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An 8-Week High-Fat Diet Induces Obesity and Insulin Resistance with Small Changes in the Muscle Transcriptome of C57BL/6J Mice

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Key Words
Metabolic syndrome · Microarray analysis · Nutrigenomics · Skeletal muscle

Abstract
Background: Skeletal muscle is responsible for most of the insulin-stimulated glucose uptake and metabolism. Therefore, it plays an important role in the development of insulin resistance, one of the characteristics of the metabolic syndrome (MS). As the prevalence of the MS is increasing, there is an urgent need for more effective intervention strategies. ***Methods:*** C57BL/6J mice were fed an 8-week low-fat diet (10 kcal%; LFD) or high-fat diet (45 kcal%; HFD). Microarray analysis was performed by using two comparisons: (1) 8-week HFD transcriptome versus 8-week LFD transcriptome and (2) transcriptome of mice sacrificed at the start of the intervention versus 8-week LFD transcriptome and 8-week HFD transcriptome, respectively. ***Results:*** Although an 8-week HFD induced obesity and impaired insulin sensitivity, HFD-responsive changes in the muscle transcriptome were relatively small (<1.3-fold). In fact, 8-weeks of aging induced more pronounced changes than an HFD. One comparison revealed the transcriptional downregulation of the mitogen-activated protein kinase cascade, whereas both comparisons showed the upregulation of fatty acid oxidation, demonstrating that the two comparison strategies are confirmative as well as complementary. ***Conclusion:*** We suggest using complementary analysis strategies in the genome-wide search for gene expression changes induced by mild interventions, such as an HFD.

Background

The metabolic syndrome (MS) is a multicomponent metabolic disorder characterized by central obesity, high blood pressure, increased plasma triglycerides, decreased plasma HDL cholesterol and insulin resistance and is associated with an increased risk for type 2 diabetes and cardiovascular diseases [1, 2]. The increasing prevalence

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of the MS is caused by a combination of lifestyle factors, such as nutrition and limited physical activity, which are known to contribute to the pathogenesis of the MS. While the etiology of the MS is complex and still not fully elucidated, central obesity and insulin resistance are considered to be potential causative factors. Additionally, obesity is considered as the principal cause of insulin resistance [3, 4]. Because skeletal muscle is the major site of insulin-stimulated glucose metabolism, it plays an important role in the development of insulin resistance and the MS. Two important ways by which central obesity can impair skeletal muscle insulin-stimulated glucose metabolism are by the ectopic storage of lipids and by alterations in the secretion of adipokines [5].

When adipose tissue expands as in obesity, plasma levels of fatty acids (FAs) and triacylglycerol (TAG) become elevated. This will lead to an increased flux of TAG and FAs into skeletal muscle [6]. Negative correlations between high levels of intramuscular TAG and insulin sensitivity have been observed in both obese subjects and subjects with type 2 diabetes [7–10]. Paradoxically, endurance training showed to improve insulin sensitivity together with increased levels of intramuscular TAG [11, 12]. Therefore, it has been proposed that not TAG, but lipid intermediates, such as long-chain fatty acyl CoAs, diacylglycerol and ceramides may act as signaling molecules interrupting insulin signaling and glucose metabolism, which eventually results in insulin resistance [13, 14]. In addition to the role of energy storage, adipose tissue functions as an endocrine organ that can respond to metabolic signals by secreting adipokines. These adipokines have different systemic effects including modulating energy metabolism in skeletal muscle. Enlargement of adipose tissue, and especially visceral fat depots, will lead to alterations in the secretion of these adipokines. For example, increased plasma leptin levels and decreased plasma adiponectin levels are reported in both obese and insulin-resistant subjects. As such, alterations in the secretion of these adipokines are linked to the impairment of insulin-stimulated glucose metabolism [15–17].

In the present study, we performed microarray analysis of skeletal muscle to get more insight into molecular mechanisms underlying the development of diet-induced obesity and insulin resistance in C57BL/6J mice. To find high-fat diet (HFD)-responsive changes, we compared the muscle transcriptome of mice fed an 8-week HFD with the muscle transcriptome of mice fed an 8-week low-fat diet (LFD). Biological processes in skeletal muscle affected by the HFD were assessed by the analyses of predefined gene sets based on gene ontology, biochemical, metabolic and signaling pathways. Since nutritional interventions induce subtle changes in gene expression [18, 19], we used a second comparison strategy in which we compared the 8-week HFD muscle transcriptome as well as the 8-week LFD muscle transcriptome with the muscle transcriptome of mice sacrificed at the start of the intervention. By paralleling the outcomes of these comparisons, we could find overlapping changes, suggesting that these changes are related to aging rather than to the HFD, as well as HFD-specific responsive changes. Finally, we evaluated the results of the two comparison strategies demonstrating their confirmative and complementary use.

Methods

Animals and Diet

Male C57BL/6J mice were obtained from Harlan (Horst, The Netherlands). At 9 weeks of age, mice were switched to a run-in diet consisting of an LFD (10 kcal% fat) for 3 weeks. Following the run-in period, mice were randomly assigned to the LFD or the HFD (45 kcal% fat) for 8 weeks. Both diets contained fat in the form of palm oil (based on D12450B and D12451; Research Diet Services, Wijk bij Duurstede, The Netherlands) as described previously [20]. Study 1*:* at the start of the intervention and after 8 weeks, mice (n = 10 per diet) were fasted for 6 h and anesthetized with a mixture of isofluorane (1.5%), nitrous oxide (70%) and oxygen (30%). Blood was collected by orbital puncture, after which the mice were sacrificed by cervical dislocation. The quadriceps muscle was dissected, snap-frozen in liquid nitrogen and stored at –80°C until further analysis. Study 2*:* after an overnight fast, a hyperinsulinemic, euglycemic clamp was performed to measure whole-body insulin resistance and tissue-specific insulin-stimulated glucose uptake (n = 5 per diet) after 8 weeks of dietary intervention. The animal studies were approved by the Local Committee for Care and Use of Laboratory Animals at Wageningen University.

