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Comparison of Hormones, Lipoproteins and Substrates in Blood Plasma in a C57bl6 Mouse Strain after Starvation and a High Fat Diet: A Metabolomics Approach

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Abstract

Male C57bl6 mice (age 8-12 weeks) were fed a high-fat diet, containing 0.25% cholesterol (Ch) and 24% energy from bovine lard for a period of approximately 40 days or were exposed to 24 hours of starvation. Exposure to 24 hours of starvation, resulted in a significant drop in plasma glucose (1.8-fold), Phospholipids (PL) (1.2-fold), Triacylglycerols (TG) (1.4-fold), Total Cholesterol (TCh) (1.4-fold), Free cholesterol (FCh) (1.4-fold) and a significant increase in Ketone bodies (9.6-fold). After 24 hours of starvation plasma Very Low Density Lipoproteins- (VLDL), Low Density Lipoproteins-(LDL) and High Density Lipoproteins-(HDL) TCh and-Phospholipids (PL) were unaffected, while VLDL TG was significantly decreased and LDL and HDL TG significantly increased. Plasma Free Fatty Acids (FFA) and Insulin were unaffected.

In the high-fat diet group plasma VLDL-, LDL- and HDL-TCh and -PL were significantly increased as compared to the values on regular chow diet before starting the diet-feeding period. Furthermore, a significant rise in VLDL-TG was found.

The high fat diet resulted in significantly elevated levels of plasma TCh (2.9-fold), FCh (3.4-fold), PL (2.5-fold) and Insulin (4.4-fold) compared to the Control group that was fed regular chow diet, while glucose and Free Fatty Acids (FFA) levels were unaffected. The increase in Insulin as a result of a fatty diet may be indicative for increased insulin insensitivity.

Keywords: Mice; Starvation; Fatty diet; Plasma; FPLC; Insulin

Introduction

Metabolic syndrome, the complex of conditions that put humans to risk for coronary heart disease and type-2 diabetes, is increasingly common in Western countries. In North America, it is estimated that over 50 million American people or 20% of the US population are affected [1]. In addition, the numbers of cases are also increasing in Europe.

Obesity is a strong factor involved in metabolic syndrome, and some researchers claim metabolic syndrome is in itself closely linked to the metabolic derangement called insulin resistance [2] in humans. Early signals of emerging obesity might be found in the blood, which functions as the body's transport medium by carrying nutrients, lipids and waste products between cells. It is known that different food regimes are able to affect the composition of the blood plasma, as this was earlier investigated in humans. However, it remains unknown to what extent these results were influenced by confounding factors, as is often the case in human studies. In order to eliminate as much confounding factors as possible, we used a mouse model.

In this study, we analyzed a number of lipoproteins and plasma substrates in a C57BL/6 strain of mice, when confronting them with different feeding regimes. Lipoproteins are water-soluble protein complexes, which consist of a hydrophobic core, containing triacylglycerols and cholesterol esters, and a hydrophilic monolayered shell, composed of phospholipids, free cholesterol and specific proteins (apolipoproteins) [3]. Five major classes of lipoproteins can be distinguished in blood plasma, including chylomicrons, very-low-density lipoproteins (VLDL), intermediate- density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). Each of these proteins has its own specific function VLDL proteins transport triglycerides and cholesterol from the liver for redistribution to various tissues, while HDL is involved in a process that is referred to as reverse cholesterol transport. In this process, HDL acquires cholesterol from peripheral tissues and then transports it to the liver for excretion. LDL represents the final product of VLDL catabolism, and is the major cholesterol-transporting lipoprotein in plasma. Accumulated levels of LDL (e.g. as result of defective apolipoprotein-receptors in patients with familial hypercholesterolemia) correlate with accelerated coronary heart disease.

During fasting, the body uses reserve stores of nutrients to supply energy required for anabolism and to sustain vital body functions. Free

fatty acids are metabolized throughout body tissues and broken down to acyl-CoA, which can be used in the tricarboxylic acid cycle for ATP production.

