Online appendix

Impact of Short-Term High-Fat Feeding on Glucose and Insulin Metabolism in Young Healthy Men

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Microarray analysis

Whole genome microarray analysis was performed on RNA extracted from insulin-stimulated skeletal muscle tissue before and after 5 days of high-fat overfeeding. Extraction of total RNA was quantified by spectrophotometric analysis and RNA quantity assessed by Agilent Bioanalyser 2100 using RNA 6000 Nano Assay Kit (Agilent Technologies).

cRNA preparation and hybridization - 1 μg of total RNA for each sample was amplified according to the Codelink^{TM} (GE Healthcare) Gene expression system: Manual Labelled cRNA Target Preparation. All cRNA samples were QC checked for consistent amplification on the Agilent Bioanalyser and quantified using absorbance at 260nm. 10μg aliquots of each cRNA sample were hybridized on whole genome human arrays (Codelink^{TM} product no.300033) according to the manufacturers protocol (CodeLink Gene Expression System: Single-Assay Bioarray Hybridization and Detection) for a 22 hr period at 37^{o}C. The slides were subsequently washed and stained with Cy5-Streptavidin prior to scanning on an Axon. 4100A scanner (Codelink application note Scanning Codelink Bioarrays on the GenePix Personal 4100A microarray scanner), with a pixel resolution setting of 5μm. Primary image analysis was performed using the Codelink expression analysis v4.1 software. QC thresholds for arrays were set to the manufacturer's default setting. Data was extracted directly into the Genespring (Agilent Technologies) format for subsequent analysis.

The data were analyzed using Genespring GX 7.3 Expression Analysis software (Agilent Technologies). Text tab delimited raw files were imported into Genespring. Standard data transformation and chip normalizations were applied in addition to non-pooled normalization for each individual subject for direct within-subject analysis. This approach was taken due to the low subject numbers and relatively high variability normally observed in human skeletal muscle tissue. Standard Genespring quality control procedures were employed to confirm appropriate normalization (box plots) and to apply sample linkage clustering for identification of suspect samples (condition tree) that were excluded from further analysis. Changes in insulin-stimulated gene expression from the control to the overfeeding diet in skeletal muscle tissue were identified using Volcano Plot analysis. Data were corrected for multiple testing corrections with adjusted *P*-values using the method of Benjamini and Hochberg with a user-defined

false discovery rate of 5% (Benjamini & Hochberg, 1995). A fold change cut-off of 1.2 was also applied. For pathway analysis, we performed Volcano Plot analysis eliminating multiple testing corrections whilst applying a t-test *P*-value of 0.05 and fold change consistent at 1.2 fold. Using this approach we identified a gene list containing genes showing an (insulin-stimulated) increase or decrease in expression following overfeeding. These two lists were imported into Ingenuity Pathway Analysis (Ingenuity Systems Inc.) which utilizes clustering algorithms to identify functional and signalling pathways overrepresented in each gene list. Specific consideration was given to the presence/absence of genes associated with insulin-mediated metabolic and signalling pathways.

Calculations of glucose turn-over rates and insulin secretion

Tracer equilibrium, i.e. constant specific activity of glucose tracer was reached in steady-state periods and glucose and insulin levels did not differ between the diet periods. During the insulin stimulated steady-state period rates of unlabelled glucose appearance (Ra), unlabelled glucose disappearance (Rd) and hepatic glucose production (HGP) were calculated using Steele's non-steady state equation (Steele R, 1959). During steady-state, Rd and HGP were calculated at 10 min intervals, and the distribution volume of glucose was set at 200 ml/kg body weight and the pool fraction as 0.65 (Cowan & Hetenyi, 1971). The glycolytic flux (GF) was calculated from appearance rate of tritiated water, and the total plasma water was assumed to be 93% of the total plasma volume (L Rossetti and A Giaccari, 1990;Del *et al.*, 1993). HGP during the insulin stimulated steady-state period was calculated as the difference between Ra and the glucose infusion rate. In cases where Ra was lower than the exogenous glucose infusion (i.e. HGP negative), the M-value calculated from the glucose infusion rate was used in the calculations. Endogenous glucose storage (EGS) was calculated as Rd – GF, and non-oxidative glucose metabolism (NOGM) as Rd – GOX. The hepatic insulin resistance index (Hep. IR index) was calculated as the product of fasting serum insulin concentration and HGP (Abdul-Ghani *et al.*, 2007).