Assessment of Body Composition

The percentages of body fat, lean body mass and total fat mass were measured with Dual-energy X-ray absorptiometry using the Lunar PIXImus® densitometer (GE Lunar, Madison, Wisc., USA) at the start of the intervention, after 4 and 8 weeks of the dietary intervention. Prior to use, the Lunar PIXImus densitometer was calibrated according to the manufacturer's instructions. Mice were anesthetized as described and measurements were performed on the whole body excluding the head area.

Plasma Insulin, Leptin and Adiponectin Measurements

Blood was collected in EDTA-containing tubes (Sarstedt AG & Co., Nümbrecht, Germany). Plasma was obtained after centrifugation at 11,000 *g* for 10 min and stored at –80°C for further analysis. Plasma insulin and leptin were measured simultaneously with the mouse serum adipokine Linco*plex* kit (Linco Research, Nuclilab, Ede, The Netherlands) using the Luminex100 system (Applied Cytometry Systems, Sheffield, UK) with Starstation software (Applied Cytometry Systems) as described by van

Schothorst et al. [21]. Plasma adiponectin was measured with the mouse Adiponectin/Acrp30 Quantikine ELISA Kit (R&D Systems, Minneapolis, Minn., USA).

Hyperinsulinemic Euglycemic Clamp

The hyperinsulinemic euglycemic clamp, preparation of tissue homogenates, analytical procedures and calculations for stimulation whole-body glucose uptake, inhibition of hepatic glucose production and tissue-specific glucose uptake were performed as described previously [22–24]. Inhibition of lipolysis was calculated as the ratio between the plasma free fatty acid levels after and before the hyperinsulinemic euglycemic clamp.

Affymetrix Microarray Analysis

Total RNA from the complete quadriceps muscle was isolated and Affymetrix GeneChip® Mouse Genome 430 2.0 arrays were used as described earlier with minor adaptations [25]. Briefly, only probe sets with an average signal intensity >20, across all arrays, were selected for further analysis. Since nutritional interventions induce subtle changes in gene expression [18, 19], we used a fold change >1.3 to find differentially expressed genes. To determine the level of significance, we used a false discovery rate (FDR) <0.05, which is used to correct for multiple testing [26]. Only genes that satisfied both criteria were considered as significantly differentially expressed. Changes in gene expression were related to functional changes by using ErmineJ (FDR <0.001) [27] and Gene set enrichment analysis (GSEA; FDR <0.05) [28]. For the microarray analyses in this study we used two different comparison strategies. Strategy 1: to identify HFD-responsive genes, we compared the muscle transcriptome of 8-week HFD mice with the muscle transcriptome of the 8-week LFD mice. Strategy 2: comparison of the muscle transcriptome of mice sacrificed at the start of the intervention (start transcriptome) with the muscle transcriptome of 8-week LFD mice as well as the muscle transcriptome of 8-week HFD mice. Changes in gene expression, overrepresented gene ontology (GO) classes and regulated gene sets that were only present in the 8-week HFD muscle transcriptome were considered as HFD-specific changes, whereas changes present in both the 8-week HFD muscle transcriptome and the 8-week LFD muscle transcriptome were considered as changes related to aging. Array data have been submitted to the Gene Expression Omnibus, GSE17576.

Verification of Microarray-Detected Changes

To verify micro-array detected changes, we measured enzyme activity levels or performed Western blotting to determine protein expression.

Enzyme Activity Measurements

Activity levels of the enzymes β-hydroxyacyl-CoA dehydrogenase (HAD; β-oxidation) and citrate synthase (CS; TCA cycle) were determined in quadriceps muscle homogenates (n = 7) as described by Den Hoed et al. [29].

Western Blotting

To determine whether microarray-detected changes in skeletal muscle could be translated to the protein level we performed Western blotting as described earlier [25]. Briefly, total protein was separated by SDS-PAGE on 4–12% Bis-Tris Criterion gels at 150 V and transferred to a polyvinylidene fluoride membrane for 90 min at 100 V. Blocking steps were performed in TBST supplemented with 5% nonfat dry milk (NFDM). Antibody (Ab) incubation steps of the membrane were performed in TBST supplemented with 5% BSA or 5% NFDM. Membranes were incubated overnight with an anti-mouse monoclonal oxidative phosphorylation Ab cocktail (1:2,000; Mitosciences, Eugene, Oreg., USA), a polyclonal p38 Ab (1:1,000; Cell Signaling, Danvers, Mass., USA) or a monoclonal phospho-p38 mitogen-activated protein kinase (MAPK) (Thr180/Tyr182) Ab (1:1,000; Cell Signaling) or a GAPDH Ab (1:3,000; Cell Signaling) at 4°C. After washing with TBST, membranes were incubated with a HRP-conjugated secondary Ab and signals were detected by ECL using Pierce reagents. Films were scanned with a GS800 densitometer (Bio-Rad) and signals were quantified with Quantity One software (Bio-Rad). As the GAPDH protein was stably expressed under the applied conditions, we used the GAPDH signal to calculate the relative protein abundance.

Statistical Analyses

All data are expressed as means ± SE. Microarray data were analyzed as described above. All further statistical analyses were performed using Prism software (GraphPad Software, San Diego, Calif., USA). An unpaired t test was used to compare differences between the LFD and the HFD mice. As data for whole-body insulin resistance and tissue-specific glucose uptake were not normally distributed, we used the non-parametric Mann-Whitney test to find differences. Repeated-measures ANOVA was used to find out whether body composition of LFD and HFD mice changes during the dietary intervention. When significant differences were found, a Tukey's post hoc test was used to determine the exact location of the difference. One-way ANOVA was used to compare the protein levels of p38 MAPK, phospho-p38 MAPK and the ratio phospho-p38 MAPK versus p38 between mice sacrificed at the start of the intervention, LFD and HFD mice. When significant differences were found, a Tukey's post hoc test was used to determine the exact location of the difference. $p < 0.05$ was considered as statistically significant.

Results

HFD Mice Develop Obesity

After a 3-week run-in period, mice were put on an LFD or HFD for 8 weeks. After 3 weeks, HFD mice had a significantly higher body mass than the LFD mice (fig. 1). Dual-energy X-ray absorptiometry was used to examine differences in body composition throughout the dietary intervention. During the first 4 weeks, both the LFD mice and the HFD mice significantly gained lean body mass (LBM) (LFD: +1.6 g; HFD: +2.4 g). From the 4th week onwards only the LFD mice significantly gained LBM (LFD: +0.8 g). No significant differences in LBM between the LFD and the HFD mice were observed. Both the LFD and the HFD mice increased their fat mass during the dietary intervention. However, the HFD mice accumulated significantly more fat mass than the LFD mice (table 1).

