Material and Methods

Animal Procedures: Mice heterozygous for IKKα were on the C57BL/6J background [1]. Recipient LDLR$^{-/-}$ (on C57BL/6 background) and C57BL/6 mice were purchased from the Jackson Laboratories. Mice were maintained in microisolator cages on a rodent chow diet containing 4.5% fat (PMI 5010, St. Louis, MO) or a Western type diet containing 21% milk fat and 0.15% cholesterol (Teklad, Madison, WI). Animal care and experimental procedures were performed according to the regulations of Vanderbilt University's Institutional Animal Care and Usage Committee.

Fetal liver cell (FLC) transplantation: FLC were isolated on day 14-16 of gestation and the gender of the embryos were determined by PCR as described [2]. Recipient LDLR$^{-/-}$ or C57BL6 mice were lethally irradiated (9Gy) and transplanted (4x10^6) with FLCs as described[3].

Peritoneal macrophage isolation and reagents. Thioglycollate-elicited peritoneal macrophages were isolated. Two days later, macrophages were incubated with serum-free media and treated with recombinant mouse EGF (R&D Systems), or recombinant human platelet-derived growth factor (PDGF-BB, eBisoscience), or insulin from bovine pancreas or Akt activator, SC79 (both from Sigma), or full-length Akt1 protein (Abcam), or treated with palmitic acid complexed to BSA (PA-BSA) or human oxidized or acetylated LDL (both from Intracel, Frederick, MD) with an ACAT inhibitor, Sandoz 58035 (Sigma), or BAY 11-7082 (EMD Millipore), or an Akt inhibitor IV or Rapamycin (EMD Millipore), or the IKKalpha/beta inhibitor wedelolactone (Enzo Life Science).

Western blotting. Cells were lysed in a lysis buffer (Cell Signaling Technology, Danvers, MA) containing protease and phosphatase inhibitors. Proteins were measured with the DC Protein assay kit (Bio-Rad Laboratories) and resolved by NuPAGE Bis-Tris electrophoresis and transferred onto nitrocellulose membranes (Amersham Bioscience). Blots were probed with rabbit antibodies to Akt, Akt1, Akt2, pan-Akt, p-Akt (both S^{473} and T^{308}), p-GSK3α/β, mTOR, Raptor, Rictor, p-mTOR S^{2448}, p-70S6K, p-4E-BP1 (Cell signaling Technology), p-SGK-R and p-PKCa (Santa CruzBiotechnology), IKKα and β-actin antibodies (Abcam) and goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Sigma). Proteins were visualized with ECL western blotting detection reagents (GE Healthcare) and quantified by densitometry using ImageJ software (NIH).

Isolation of the mTOR complexes and Akt kinase assay. mTOR complexes were immunoprecipitated using CHAPS buffer. Briefly, the cells were lysed and mTOR complexes were precipitated by goat antibodies to mTOR (R&D System) or goat antibodies to Rictor (S20), or mouse monoclonal to IKKα (both from Santa Cruz Biotechnology). The precipitates were incubated with full sized Akt1 and the samples were resolved and the Akt S^{437} activity was detected by immunobloting. mTORC2 kinase activity was measured as recommended [4, 5].

Apoptosis assessment. Cultured cells in Laboratory-Tek chambers (Nalge Nunc International) or 5-micron cryosections from the proximal aorta were fixed in 4% paraformaldehyde in PBS, treated with 3% citric acid and apoptotic cells were detected by the *in situ* cell death detection kit (Roche Applied Science). TUNEL-positive (TUNEL+) cells were counted in 4 different sections of each aorta as described[6]. The number of TUNEL+ cultured cells were counted as a percentage of the total number of cells in at least four separate fields (containing ≈1,000 cells) from duplicate chambers.

Analysis of Serum Lipids and Aortic Lesions: Serum total cholesterol and triglyceride levels were determined after overnight fasting by the enzyme method as described[7]. Aortas were flushed through the left ventricle and the entire aorta was dissected for en face analysis as described [6]. Cryosections of the proximal aorta were analyzed using an Imaging system KS 300 (Kontron Electronik GmbH.).

Statistical Analysis: Data are provided as means ± SEM. The statistical differences in mean serum lipids and aortic lesion areas between the groups were determined using a SPSS Statistics Premium 22 (IBM, Armonk, NY). A difference was considered to be statistically significant at a *P*-value less than 0.05 by Mann-Whitney Rank Sum Test.

References:

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