During prolonged fasting brain, heart and muscle shift to using ketone bodies instead of free fatty acids as energy and glucose as energy source of the brain. In this situation, free fatty acids are channeled to ketogenesis through ß-oxidation when the body is in short supply of carbohydrates. This also occurs under conditions of high circulating glucagons or low circulating insulin (e.g., diabetes mellitus) [4].

On the other hand, free fatty acids are used for synthesis of triacylglycerols that are packed in lipoproteins, when carbohydrate supply is sufficient (e.g. in case food is abundant) or under conditions of low circulating levels of glucagons or high insulin concentrations [4]. An excess of triacylglycerols may lead to hepatic triacylglycerol accumulation, eventually leading to obesity. Obesity will result in an increased release of free fatty acids from adipose tissues, leading to higher presence in blood plasma during transport between different body compartments. An increased adipocyte mass and increased hydrolysis of triacylglycerols through increased secretion of hormone sensitive lipase (HSL) will contribute to the elevated flux of free fatty acids in the blood.

In order to test the validity of the results of human models, we tested whether different diet patterns (the abundance of food and food restriction) lead to different blood profiles in a specific mouse model. Several different blood parameters were determined: lipoproteins, blood substrates and insulin levels.

Material and Methods

In order to determine the blood composition under different feeding regimes, we administered two different diets on a specific mouse model, the inbred mouse strain C57BL/6 and compared these with two control groups. The C57BL/6 strain was used as it has extensively been studied as model of genetic control of diet-induced atherosclerosis (REFS). Two regimes were compared: 1. a low-dietary situation in which the mice were fasting for 24 hours and then were sacrificied (Treatment A) and 2. a high-dietary situation in which mice received a fatty diet during 40 days and then were sacrificied (Treatment B). Each treatment was compared to its own control group (thus, Control A and Control B) that lasted as long as its corresponding treatment. In total, 25 rodents were used: 6 mice in each of the control groups, 6 under treatment A and 7 under treatment B.

Animals

Mice were housed in a temperature-controlled room (23°C) on a 10-hour dark/14-hour light cycle. Purebred male wild-type C57bl/6 mice (age 8-12 weeks), obtained from Charles River (Maastricht, The Netherlands) were used. After anesthesia with isoflurane, blood was collected from the orbital sinus. The animal experiments were approved by the animal experimentation committee of the Leiden University Medical Center (The Netherlands).

Diet

Mice under treatment A were fed a standard lab chow (SDS.3, Special Diet Services, Witham, UK) containing about 4.3 energy percent fat (Table 1). The fatty diet that was fed to mice under treatment B contained 21.4% protein, 36% carbohydrates, 24% fat, 6% fibers and 5.7% water (weight-percentages)). Before the experiment started, animals of both treatments received unrestricted amounts of food and water. Mice in both control groups fasted for 4 hours before the start of the experiment in order to standardize their metabolic rate.

Proximate Analysis	**Regular (SDS.3)**	**Proximate Analysis**	**Fatty diet (4032.05)**
Moisture (%)	10.00	Moisture (%)	5.74
Crude Oil (%)	4.25	Crude Fat (Bovine Lard) (%)	24.00
Crude Protein (%)	22.39	Crude Protein (%)	21.44
Crude Fiber (%)	4.21	Crude Fibre (%)	6.16
Ash	7.56	Ash	2.25
Nitrogen Free Extract	51.20	Nitrogen Free Extract	36.19
-----	-	Cholesterol	0.25
Total	99.61	TOTAL	96.03
Measured Energy (Bombcalorimetry, [kJ/g dm])	16.86	Measured Energy (Bombcalorimetry, [kJ/g dm])	21.46

Table 1: Food constitution of the mice chow: "Regular" for Control group (Special Diet Services, SDS No.3, Witham, UK) and the "Fatty diet" (Arie Blok, food code 4032.05, Woerden, The Netherlands) based on bovine lard and 0.25% cholesterol.