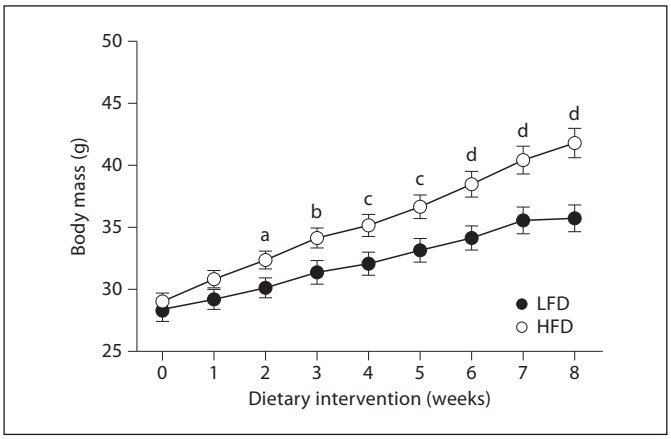

Fig. 1. Body mass development of LFD and HFD mice during 8 weeks of dietary intervention. Values are means ± SE (n = 10). Differences as obtained by using an unpaired t test: [a]$p < 0.10$, [b]$p < 0.05$, [c]$p < 0.01$ and [d]$p < 0.001$.

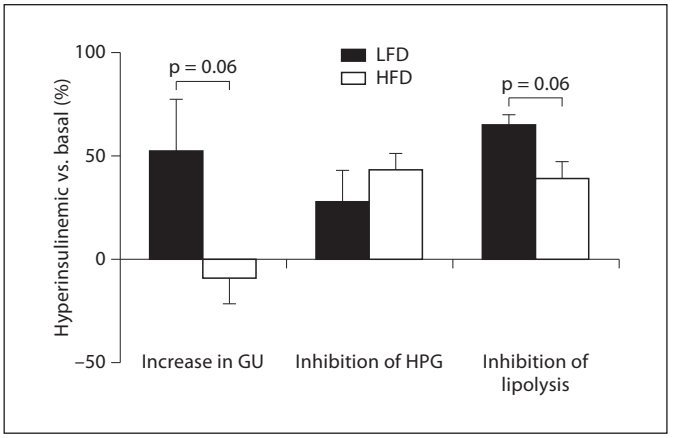

Fig. 2. Increase in whole-body glucose utilization (GU) and inhibition of hepatic glucose production (HPG) and lipolysis after hyperinsulinemic conditions in LFD and HFD mice. Values are means ± SE (n = 5) and p values were obtained by using the Mann-Whitney test.

Table 1. Body composition of LFD and HFD mice during the dietary intervention

	0 weeks	4 weeks		8 weeks	
		LFD	HFD	LFD	HFD
Total fat mass, g	6.8 ± 0.3	9.8 ± 0.6°°°	12.4 ± 0.6**, °°°	14.5 ± 0.8°°°	20.1 ± 0.9***, °°°
Lean body mass, g	22.7 ± 0.4	24.3 ± 0.5°°°	25.1 ± 0.6°	25.1 ± 0.6°	25.8 ± 0.9
Fat, %	23.0 ± 0.7	28.4 ± 1.2°°°	36.3 ± 1.1**, °°°	33.0 ± 1.1°°°	44.0 ± 0.9***, °°°

Body composition was measured by dual-energy X-ray absorptiometry scanning at the start, after 4 and 8 weeks of the dietary intervention. Values are means ± SE (week 0: n = 20 and weeks 4–8: n = 10). ** $p < 0.01$ and *** $p < 0.001$ indicate significant differences between LFD and HFD mice as obtained by an unpaired t test; ° $p < 0.05$ and °°° $p < 0.001$ indicate significant differences between time points (0 weeks vs. 4 weeks and 4 weeks vs. 8 weeks) by repeated-measures ANOVA.

Plasma Adiponectin, Insulin and Leptin Levels

Table 2 shows that at 8 weeks of dietary intervention, adiponectin plasma levels were significantly lower in HFD mice than in LFD mice. Leptin levels were significantly higher in the HFD mice in comparison with the LFD mice. The difference in fat accumulation strongly correlated with plasma leptin levels (Pearson correlation = 0.713; $p < 0.001$; 95% confidence interval: 0.39–0.88). Furthermore, we observed higher plasma insulin levels in the HFD mice than in the LFD mice.

An 8-Week HFD Induces Insulin Resistance

After 8 weeks of dietary intervention, whole-body insulin resistance was measured with a hyperinsulinemic euglycemic clamp. Whereas in the LFD mice whole-body glucose utilization increased by 50% after insulin infusion, in the HFD mice whole-body glucose utilization remained unaffected (p = 0.06). Insulin-induced inhibition of hepatic glucose production was not different between LFD and HFD mice, indicating that the liver was still insulin sensitive in HFD mice. A clear tendency towards decreased inhibition of lypolysis by insulin was observed in the HFD mice (p = 0.06), indicating insulin resistance of adipose tissue (fig. 2). Together these results point to peripheral insulin resistance. Tissue-specific glucose uptake was determined under hyperinsulinemic euglycemic conditions after a bolus of 2-deoxy-D-[^{3}H] glucose. Glucose uptake was significantly lower in the visceral fat depots of

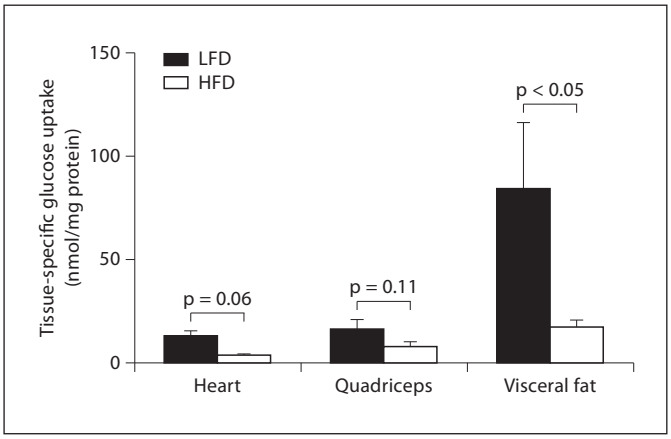

Fig. 3. Tissue-specific glucose uptake in heart, quadriceps and visceral fat deposits of LFD and HFD mice. Values are means ± SE (n = 5). p values were obtained using the Mann-Whitney test.

