Electronic supplementary material

*Generation and use of recombinant adenoviruses* cDNA encoding HA-tagged diacylglycerol kinase ε (DAGKε) was a gift from S. Prescott (Huntsman Cancer Institute, Salt Lake City, UT, USA) and has been extensively characterised [1]. HA-tagged IRS-1 cDNA was a gift from D. James (Garvan Institute, Sydney, NSW, Australia). The pAdEasy system [2] was used to generate recombinant adenoviruses for the expression of these constructs as previously described [3, 4]. A pAdEasy-derived virus expressing green fluorescent protein (GFP) alone was used in control infections. Cells were infected for 6 h with adenoviruses, after which medium was removed and linoleate pre-treatment carried out. In experiments where cells were co-infected with viruses for the overexpression of DAGKε and IRS-1, DAGKε virus was used at a fivefold excess over IRS-1 virus to ensure that any HA-IRS-1 detected was from DAGKε-overexpressing cells.

*Immunoprecipitation and immunoblot analysis* After experimental treatments, cells were washed three times in ice-cold PBS. Cell lysis, protein determinations, IRS-1 immunoprecipitations and immunoblotting were carried out as previously [5]. Membranes were probed with antibodies against IRS-1 (sc-559; Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphotyrosine (RC20H; BD Biosciences, San Jose, CA, USA), p85 (06-195; Upstate Biotechnology, Lake Placid, NY, USA), β-actin (clone AC74; Sigma Chemical, St Louis, MO, USA) or haemagglutinin (HA;
clone 3F10; Roche, Nutley, NJ, USA). Proteins were visualised with enhanced chemiluminescence detection kit (Perkin Elmer, Boston, MA, USA).

**Lipid determination by thin layer chromatography** Cellular DAG was measured by a method adapted from Nakamura and Handa [6]. Myotubes in 10 cm dishes were treated in the absence or presence of linoleate and washed with ice-cold PBS. For the investigation of lipid compartmentalisation, cells were homogenised and subjected to sucrose gradient flotation [7, 8]. Folch extraction was performed to isolate lipids [9] from cells or gradient fractions. Dried lipid fractions were resuspended with 100 µl 2:1 chloroform:methanol (vol./vol.) and spotted onto silica gel 60 F_{524} chromatography plates (Merck, Kilsyth, VIC, Australia). Thin-layer chromatography (TLC) was performed as described [6] and lipids stained with Coomassie brilliant blue R-250 (Bio-Rad, Regents Park, NSW, Australia). DAG and triacylglycerol (TG) were identified by comparison with authentic standards (dilinoleoyl-DAG and triolein; Sigma). For the investigation of PtdH, myotubes were labelled for 24 h with medium containing 3.2 MBq/ml $^{32}$P orthophosphate (Amersham, Little Chalfont, Bucks, UK). The labelling medium was removed and the cells incubated in the absence or presence of linoleate. Phospholipids were separated by two-dimensional TLC [10]. Lipids were visualised by autoradiography and identified by comparison to standards.

**Mass spectrometry** Electrospray ionisation mass spectrometry was performed on a Waters Quattro Micro equipped with an electrospray ion source and controlled by Micromass Masslynx version 4.0 software (Waters, Manchester, UK). PtdH
molecular species were analysed in negative ion mode with capillary voltage set to –3000 V, cone voltage –50 V, source temperature 80°C and desolvation temperature 120°C. Nitrogen was used as the drying gas at a flow rate of 320 ml/min. Phospholipids were diluted to a final concentration of 40 pmol/µl using ultra-pure grade methanol:chloroform 2:1 (vol./vol.) containing 625 fmol/µl internal standard (diheptadecanoyl PtdH, 17:0/17:0) and 1.4 % ammonia, pH10. Samples were infused (10 ml/min) using the instrument’s syringe pump and phospholipids were detected in the mass to charge ratio (m/z) range of 640 to 750. Scans for precursors of glycerophosphate anions (m/z 153.0) were obtained using argon as the collision gas at 400 kPa and a laboratory-frame energy of 50 eV. Precursor ion scans for the fatty acid carboxylate anions were performed using the same conditions. Typically, 120 spectra were averaged for each glycerophosphate precursor ion scan. Each mass spectrum was normalised to the internal standard after correction for isotope contributions and each PtdH molecular species presented as a percentage of total phosphatidic acid. Fatty acid precursor ion scans were performed for identification purposes only; accordingly, the minimum number of scans was averaged in order to obtain clean spectra. Tandem mass spectrometry was performed on isobaric phospholipids and the relative contribution of each isobaric species was determined from the ratio of fatty acid ion abundances.

Comparison of PtdH species in muscle from mice fed standard chow and high-fat diet
All experimental procedures were approved by the Animal Experimentation Ethics Committee (Garvan Institute of Medical Research/St Vincent’s Hospital, Sydney,
in accordance with National Health and Medical Research Council of Australia Guidelines. Male C57B/6 mice (7 weeks old; Animal Resources Centre, Perth, WA, Australia) had free access for 5 weeks to standard laboratory chow or a diet high in polyunsaturated fat that was prepared in-house [11], adapted from diet A12451 (Research Diets, New Brunswick, NJ, USA) by replacing lard with safflower oil. Mice were then subjected to an intraperitoneal glucose tolerance test (ipGTT, 2 g/kg glucose) [12]. Mice were killed 1 week later and quadriceps muscle samples rapidly removed, frozen in liquid nitrogen and subsequently powdered. Folch extracts were prepared (28 mg tissue/ml) in the presence of an internal standard (PtdH 17:0/17:0) for mass spectrometry as above.

References