Hormones and Substrates in Blood

Upon blood extraction plasma concentrations of total plasma glucose, free fatty acids, triacylglycerols and total cholesterol were determined via commercially available kits (Sigma, Diagnostics, St. Louis, MO; Roche Molecular Biochemicals GmbH, Mannheim, Germany; and Wako Chemicals GmbH, Neuss, Germany) according to the manufacturer's instructions. The concentrations of free cholesterols in serum were determined by enzymatic colorimetric assays with 0.025 U/ml cholesterol oxidase (Sigma) and 0.065 U/ml peroxidase (Roche Diagnostics, Mannheim, Germany) in reaction buffer (1.0 KPi buffer, pH=7.7 containing 0.01 M phenol, 1 mM 4-amino-antipyrine, 1% polyoxyethylene-9-laurylether, and 7.5% met hanol). Total cholesterol content was determined after addition of 15 µg/mL cholesterylesterase (Roche Diagnostics). Plasma phospholipids concentrations were determined using commercial enzymatic colorimetric assays (Wako chemicals GmbH, Neuss, Germany). Precipath L was used as an internal standard. Plasma insulin was measured by radioimmunoassays (RIA), using rat insulin standards (Sensitive Rat Insulin Assay, Linco Research, Inc. St. Charles, MO; 100% cross-reaction with mouse and human insulin).

Fast Performance Liquid Chromatography (FPLC)

The distribution of total cholesterol, triacylglycerols and phospholipids over the different lipoproteins in plasma was analyzed by fractionation of 30 µl of pooled plasma of each mouse using a Superose 6 column (3.2 × 300 mm, Smart System; Pharmacia, Uppsala, Sweden). Total lipid content of the effluent was determined using enzymatic colorimetric assays taking the efficiency of recovery from

the column into account. The concentrations of free cholesterol and the total cholesterol content were determined as described above. In addition, the concentration of triacylglycerols (Roche Diagnostics) and choline containing phospholipids (Wako chemicals GmbH, Neuss, Germany) were determined.

Calculations and Statistics

For all measured parameters, the mean value of the control mice group was compared to the mean value of the starvation mice group and the high fat diet group. Statistics were performed via SAS (Statistical Analyzing Software) using a one-way ANOVA for differences between the control groups, starvation and high fat diet groups and $P \leq 0.05$ was considered as statistically significant. Normality of the data and homogeneity of variances were checked by Kolmogorov-Smirnov and Fmax tests, respectively. Also a correction for multiple testing was applied.

Results

Lipoproteins

In order to investigate the effects of different feeding regimes on plasma lipids, we determined the distribution of lipids over different lipoproteins (Tables 2 and 3) (Figure 1).

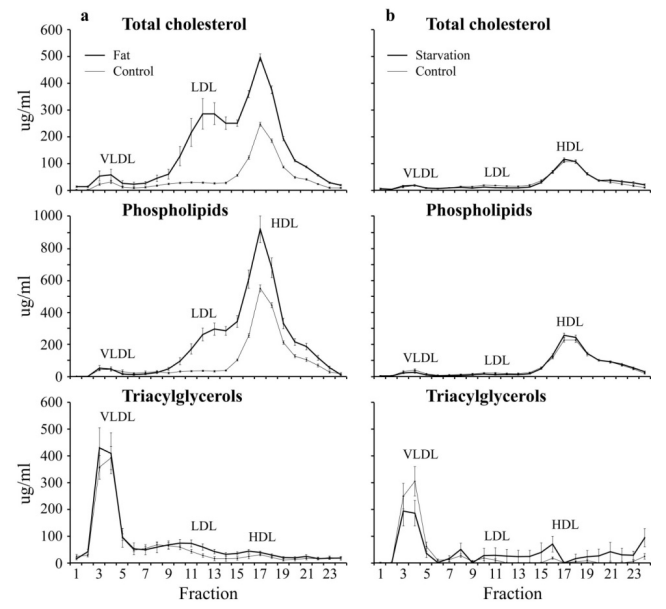

Figure 1: Lipoprotein distribution-profiles. Effect of starvation and a high fat diet on cholesterol, phospholipids and triacylglycerol levels in plasma and the effect on the distribution of those lipids over different lipoproteins in plasma; Fractions 3-7 represent VLDL, fractions 8-15 LDL and fractions 15-19 represent HDL (Mean ± SD). Number of animals Control A (n=6), Treatment A (n=6), Control B (n=6), Treatment B (n=7).