Table 2. Plasma adiponectin, insulin and leptin levels in 8-week LFD and 8-week HFD mice

	LFD	HFD	p
Adiponectin, μg/ml	5.7 ± 0.1	5.1 ± 0.2	≤0.05
Insulin, ng/ml	0.9 ± 0.1	1.3 ± 0.2	≤0.05
Leptin, ng/ml	18.4 ± 3.0	37.4 ± 5.8	≤0.01

Values are means ± SE (n = 10). p values were obtained with an unpaired t test.

Table 3. Changed gene sets in HFD transcriptome as compared with the LFD transcriptome

	n	ES	NES	FDR
Upregulated cellular processes				
Mitochondrial FA oxidation[1]	29	0.72	2.36	<0.001
ECM receptor interaction[2]	80	0.51	2.11	0.006
FAβ-oxidation meta[3]	34	0.59	2.06	0.008
Mitochondrial FAβ-oxidation[3]	15	0.71	2.02	0.009
Tissues blood and lymph 1[3]	32	0.60	2.02	0.008
FAβ-oxidation[3]	29	0.60	1.95	0.012
FA metabolism[2]	36	0.53	1.84	0.041
Downregulated cellular processes				
RNA polymerase[2]	20	–0.70	–2.26	0.001

GSEA was applied to identify upregulated and downregulated processes after an 8-week HFD. Presented are regulated processes with an FDR <0.05. An FDR was calculated to adjust for testing multiple hypotheses. n = Number of genes; ES = enrichment score for the gene set that reflects the degree to which a gene set is overrepresented at the top or bottom of the ranked list; NES = normalized enrichment score, i.e. the normalized ES to account for the size of the gene set.

[1] Source of the gene set: SK manual. [2] Source of the gene set: KEGG. [3] Source of the gene set: GENMAPP.

the HFD mice as compared with the LFD mice. Glucose uptake was also lower in the heart and skeletal muscle; however, this was not statistically significant (fig. 3).

Microarray Analysis

We showed that an 8-week HFD induces obesity, insulin resistance and disturbed plasma levels of adiponectin, insulin and leptin in C57BL/6J mice. To get more insight into the molecular mechanisms underlying the development of diet-induced obesity and insulin resistance, we performed microarray analysis of skeletal muscle. To find HFD-responsive changes, we compared the muscle transcriptome of mice fed an 8-week HFD with the muscle transcriptome of mice fed an 8-week LFD. Since nutritional interventions induce subtle changes in gene expression [18, 19], we used a second comparison strategy in which we compared the 8-week HFD muscle transcriptome as well as the 8-week LFD muscle transcriptome with the muscle transcriptome of mice sacrificed at the start of the intervention (start transcriptome). Regarding strategy 2, changes that were only present in the 8-week HFD muscle transcriptome were considered as HFD-specific changes, whereas changes that were found to be overlapping between the 8-week LFD muscle transcriptome and the 8-week HFD muscle transcriptome were considered to be related to aging. To identify differentially expressed genes we used a fold change >1.3 and an FDR <0.05. ErmineJ was used to find overrepresented GO classes (FDR <0.001) and GSEA was applied to find regulated cellular processes (FDR <0.05).

FA Oxidation Is the Only Responsive Pathway in HFD Mice Compared with LFD Mice

Comparing the 8-week HFD muscle transcriptome with the 8-week LFD muscle transcriptome using the criteria of a fold change >1.3 and an FDR <0.05, we found no differentially expressed genes. Thus, an 8-week HFD induced changes in gene expression level that are relatively small. ErmineJ showed that only 3 GO classes, i.e. cytokine production (GO: 0001816), regulation of cellular biosynthetic process (GO: 0031326) and steroid metabolic

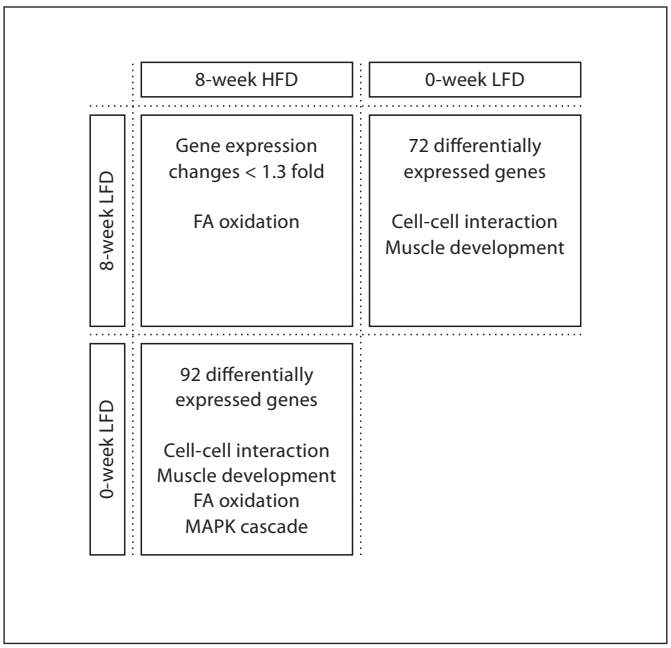

Fig. 4. Overview of the microarray analysis as performed by two different comparison strategies. Comparison strategy 1: to find HFD-responsive changes, we compared the 8-week HFD muscle transcriptome versus 8-week LFD muscle transcriptome. Comparison strategy 2: the 8-week HFD muscle transcriptome as well as the 8-week LFD muscle transcriptome with the muscle transcriptome of mice sacrificed at the start of the intervention.

process (GO: 0008202) were overrepresented in the 8-week HFD muscle transcriptome. Using GSEA, we found that 7 gene sets were upregulated in the 8-week HFD transcriptome. Of these 7 upregulated gene sets, we could relate 5 gene sets to FA oxidation. The only gene set that was downregulated was named RNA polymerase (table 3).

