF-7 cells stimulated with 2 mM AICAR (Fig. 6A). LKB1 activity remained comparable with control in MCF-7 cells transfected with COX-2 and stimulated with 2 mM AICAR. The phospho-Thr<sup>172</sup>-AMPKα content fell (p < 0.01) in MCF-7 cells transfected with COX-2 and supplemented with 100 μM arachidonic acid (Fig. 6B, lane 5). In mock-transfected MCF-7 cells incubated with 100 μM arachidonic acid, the phospho-Thr<sup>172</sup>-AMPKα levels were indistinguishable from the control (p > 0.05) (Fig. 6A).

Ibuprofen, a known inhibitor of COX-2, restored the kinase activity of LKB1 demonstrated by phospho-Thr<sup>172</sup>-AMPKα content (Fig. 6B, lane 6). These data are consistent with the inhibition of LKB1 by endogenously generated reactive lipid species. We verified that MCF-7 cells transfected with COX-2 made more PGE<sub>2</sub> and PGD<sub>2</sub> than mock-transfected cells when incubated with 100 μM arachidonic acid (Table 1). NS-398, a specific COX-2 inhibitor, lowered PGE<sub>2</sub> and PGD<sub>2</sub> formation by cells transfected with COX-2 but not mock-transfected cells. MCF-7 cells constitutively
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FIGURE 5. Electrophilic lipids inhibit phosphorylation of acetyl-CoA carboxylase and S6 kinase downstream from their inhibition of LKB1 → AMP kinase. A, immunoblot of total ACC and phospho-Ser79-ACC in lysates from MCF-7 cells incubated with 60 µM 12-PGJ2, 4-HNE, or Me2SO (0.1%) vehicle control for 4 h and then with 2 mM AICAR for 30 min to stimulate the LKB1 → AMPKα signaling pathway and subsequent ACC phosphorylation. Representative lipids that inhibited LKB1, at the apex of the signaling pathway, also attenuated the phosphorylation of ACC, as determined by Western blotting. Values are mean ± S.E., n = 3.

DISCUSSION

Recently, LKB1 was identified as the gene responsible for Peutz-Jeghers syndrome (18, 19), which predisposes to tumors of the digestive tract, reproductive organs, and breast. Cancer incidence in Peutz-Jeghers syndrome is 5–18-fold greater than in the general population (29), and it is the only familial cancer syndrome attributed to loss-of-function mutations in a serine/threonine kinase. The cellular substrates and biological roles of LKB1 were unknown until 2003–2004, when investigators discovered that it phosphorylated AMPK and functioned as a unique AMP-kinase kinase (20–24).

LKB1 has two vital cellular roles as follows: tumor suppressor and regulator of anabolic/catabolic homeostasis. LKB1 achieves its purpose by negatively regulating the phosphatidylinositol 3-kinase-AKT pathway (Fig. 1). When activated by their respective stimuli, LKB1 and AKT send opposing signals that determine the ratio of the active/inactive forms of TSC1/2, mTOR, and ribosomal S6K. This, in turn, balances anabolic and catabolic processes in order to maintain cellular energy homeostasis (ATP levels) (23). Our data show that exogenous electrophilic lipids, as well as endogenous catalysis by cellular COX-2, can inhibit LKB1 activity and shift the equilibrium in the pathway toward phosphorylation and activation of ribosomal S6K. If this should occur when cells have too little ATP to support proper translation of RNA into proteins, it might facilitate tumor progression. Dysregulation of protein translation is important in several cancers (30–32). Low expression of LKB1 in breast tumors is associated with poor prognosis and survival (33) and with the transition from pre-malignant to malignant tumor growth in lung cancer (34). Most interestingly, several studies show that hamartomas overexpress COX-2 (35, 36), and its expression facilitates tumorigenesis via a hamartoma-adenoma-carcinoma sequence (37, 38). Although speculative at this point, our results suggest that inhibition of LKB1 because of its chemical inactivation by reactive lipid species or overexpression of COX-2, as depicted in Fig. 6, may have consequences similar to loss of expression of LKB1 or mutational inactivation.

LKB1 is a tumor suppressor that often retains one wild type allele, deviating from Knudson’s two-hit hypothesis. This suggests that LKB1 is a potential candidate for inactivating processes that are distinct from genetic or epigenetic lesions. Furthermore, we reasoned that LKB1 would be covalently modified and inactivated by cyclopentenone PG
Likewise, a particular electrophilic lipid, or overexpression of COX-2, the biological milieu may contain multiple serine/threonine kinases do not react indiscriminately with PGA1-ami-
cal effect. 4-HNE, cyclopentenone PGs, and 8-iso-cyclopentenone PGs, various ketone will contribute, stoichiometrically, to any pathophysiolog-
i.e., multiple lipids with

Acknowledgment—We thank Dr. Tomi Makela for providing the plasmids encoding LKB1.

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