In Figure 1 the distribution profiles of Lipoproteins are provided. It is shown that in the case of standard lab chow diet total cholesterol and phospholipids are primarily transported by HDL and after a high fat diet these lipids are also transported by LDL. VLDL transports triacylglycerol under all feeding regimes.

Blood plasma parameter	Lipoprotein	Control A (n=6)	Treatment A (n=6)	P-value
Total Cholesterol (ug/ml)	VLDL LDL	52.0 ± 24.5	55.6 ± 27.5	P ≤ 0.806 n.s
	HDL	106.2 ± 24.6	64.6 ± 25.9	P ≤ 0.013*
		440.5 ± 66.7	453.4 ± 59.6	P ≤ 0.719 n.s
Phospholipids (ug/ml)	VLDL	105.6 ± 51.2	70.1 ± 45.4	P ≤ 0.212 n.s
	LDL	128.3 ± 35.9	82.8 ± 27.0	P ≤ 0.024*
	HDL	963.2 ± 144.8	1023.3 ± 135.2	P ≤ 0.456 n.s
Triacylglycerols (ug/ml)	VLDL	318.9 ± 152.5	215.9 ± 159.3	P ≤ 0.261 n.s
	LDL	30.6 ± 26.1	88.9 ± 160.5	P ≤ 0.400 n.s
	HDL	17.5 ± 18.4	109.0 ± 170.8	P ≤ 0.221 n.s

Table 2: Levels of total cholesterol, phospholipids and triacylglycerols over different lipoproteins, Control A vs. Treatment A (fasting) (Means ± SD) ($P \leq 0.05$ is significant).

Blood plasma parameter	Lipoprotein	Control B (n=6)	Treatment B (n=7)	P-value
Total Cholesterol (ug/ml)	VLDL LDL	71.9 ± 38.7	171.9 ± 157.1	P ≤ 0.159 n.s
	HDL	186.7 ± 30.7	1297.3 ± 640.7	P ≤ 0.001**
		815.9 ± 67.9	1968.7 ± 166.2	P ≤ 0.0001
Phospholipids (ug/ml)	VLDL	137.3 ± 49.4	129.3 ± 99.0	P ≤ 0.861 n.s
	LDL	289.1 ± 61.4	1499.9 ± 480.8	P ≤ 0.0001***
	HDL	1701.1 ± 180.1	2948.5 ± 714.9	P ≤ 0.002**
Triacylglycerols (ug/ml)	VLDL	453.2 ± 120.2	461.2 ± 261.0	P ≤ 0.947 n.s
	LDL	120.5 ± 70.2	197.4 ± 78.0	P ≤ 0.093 n.s
	HDL	62.6 ± 30.2	93.7 ± 22.0	P ≤ 0.055

Table 3: Levels of total cholesterol, phospholipds and triacylglyeceols over different lipoproteins, Control B vs. Treatment B (high fat diet) (Means ± SD) ($P \leq 0.05$ is significant).

Animals from Treatment A (fasting) showed a significant decline of total cholesterol and phospholipids compared to its control group (A), but only in the LDL lipoprotein (Table 2). No significant change of triacylglycerides could be encountered in any of the three lipoproteins (Tables 2 and 3).

As expected, Figure 1 demonstrates that total cholesterol in plasma from mice after a high fat diet (Treatment B) was significantly higher in the lipoprotein LDL and HDL respectively, when compared to its control, Control B ($P \leq 0.001$ and $P \leq 0.0001$ respectively, Table 3). The same applies for phospholipids, which were also significantly higher in LDL and HDL ($P \leq 0.0001$, $P \leq 0.002$, Table 2). There was no clear difference in plasma levels of triacylglycerols between Treatment B and Control B, however a trend towards a significant increase of triacylglycerols in the lipoprotein HDL was observed (Table 3).