Descriptors for Cell-Cell Interaction and Development Are Regulated in Muscle of Both 8-Week LFD Mice and 8-Week HFD Mice, whereas the MAPK Cascade and FA Oxidation Are Only Regulated in 8-Week HFD Mice

When we compared the 8-week LFD muscle transcriptome to the start transcriptome, 72 genes were identified as differentially expressed (fold change >1.3 and FDR <0.05). ErmineJ showed that 20 GO classes were overrepresented in the 8-week LFD muscle transcriptome. This list contained descriptors for development, signaling and cell-cell interaction (table 4). GSEA revealed that only 2 gene sets were upregulated in the 8-week LFD muscle transcriptome. Nine gene sets were downregulated, including 4 genes sets corresponding to cell-cell interaction and 2 gene sets named 'Tissue blood and lymph' (table 5).

Comparison of the 8-week HFD muscle transcriptome with the start muscle transcriptome resulted in the identification of 92 differentially expressed genes (fold change >1.3 and FDR <0.05). Using ErmineJ, we found that 48 GO classes were overrepresented in the 8-week HFD muscle transcriptome. This included descriptors for cell-cell interaction, contraction, development, FA oxidation and the MAPK cascade (table 4). GSEA showed that 9 gene sets were upregulated in the 8-week HFD muscle transcriptome. This list contained 5 gene sets that were related to FA oxidation. The other 4 gene sets corresponded to protein metabolism and PPAR signaling. Four gene sets related to cell-cell interaction, 1 gene set describing the MAPK cascade and 1 gene set named 'Tissue blood and lymph' were downregulated (table 5).

When we paralleled the outcomes of the comparison 8-week LFD muscle transcriptome versus start transcriptome with the comparison 8-week HFD muscle transcriptome versus start transcriptome we observed that a total of 39 genes showed an overlap. From the overrepresented GO classes we identified 9 GO classes, including striated muscle development and descriptors for cell-cell interaction, that were overlapping (table 4). From the outcomes of GSEA, we found that 1 upregulated gene set and 5 downregulated gene sets were overlapping. Three downregulated gene sets corresponded to cell-cell interaction and 1 gene set was 'Tissue blood and lymph'.

Altogether, the outcomes of microarray analyses of skeletal muscle can be summarized as follows: HFD-specific changes in gene expression are smaller than 1.3 fold; 8-weeks of aging induces more pronounced changes in the muscle transcriptome than an HFD; the muscle responds to an 8-week HFD with changes in the regulation of FA oxidation and MAPK cascade; changes in the regulation of cell-cell interaction and muscle development are observed in both LFD and HFD mice and are thus related to aging. An overview of the microarray-detected changes is shown in figure 4. A complete list of regulated genes is available in online supplementary tables 1 and 2 (see www.karger.com/doi/10.1159/000308466).

Verification of Microarray-Detected Changes

To verify microarray-detected changes, we measured enzyme activities or performed Western blotting to analyze protein expression. First, we verified the transcription upregulation of FA oxidation in 8-week HFD mice versus 8-week LFD mice. Activity of HAD (β-oxidation) in HFD mice was comparable to LFD mice (7.2 ± 2.0 vs.

Table 4. Overrepresented GO classes in the LFD and the HFD transcriptome as compared with the start transcriptome

GO ID	GO class	n	Raw score 8-week LFD	FDR	Raw score 8-week HFD	FDR
GO:0030199	collagen fibril organization	14	2.03	1.2E-10		
GO:0006817	phosphate transport	61	1.53	1.5E-10	1.08	7.48E-11
GO:0030111	regulation of Wnt receptor signaling pathway	19	1.22	8.0E-04		
GO:0030198	extracellular matrix organization and biogenesis	50	1.21	2.0E-10	0.85	8.54E-11
GO:0043062	extracellular structure organization and biogenesis	85	0.94	2.4E-10	0.77	1.09E-10
GO:0007160	cell-matrix adhesion	55	0.85	4.0E-10		
GO:0031589	cell-substrate adhesion	59	0.81	1.2E-09		
GO:0042060	wound healing	72	0.80	1.7E-10		
GO:0009968	negative regulation of signal transduction	81	0.78	4.6E-04		
GO:0016055	Wnt receptor signaling pathway	107	0.75	1.3E-10	0.66	2.49E-04
GO:0008361	regulation of cell size	91	0.74	4.3E-04		
GO:0007229	integrin-mediated signaling pathway	74	0.73	9.0E-04		
GO:0016049	cell growth	79	0.72	5.0E-04		
GO:0021700	developmental maturation	79	0.72	5.4E-04		
GO:0007519	striated muscle development	103	0.71	3.0E-10	0.68	2.72E-04
GO:0046849	bone remodeling	89	0.71	7.0E-04		
GO:0015674	di-, tri-valent inorganic cation transport	112	0.68	6.0E-10	0.63	6.47E-04
GO:0043085	positive regulation of enzyme activity	118	0.66	9.4E-04	0.65	2.99E-10
GO:0008202	steroid metabolic process	122	0.64	6.6E-04	0.82	7.04E-11
GO:0051338	regulation of transferase activity	121	0.64	7.5E-04	0.70	1.99E-10
GO:0006635	FA β-oxidation	12			2.39	5.70E-11
GO:0019395	FA oxidation	19			1.72	9.20E-11
GO:0009636	response to toxin	18			1.40	3.99E-04
GO:0046486	glycerolipid metabolic process	21			1.18	6.30E-04
GO:0006941	striated muscle contraction	30			1.10	1.20E-09
GO:0016126	sterol biosynthetic process	25			1.06	8.16E-04
GO:0008203	cholesterol metabolic process	54			1.00	1.50E-10
GO:0060047	heart contraction	41			0.97	2.39E-10
GO:0000160	two-component signal transduction system (phosphorelay)	33			0.96	7.29E-04
GO:0016125	sterol metabolic process	61			0.94	6.30E-11
GO:0006469	negative regulation of protein kinase activity	35			0.90	7.97E-04
GO:0006936	muscle contraction	69			0.88	5.98E-10
GO:0006694	steroid biosynthetic process	61			0.87	1.33E-10
GO:0030324	lung development	59			0.85	9.97E-11
GO:0030323	respiratory tube development	60			0.84	1.20E-10
GO:0008015	circulation	84			0.83	1.71E-10
GO:0006766	vitamin metabolic process	56			0.79	7.12E-04
GO:0043405	regulation of MAPK activity	55			0.78	4.98E-04
GO:0042445	hormone metabolic process	71			0.78	2.39E-04
GO:0006869	lipid transport	64			0.76	4.12E-04
GO:0046942	carboxylic acid transport	55			0.75	5.44E-04
GO:0006725	aromatic compound metabolic process	102			0.74	7.97E-11
GO:0048637	skeletal muscle development	61			0.74	8.72E-04
GO:0000165	MAPKKK cascade	108			0.73	6.64E-11
GO:0045859	regulation of protein kinase activity	115			0.72	5.98E-11
GO:0006006	glucose metabolic process	80			0.72	2.60E-04
GO:0002526	acute inflammatory response	67			0.71	5.13E-04
GO:0043549	regulation of kinase activity	120			0.71	3.99E-10
GO:0030098	lymphocyte differentiation	81			0.70	6.13E-04
GO:0045860	positive regulation of protein kinase activity	74			0.70	6.95E-04
GO:0006470	protein amino acid dephosphorylation	93			0.68	4.43E-04
GO:0051347	positive regulation of transferase activity	79			0.68	8.91E-04
GO:0002252	immune effector process	106			0.67	5.28E-04
GO:0012502	induction of programmed cell death	112			0.64	5.98E-04
GO:0002521	leukocyte differentiation	113			0.63	4.27E-04
GO:0016311	dephosphorylation	105			0.63	5.61E-04
GO:0031326	regulation of cellular biosynthetic process	124			0.63	3.86E-04
GO:0019318	hexose metabolic process	111			0.63	4.60E-04
GO:0005996	monosaccharide metabolic process	113			0.62	9.10E-04