IVGTT: The area under the curve (AUC) was calculated using a trapezoidal method for glucose, insulin and C-peptide during the IVGTT. The incremental first phase insulin response (FPIR) during the IVGTT (0-10 min) was calculated as [$AUC_{insulin(0\text{-}10min)}$ -

$AUC_{basal(ins\ 0\ *\ 10)}$]. The disposition index (Di) is an expression of beta-cell function in relation to insulin action. The index is using PHI_1 as a measure of insulin secretion calculated from the IVGTT (0-10 min) as [$AUC_{insulin}$ / $AUC_{glucose}$]. The disposition index for muscle insulin action is calculated as [PHI_1 * Si] were Si is calculated as [$R_{d\_insulin\ stim.}$ - R_{d_basal} / $Insulin_{insulin\ stim.}$ – $Insulin_{basal}$]. The disposition index based on hepatic insulin resistance is calculated as [PHI_1 / Hep. IR index].

Analytical procedure

Blood samples for serum insulin and C-peptide were centrifuged immediately at 4 °C and stored at -80 °C. Serum insulin and C-peptide concentrations were determined by AutoDELPHIA™ Time-resolved fluroimmunoassay (Perkin Elmer-Wallac Oy, Turku, Finland). Plasma adiponectin was measured by Radio Immuno Assay (RIA). FFA was quantified by an *in vitro* enzymatic colorimetric method (Wako, VA, USA). Tritiated glucose and water was determined as previous described (Hother-Nielsen O & Beck-Nielsen H, 1990).

Online appendix Table 1. Insulin-mediated metabolic and signalling pathways represented in the list of genes changed in response to 5 days of high-fat overfeeding in skeletal muscle tissue.

	HFHC diet	
	P-value	Ratio*
Insulin receptor signalling	NS	4/133
NFκB signalling	NS	5/143
PPARα/RXRα signalling	NS	6/176
PPAR signalling	NS	3/95
PTEN signalling	-	2/92
PI3K/AKT signalling	-	0/176
Actin cytoskeleton signalling	NS	7/267
FXR/RXR activation	NS	3/96
Glycerolphospholipid metabolism	NS	3/179
Glycolysis/Gluconeogenesis	-	1/142
Fatty acid metabolism	NS	1/187
Oxidative Phosphorylation	-	0/158

* Ratio = number of genes changed in response to overfeeding in relation to the total number of genes involved in the pathway.

NFκB (Nuclear factor kappa B), PPARα (Peroxisome Proliferator-activated Receptor α), RXR (Retinoic X Receptor), PTEN (Phosphatase and Tensin Homolog), FXR (Farnesoid X Receptor)

Reference List

Abdul-Ghani MA, Matsuda M, Balas B, & DeFronzo RA (2007). Muscle and Liver Insulin Resistance Indexes Derived From the Oral Glucose Tolerance Test. *Diabetes Care* **30**, 89-94.

Benjamini Y & Hochberg Y (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Roy Statist Soc Ser B 57* **57**, 289-300.

Cowan JS & Hetenyi G (1971). Glucoregulatory responses in normal and diabetic dogs recorded by a new tracer method. *Metabolism* **20**, 360-372.

Del PS, Bonadonna RC, Bonora E, Gulli G, Solini A, Shank M, & Defronzo RA (1993). Characterization of cellular defects of insulin action in type 2 (non-insulin-dependent) diabetes mellitus. *J Clin Invest* **91**, 484-494.

Hother-Nielsen O & Beck-Nielsen H (1990). On the determination of basal glucose production rate in patients with Type 2 (non-insulin-dependent) diabetes mellitus using primed-continuous 3-^{3}H-glucose infusion. *Diabetologia* **33**, 603-610.

L Rossetti and A Giaccari (1990). Relative contribution of glycogen synthesis and glycolysis to insulin-mediated glucose uptake. A dose-response euglycemic clamp study in normal and diabetic rats. *J Clin Invest 1990 June; 85(6):* **85**, 1785-1792.

Steele R (1959). Influences of glucose loading and of injected insulin on hepatic glucose output. *NY Acad Sci* **82**, 420-430.