ONLINE APPENDIX—Supplemental Data

Analysis: ***Breath samples***. Expired volumes of air in the Douglas bags were measured with a chain-suspended Collins spirometer, and expiratory air was analyzed for O_2 (Servomex S-3A; Servp,ex. Crowborough, UK) and CO_2 (Beckman LB2; Beckman, Irvine, CA, USA).

Blood samples. Blood glucose and lactate concentration were measured on an ABL 615 (Radiometer Medical A/S, Copenhagen, Denmark). Plasma long-chain fatty acid (LCFA) concentration (NEFA C kit, Wako Chemicals GmbH), plasma glycerol (Beckman Coulter, Ireland) and plasma triacylglycerol (TG) (triacylglycerol GPO-PAP kit) were measured using enzymatic colorimetric methods (Hitachi 912 automatic analyzer, Boehringer, Mannheim). Plasma TG was calculated as plasma TG minus free plasma glycerol. Plasma concentration of insulin and tumor necrosis factor (TNF) α were measured by enzyme-linked immunosorbent assay (ELISA, DakoCytomation K6219). Estradiol, progesterone and adiponectin were measured on an AutoDELFIA (Perkin Elmer) automated analyzer. Venous plasma resistin concentration was measured by sandwich enzyme immunoassays (SPI bio #A05177). Plasma epinephrine and norepinephrine were determined by radioimmunoassay (2-CAT RIA ^{125}I RIA kit, Labor Diagnostika Nord GmbH & Co. KG).

Stable isotopes enrichments were measured using liquid chromatography-mass spectrometry (Finnegan aQa) as previously described (1).

Muscle glycogen concentration was determined as glycosyl units after acid hydrolysis of freeze-dried and dissected muscle tissue by a fluorometric method (2).

Lysate. Freeze-dried and dissected muscle tissue or wet adipose tissue was homogenized as described (3). Homogenates were rotated end-over-end for 1h at 4ºC and then cleared by centrifugation for 20 min (17500 g, 4ºC). Protein content in the supernatant (the lysate) was measured by the bicinchoninic acid method (Pierce, Rockford, IL). The lysates were boiled in Laemmli buffer before being subjected to SDS-PAGE and immunoblotting.

Western blotting. Muscle lysate proteins were separated by SDS-PAGE and Western blotting followed by immunodetection as described previously (4).

Antibody used. The antibodies used for protein detection were anti-Munc 18c (Santa Cruz Biotechnology, CA, USA), anti-GLUT4 (Chemicon, CA, USA), anti-HKII (HXK21-A) (Alpha Dignastics, TX, USA), anti-Actin (Sigma Aldrich, St. Louis, MO) anti-Akt 1/2 and anti-AS160 (Upstate Biotechnology, NY, USA). The antibodies used for detection of protein phosphorylation were anti-Akt Ser^{473} (9271), anti-phospho- (Ser/Thr) Akt substrate for detection of Akt phosphorylation sites on AS160 (9611), AMPK Thr^{172} phospho specific antibody (Cell signalling technology, MA, USA) and anti-Akt Thr^{308} (06-678) (Upstate Biotechnology, NY, USA).

RNA isolation, reverse transcription and cDNA content. Total RNA was isolated from ~20 mg of muscle tissue by a modified guanidinium thiocyanate-phenol-chloroform extraction method as previously described (5). Superscript II RNase H$^-$ system and Oligo dT (Invitrogen, CA, USA) were used to reverse transcribe the mRNA to cDNA as previously described (5). The cDNA samples originating from 2µg of total RNA were diluted in nuclease-free H_2O to a total volume of 150µl. The amount of single-stranded DNA (ssDNA) was determined in each cDNA sample using OliGreen reagent (Molecular Probes, The Netherlands) according to Lundby *et al* (6).

Real time PCR. mRNA content was determined by PCR using the fluorogenic 5' nuclease assay with TaqMan probe (ABI PRISM 7900 Sequence Detection System, Applied Biosystems, CA, USA) as previously described (6). The sequences for detecting a fragment of the HKII mRNA

have been published previously (7). The TaqMan probe was 5'-6-carboxyfluorescein (FAM) and 3'-6-carboxy-N,N,N',N'-tetramethylrhodamine (TAMRA) labelled. The obtained cycle threshold (Ct) values reflecting the initial content of the transcript in the samples were converted to an arbitrary amount by using standard curves obtained from a serial dilution of a representative pooled sample. For each sample, the amount of a given target cDNA was normalized to the ssDNA content of the RT sample.

GS activity was measured in muscle homogenates by using a Unifilter 350 microtiter plate assay (Whatman; Frisenette, Ebeltoft, Denmark) essentially as described by Thomas *et al* (8) modified for microtiter plate assay.

IRS-1 associated PI3-kinase activit.y Activity was determined using thin layer chromatography as described previously (3).

ATPase and capillary stainings. Serial cross-sections (10 µm) were cut and stained for myofibrillar ATPase to identify type 1, 2A,2X muscle fibers (9). On an additional cross-section, capillaries were stained using the method by Qu *et al* (10). Muscle fiber types composition and capillary density were analysed using the TEMA image analyses software (ChechVision, Støvring, Denmark).