ErmineJ was used to identify significantly overrepresented GO classes in the muscle of LFD mice and HFD mice, respectively. For the concept biological processes we selected classes with an FDR <0.001. For this analysis only classes containing 8–125 genes were taken into account. n = Number of genes in GO class.

Table 5. Changed gene sets in the LFD and the HFD transcriptome as compared with the start transcriptome

	n	8-week LFD			8-week HFD		
		ES	NES	FDR	ES	NES	FDR
Upregulated cellular processes							
Proteasome[2]	24	0.63	2.08	0.017	0.70	2.16	<0.001
Olfactory transduction[2]	28	0.59	2.00	0.028			
Mitochondrial FA oxidation[1]	29				0.76	2.47	<0.001
FA β-oxidation meta[3]	34				0.61	2.08	0.003
PPAR signaling pathway[2]	63				0.53	2.04	0.005
FA metabolism[2]	36				0.56	1.92	0.016
Mitochondrial FA β-oxidation[3]	15				0.71	1.93	0.018
FA β-oxidation[3]	29				0.57	1.89	0.026
Tyrosine metabolism[2]	46				0.52	1.86	0.030
Proteasome degradation[3]	59				0.46	1.80	0.048
Downregulated cellular processes							
Cell communication[2]	91	−0.66	−2.73	<0.001	−0.56	−2.34	<0.001
ECM receptor interaction[2]	80	−0.67	−2.68	<0.001	−0.57	−2.30	<0.001
Focal adhesion[2]	170	−0.57	−2.57	<0.001	−0.48	−2.20	<0.001
Focal adhesion[3]	182	−0.56	−2.52	<0.001	−0.46	−2.08	0.002
MAPK cascade[3]	30				−0.60	−2.00	0.007
Tissue blood and lymph 1[3]	15	−0.77	−2.09	0.001	−0.69	−1.94	0.016
Prostaglandin synthesis regulation[3]	31	−0.63	−2.10	0.001			
Tissue blood and lymph 2[3]	32	−0.58	−1.92	0.012			
Inflammatory response pathway[3]	40	−0.56	−1.93	0.013			
Induction of apoptosis through Dr3 and Dr4 and 5 death receptors[4]	27	−0.60	−1.89	0.017			

GSEA was applied to identify upregulated and downregulated processes after an 8-week LFD and after an 8-week HFD, respectively. Presented are regulated processes with an FDR <0.05. An FDR was calculated to adjust for multiple hypotheses testing. n = Number of genes; ES = enrichment score for the gene set that reflects the degree to which a gene set is overrepresented at the top or bottom of the ranked list; NES = normalized enrichment score, i.e. the normalized ES to account for the size of the gene set.

[1] Source of the gene set: SK manual. [2] Source of the gene set: KEGG. [3] Source of the gene set: GENMAPP. [4] Source of the gene set: BIOCARTA.

6.8 ± 1.4 μmol/min/g protein with p = 0.88 in HFD vs. LFD). Also, the activity of CS (TCA cycle) in HFD mice was not different from LFD mice (24.1 ± 3.5 vs. 21.5 ± 3.0 μmol/min/g protein with p = 0.58 in HFD vs. LFD). Furthermore, protein levels of subunits of the 5 complexes of oxidative phosphorylation were not detectably changed in the HFD mice as compared with the LFD mice (complex I: 0.64 ± 0.10 vs. 0.60 ± 0.09; complex II: 1.00 ± 0.07 vs. 0.98 ± 0.12; complex III: 0.85 ± 0.07 vs. 0.86 ± 0.13; complex IV: 0.85 ± 0.14 vs. 0.80 ± 0.13 and complex V: 0.84 ± 0.08 vs. 0.87 ± 0.09).

