Phosphorylation of LKB1 at Serine 428 by Protein Kinase C-ζ Is Required for Metformin-Enhanced Activation of the AMP-Activated Protein Kinase in Endothelial Cells

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Supplemental Materials

Human umbilical vein endothelial cells (HUVEC) were obtained from Cascade biologics (Portland, OR). Bovine aortic endothelial cells (BAEC) and cell culture media were obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). HeLa-S3 and A549 cells were from ATCC (Manassas, VA). The SAMS peptide, PKC-ζ peptides, LKB1-specific peptide (LKB1tide) and human LKB1 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies against phospho-AMPK (Thr172), AMPKα, phospho-LKB1 (Ser428), phospho -PKC-ζ (Thr410/403), catalytic subunit of PKA (PKAc), phospho-PKAc (Thr197), RSK1/RSK2/RSK3, and phospho-RSK3 (Thr356/Ser360) were obtained from Cell Signaling Inc. (Beverly, MA). Antibodies against PKC-ζ and LKB1 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and antibodies against AMPKα1, AMPKα2 and H2AX were from Bethyl Laboratories, Inc. (Montgomery, TX). Anti-human alkaline phosphatase/ALPL antibody was from R&D Systems (Minneapolis, MN). Anti-lactate dehydrogenase antibody was

from Rockland Inc. (Gilbertsville, PA). Protein A Sepharose™ CL-48 was from Amersham Biosciences (Uppsala, Sweden). Cell permeable myristoylated PKC-ζ pseudosubstrate (PS) was from Biosource International (Camarillo, CA). Other chemicals and organic solvents of the highest grade were obtained from Sigma.

Methods

Immunoprecipitation and Western Blotting: The proteins were immunoprecipitated with specific antibodies and Western blotted onto nitrocellulose membranes, and detected by specific antibodies, as described previously[1]. The intensity (area x density) of the individual bands on Western blots was measured by densitometry (model GS-700, Imaging Densitometer; Bio-Rad). The background was subtracted from the calculated area.

LKB1 Activity Assay: LKB1 was immunoprecipitated from non-treated (control) or treated cells with an antibody against LKB1 (Santa Cruz, catalogue number Sc-5638, D-19) overnight at 4^{o}C in the presence of protein A-G agarose. LKB1 activity present in the immunoprecipitates was determined by its ability to activate recombinant AMPK as described previously. Briefly, LKB1 immunoprecipitates were incubated with recombinant AMPK (α1β1γ1, 20 μg/ml) prepared as described previously [4, 5] in reaction buffer for 30 min at 37^{o}C. Following brief centrifugation, LKB1 activity in the supernatants was measured using the AMPK-dependent ^{32}P incorporation into SAMS peptide assay as described above[6]. LKB1-MO25α-STRADα complexes were bacterially

expressed and purified up to the Heparin-ste[7]. The LKB1 S431A mutant was constructed by site-directed mutagenesis and sequence was verified by DNA sequencing.

AMPK Activity Assay: Total AMPK was immunoprecipitated from 500 μg protein using an antibody against AMPKα, and AMPK activity was assessed by determining the incorporation of ^{32}P into the synthetic SAMS peptide as described[2, 8].

Protein Kinase C-ζ activity: PKC-ζ was first immunoprecipitated by PKC-ζ-specific antibody and PKC-ζ activity was assayed by PKC-ζ-specific peptides, as described previously [9].

Immunocytochemical staining of LKB1: The HUVEC and A549 cells transfected with LKB1 wild type and mutated plasmids were cultured on coverslips and then fixed with 4% paraformaldehyde. After blocking, the HUVEC were incubated with a goat anti-LKB1 antibody (Santa Cruz, Biotechnology, INC., Santa Cruz, CA) overnight. Since all tested commercially available antibodies against LKB1 did not work in A549 cells and LKB1 plasmids encoded an N-terminal His Tag, the LKB1 in A549 were detected by using a mouse anti-His Tag antibody (Upstate Cell Signaling Solutions, Temecula, CA). After three washes, the slides were incubated with a FITC-conjugated donkey anti-rabbit and a FITC-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch Lab, Inc., West Grove, PA), respectively, at a dilution of 1:150 for 1h. The slides were then rinsed, counter-stained with 4'-6-diamidino-2-phenylindole (DAPI), mounted in

Vectashield™ mounting media (Vector Laboratories, Burlingame, CA), and viewed on a SLM 510 laser scanning confocal microscope (Carl Zeiss Meditec, Inc., Jena, Germany).

Preparation of sub-cellular fractions: Cellular cytosolic, membrane and nuclear fractions were prepared as described previously[10,11].

Statistical Analysis: Values are expressed as mean ± S.E.. All of the data were analyzed with a one-way analysis of variance (ANOVA) followed by Bonferroni post hoc analyses, except for those obtained from the time-course, which were analyzed with repeated-measeures ANOVA. A p value of < 0.05 is considered statistically significant.

References

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