IMTG was determined by biochemical methods on freeze-dried and dissected tissue as described previously (11;12). Determination and visualization of IMTG content in specific muscle fiber types were detected by Oil Red O (ORO) staining combined with myosin monoclonal antibody immunostaining as described previously (13). The muscle cross sections were fixed with 0,1% Triton-X100 in 4% paraformaldehyde (PFA) before immunostaining with primary myosin heavy chain antibody (mouse monoclonal anti-MHC-1 (A4.840)) and secondary antibody ((FITC) conjugated anti-mouse, Alexa 488 (A-21042)). Then, the muscle cross sections were immersed in a solution of Oil Red O. Using a fluorescence microscope (Axioplan 2, Zeiss) connected to a camera (Coolsnap Monochrome, Zeiss) with Texas red and a FITC excitations filter, Oil Red O and MHC-stained muscle cross sections were excited and the signal was quantified using computer software (Metavue version 6.0) by the same blinded observer in all subjects. Areas with crystallized Oil Red O and labelled phospholipids in the membrane were avoided.

LCFA-CoA was determined in freeze-dried and dissected muscle tissue by the fluorometric method as described previously (14).

Muscle DAG and ceramide were extracted in chloroform-methanol (2:1, v/v) from freeze-dried and dissected muscle tissue (15) and separated by thin-layer chromatography. Lipid bands were developed at 120°C in 20 min and visualized and quantified by a Kodak Image Station E440CF (Kodak, Glostrup, Denmark).

Glucose transport in giant sarcolemmal vesicles (GSV) in rats. To investigate the direct effect of fatty acids (linoleic acid) on transsarcolemmal glucose transport, GSV were prepared from rat gastrocnemius muscle and 2-deoxy glucose (2DG) transport into GSV was measured as previously described (16). It was earlier demonstrated that the interstitial concentration of fatty acids amounts to approx. 1/5 of the plasma concentration in the fasting and postprandial state (17). Because the plasma concentration of fatty acids amounted to approx. 2000 $\mu mol \cdot l^{-1}$ in the present study, the GSV were incubated in linoleic acid at the following concentrations: 0,112, 225, 450, 750 $\mu mol \cdot l^{-1}$ or with 50 µM cytochalasin B (positive control). The fatty acids and cytochalasin B were complexed to 190 $\mu mol \cdot l^{-1}$ bovine serum albumin (BSA), as it has previously been demonstrated that the concentration of albumin in the interstitial fluid of skeletal muscle on average was 190 $\mu mol \cdot l^{-1}$ (18). Incubation media was added as 5 µl into 40 µl vesicles, for 10-15 min prior to transport measurements. Glucose transport was measured as the

uptake of 2 DG (5mM) measured over 2 min at room temperature, using mannitol (35mM) as extra-vesicular marker with final concentrations and activities of 5 mM 2DG, 0.5 $\mu Ci \cdot ml^{-1}$ 3H-2DG, 35 mM Mannitol and 0.1 $\mu Ci \cdot ml^{-1}$ ^{14}C Mannitol. Number of vesicles was counted under a microscope using computer software (Metavue version 7.0). Pretesting experiments showed that the final BSA (1.4 vol%) and ethanol (1.25 vol%) concentration did not disrupt vesicle integrity (data not shown).

Calculations--*Insulin sensitivity* on a whole-body level was expressed as area under the curve (AUC) of the glucose infusion rate during the last 30 min of the clamp. Glucose uptake across the thigh was calculated as the arterial-venous (a-v) glucose difference times blood flow (Fick's Principle) and insulin stimulated glucose uptake across the leg was expressed as AUC during the last 30 min of the clamp minus the basal individual level prior to the clamp (AUC).

The endogenous glucose production was calculated during the last 30 min of the clamp by subtracting the rate of glucose appearance (R_a), calculated using a single pool non-steady-state model modified after the Steele equation procedure, from the exogenous GIR as previously described (1).

The equilibrium concentration of unbound and bound linoleic acids was calculated by the stepwise equilibrium constant method (19), using the dissociation constants for the linoleic acids-albumin complex described by Spector et al (20).

Results. Percentage of type 1 fibers expressed relative to number (number %) and area (area %), was greater in women than in men (Supplementary Table 1). Mean area of type 1, type 2A and 2X fibers was smaller in women than in men. The capillary density (cap mm^2) was higher in women than in men (Supplementary Table 1*).*

References

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Supplementary Table 1. Fiber type composition and capillary density in women and men.

	Women (n=8)		Men (n=7)
Fiber type composition (number %)			
Type 1	68±3	#	57±4
Type 2A	22±3		27±5
Type 2X	9±2		14±3
Fiber type composition (area %)			
Type 1	71±3	#	56±4
Type 2A	20±3		29±5
Type 2X	7±2	(#)	13±3
Mean area per fiber (μm^2)			
Type 1	4259±226	#	5158±335
Type 2A	4086±242	#	5415±294
Type 2X	3787±309	#	4751±246
Capillary density			
Capillary per type 1 fibers	7.3±0.3	#	6.5±0.3
Capillary per mm^2	572±28	#	483±18

Data are means ± SE. # $p<0.05$, (#) $p=0.08$ sex difference

Supplementary Figure 1. The relationship between muscle hexokinase II protein expression and insulin stimulated leg glucose uptake expressed per kg lean leg mass, in women and men. *A*. The relationship in women, r=0.74, p<0.035, n=8. *B*. The relationship in men, r=-0.13, n=8.