Secondly, we verified the transcriptional downregulation of the MAPK cascade in muscle of 8-week HFD mice versus mice sacrificed at the start of the intervention. The gene with the strongest regulation of the MAPK cascade was found to be *Mek6* which is actually involved in the activation p38 MAPK via phosphorylation [30]. To find out whether downregulation of *Mek6* gene expression influenced the activity of the p38 MAPK cascade, we analyzed protein levels of p38 MAPK as well as phospho-p38 MAPK in protein extracts of mice sacrificed at the start of the dietary intervention, in 8-week LFD mice and 8-week HFD mice. Western blotting showed that the 8-week HFD significantly decreased p38 MAPK protein levels in skeletal muscle. The phospho-p38 MAPK protein level was not different in the HFD mice as compared to mice sacrificed at the start of the dietary intervention and 8-week LFD mice. However, activation of p38 MAPK, which is expressed as the ratio phospho-p38 MAPK versus p38-MAPK, tended to be higher in the HFD mice than in mice sacrificed at the start of the intervention (p = 0.09) (fig. 5).

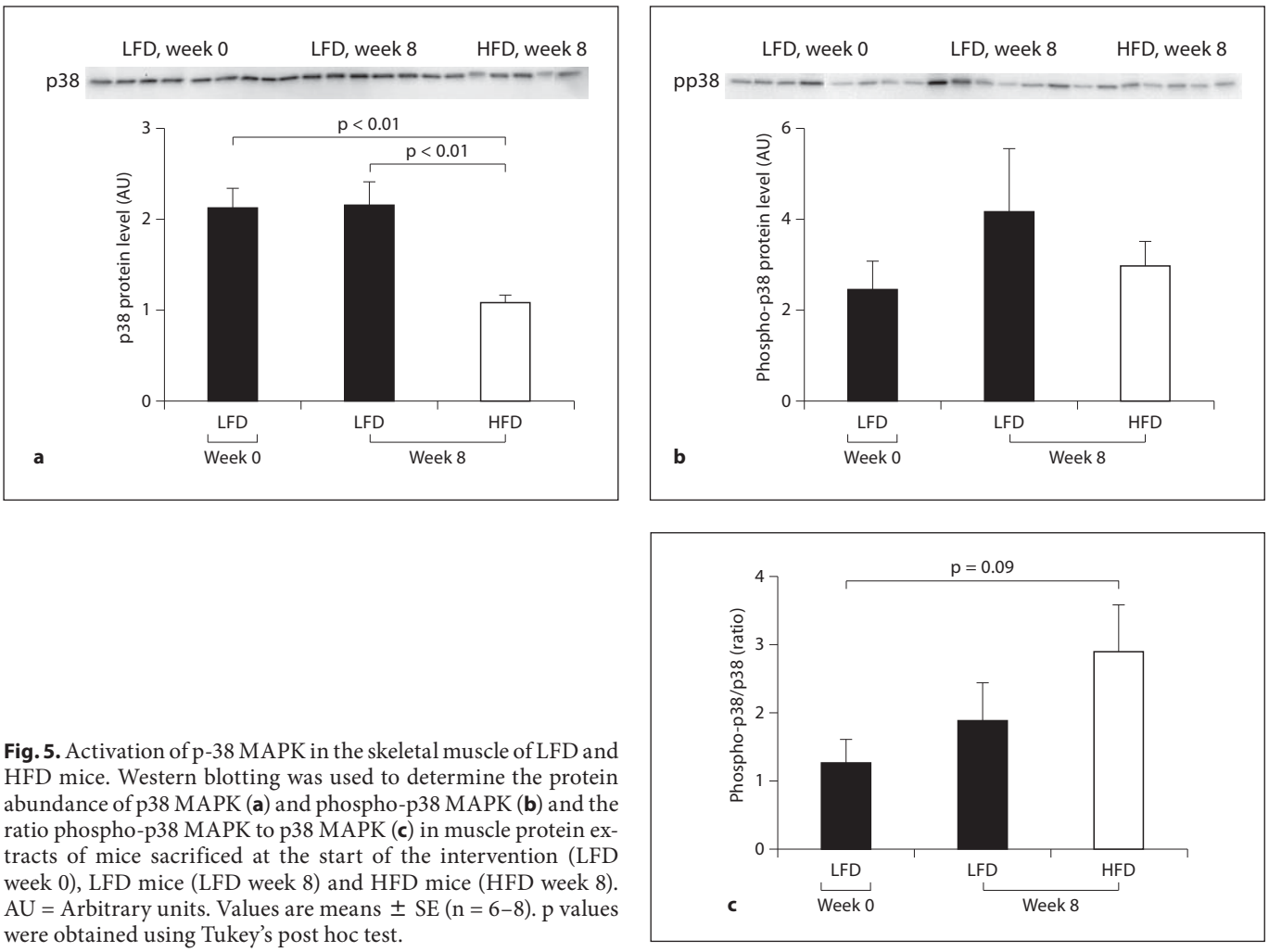

Fig. 5. Activation of p-38 MAPK in the skeletal muscle of LFD and HFD mice. Western blotting was used to determine the protein abundance of p38 MAPK (**a**) and phospho-p38 MAPK (**b**) and the ratio phospho-p38 MAPK to p38 MAPK (**c**) in muscle protein extracts of mice sacrificed at the start of the intervention (LFD week 0), LFD mice (LFD week 8) and HFD mice (HFD week 8). AU = Arbitrary units. Values are means ± SE (n = 6–8). p values were obtained using Tukey's post hoc test.

Discussion

In the present study, we demonstrated that an 8-week HFD induces obesity, reduces whole-body insulin sensitivity and decreases insulin sensitivity of heart, muscle and visceral fat deposits. Furthermore, plasma levels of the adipokines leptin and adiponectin were significantly increased and decreased, respectively. To get more insight into the underlying molecular mechanisms, we performed microarray analysis of skeletal muscle using a unique combination of two comparison strategies. First, we compared the 8-week HFD muscle transcriptome with the 8-week LFD muscle transcriptome. We found that differences in gene expression were relatively small and FA oxidation was the only regulated pathway. Secondly, we compared the start transcriptome with the 8-week HFD muscle transcriptome and the 8-week LFD muscle transcriptome, respectively. Irrespective of the diet, we found that cell-cell interaction and development were regulated when considering the possible effect of 8 weeks of aging. Interestingly, the upregulation of FA oxidation and the downregulation of the MAPK cascade appeared to be specific to the HFD intervention.

Obesity is the outcome of a prolonged positive energy balance caused by energy intake exceeding energy expenditure. The excess of energy is stored as TAG in adipose tissue. Adipose tissue secretes a variety of adipokines like leptin and adiponectin. Leptin serves as a metabolic signal of energy sufficiency regulating energy homeostasis. Furthermore, leptin also regulates peripheral metabolic mechanisms in muscle, liver, pancreas and other tissues. Adiponectin has been implicated as a modulator of glu-

cose and lipid metabolism in muscle and liver [17]. The observed strong correlation between obesity and leptin is in line with results of earlier studies describing that diet-induced obesity is accompanied by a state of leptin resistance [31]. Low circulating adiponectin levels have been demonstrated in diet-induced obesity in rodents as well as in human subjects [15]. Although only slightly lower, adiponectin levels were significantly different in HFD mice as compared to LFD mice in the present study.

Insulin resistance is manifested by decreased insulin-stimulated glucose uptake and metabolism in fat deposits and skeletal muscle and by an impaired suppression of hepatic glucose production [5]. Indeed, the HFD impaired the insulin-stimulated uptake of glucose in visceral fat deposits and muscle. Park et al. [32] showed that a 3-week HFD based on corn oil induces the development of insulin resistance simultaneously in the liver, adipose tissue and skeletal muscle in young adult C57BL/6 mice, whereas Kraegen et al. [33] found that diet-induced insulin resistance develops in liver and adipose tissue before it develops in skeletal muscle during a safflower oil-based HFD intervention in adult male Wistar rats. Here, we showed that an 8-week palm oil based HFD does not induce insulin resistance in the liver. Possible explanations for these different findings are differences in the studied species, age of the animals, but also the composition of the diet.

Previously, we showed that both a 3-day HFD as well as a 4-week HFD significantly changes the expression level of more than a thousand genes in the quadriceps muscle of mice [25]. Since the 8-week HFD mice developed obesity and insulin resistance, we expected to find a considerable number of differentially expressed genes in the skeletal muscle of these mice as well. However, no genes were found with a significantly changed expression level of more than 1.3 fold. Despite the relatively small changes in gene expression, FA oxidation was identified by both microarray analysis strategies as one of two responsive pathways in muscle of HFD mice. Although mitochondrial dysfunction has been observed in insulin resistance and type 2 diabetes, we observed that an HFD induces an upregulation of FA oxidation suggesting a metabolic adaptation. This is in line with numerous studies [25, 34–36]. Hoeks et al. [34] described that an 8-week HFD did not change mitochondrial function in rats as assessed by respirometry. In fact, they observed a tendency towards a higher respiratory control rate on lipid substrate, suggesting increased mitochondrial FA oxidative capacity [34]. Iossa et al. [35] also reported an increased lipid oxidative capacity in HFD rats. Two other groups, who assessed several markers for mitochondrial FA oxidative capacity during HFD interventions, observed an increased rather than a decreased mitochondrial FA oxidative capacity [25, 36]. Altogether, these results suggest that skeletal muscle tissue can metabolically adapt to an increased lipid load. As such, the muscle can buffer the increased lipid supply. However, in this study we showed that the transcriptional upregulation of FA oxidative genes did not result in increased activity of HAD, an enzyme involved in β-oxidation. Additionally, CS activity (marker for the TCA cycle) and oxidative phosphorylation protein expression in HFD mice was comparable to LFD mice. Finally, the mitochondrial copy number, which is another indirect marker for mitochondrial oxidative capacity, was not changed in the HFD mice (data not shown). These results suggest that the capacity to oxidize lipids was still sufficient to handle the increased fat supply. Whether an HFD intervention results in impaired oxidative capacity in the long run is currently under investigation.

The other HFD-responsive pathway was identified as an MAPK cascade. MAPK cascades are involved in the initiation of various cellular processes, such as proliferation, differentiation and development, in response to extracellular stimuli [37–39]. We observed a downregulation of the MAPK cascade in muscle of HFD mice. The gene with the strongest regulation was identified as the *Mek6* gene (1.59-fold; FDR <0.001). *Mek6* is involved in the activation of the p38 MAPK cascade via the phosphorylation of p38 MAPK [30]. Since different in vitro studies have shown that activation of the p38 MAPK cascade decreases the uptake of glucose under the influence of insulin or exercise, it has been hypothesized that p38 MAPK is a negative regulator of the stimulated uptake of glucose in muscle [40–42]. Corresponding to the decreased gene expression level of *Mek6*, we found that p38 MAPK protein levels were decreased after an 8-week HFD. Since phospho-p38 MAPK protein levels were not changed, we observed increased activation of the p38 MAPK cascade in muscles of HFD mice. This is in line with results of Li et al. [43], who observed increased p38 MAPK activation in heart tissue of HFD rats. Since in the present study the HFD mice were also insulin resistant, we consider these results support the hypothesis that p38 MAPK may act as a negative regulator of stimulated uptake of glucose.

We showed that 8-weeks of aging induce more pronounced changes in the muscle transcriptome than an HFD. We detected changes in the regulation of cell-cell interaction in the muscle transcriptome of both LFD

and HFD mice. Interactions between cells as well as interactions between cells and the extracellular matrix are critical for cellular development, organization, maintenance and function of a certain tissue [44]. We also observed that genes involved in muscle development-related processes were changed in LFD mice as well as HFD mice. Indeed, throughout the 8-week intervention period, LFD and HFD mice showed an increase in LBM as evidenced by dual-energy X-ray absorptiometry. Since the mass of the skeleton increased only marginally (~0.09 g, data not shown), we consider the increase in the amount of LBM as an actual increase in muscle mass. Thus, within a period of 8 weeks, irrespective of diet, mice develop more muscle mass as reflected by the muscle transcriptome.

In conclusion, an 8-week HFD induces obesity and reduced insulin sensitivity in C57BL/6J mice. However, HFD-responsive changes in the muscle transcriptome were relatively small. In fact, 8 weeks of aging induced more pronounced changes in the muscle transcriptome than an HFD. Only one strategy revealed the transcriptional downregulation of the MAPK cascade, whereas both strategies showed the upregulation of FA oxidation, demonstrating that the two comparison strategies are confirmative as well as complementary. Therefore, we suggest using complementary analysis strategies in the genome-wide search for gene expression changes induced by mild interventions such as an HFD.

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