Blood Substrates and Insulin

Compared to its control group (Control A), mice on a low diet (Treatment A) had significant decreased levels of glucose: a decrease of 44.6% ($P \leq 0.0001$, Table 4). Insulin in the fasting state decreased by 12.2%, but this was not significantly different ($P \leq 0.679$, Table 4). In contrast, there was a 9.6 fold increase in ketone bodies (863%, $P \leq 0.004$, Table 4) immediately after 24 hours of food deprivation. In addition, a decrease of respectively triacylglycerols, total cholesterol and free cholesterol ($P \leq 0.003$, 0.0001, 0.0001, respectively, Table 4), was observed.

Mice with the high fat diet (Treatment B) displayed a 336 % increase ($P \leq 0.042$, Table 5) in insulin levels, while their glucose levels were unaffected ($P \leq 0.352$, Table 5).

Blood-parameter	Control A	Treatment A	P-value
	6 mice	6 mice	
Insulin (µg/l)	0.41 ± 0.24	0.36 ± 0.16	P ≤ 0.679 n.s
Glucose (mmol/l)	8.96 ± 0.19	4.96 ± 0.35	P ≤ 0.0001***
Phospholipids (µg/ml)	1885 ± 114	1632 ± 119	P ≤ 0.004**
Free fatty acids (mmol/l)	1.58 ± 0.16	1.47 ± 0.21	P ≤ 0.338 n.s
Triacylglycerols (µg/ml)	943 ± 151	672 ± 82	P ≤ 0.003**
Total Cholesterol (µg/ml)	877 ± 72	634 ± 52	P ≤ 0.0001***
Free Cholesterol (µg/ml)	205 ± 9.6	120 ± 8.8	P ≤ 0.0001***
Ketone-bodies	0.447 ± 0.183	4.305 ± 0.460	P ≤ 0.004**

Table 4: Blood plasma parameters from the starvation experiment (Control A vs. Treatment A) (Means ± SD) ($P \leq 0.05$ is significant)

Blood-parameter	Control B	Treatment B	P-value
	6 mice	7 mice	
Insulin (µg/l)	0.25 ± 0.07	1.09 ± 0.89	P ≤ 0.042*
Glucose (mmol/l)	9.01 ± 0.12	9.2 ± 0.34	P ≤ 0.352 n.s
Phospholipids (µg/ml)	1541 ± 187	3884 ± 500	P ≤ 0.0001***
Free fatty acids (mmol/l)	1.54 ± 0.13	1.67 ± 0.23	P ≤ 0.261 n.s
Triacylglycerols (µg/ml)	631.9 ± 103	723.9 ± 300	P ≤ 0.491 n.s
Total Cholesterol (µg/ml)	802.2 ± 81.2	2309 ± 564.8	P ≤ 0.0001***
Free Cholesterol (µg/ml)	151.7 ± 29.3	508.5 ± 174.2	P ≤ 0.0001***
Ketone-bodies	Not determined	Not determined	Not determined

Table 5: Blood plasma parameters from the fatty diet experiment (Control B vs. Treatment B) (Means ± SD) ($P \leq 0.05$ is significant).

In addition, phospholipids strongly increased by 152 % ($P \leq 0.0001$). Total cholesterol increased significantly by 187.8 % ($P \leq 0.0001$) and free cholesterol increased significantly by 235.2 % ($P \leq 0.0001$) compared to its control group (B, Table 5).

Discussion

Our results demonstrate a strong elevation of ketone bodies and a decrease of glucose level in the blood plasma of mice under low-dietary conditions (fasting 24 hours, Treatment A). This is the typical result of the metabolic change of the body to use ketone bodies and free fatty acids as an energy source instead of carbohydrates. Our findings are consistent with the results from Heijboer et al. [5] in which plasma glucose concentrations decreased and plasma ketone bodies increased in mice that fasted for 16 hours.

Mice on a fat diet (Treatment B) showed a significant increase of free cholesterol (235%) and phospolipids (152%) in their plasma. This is consistent with the earlier findings of Vikstedt et al. [6] who also observed an increase: 81% for free cholesterol and 78% for phospholipids.

Levels of triacylglycerols in blood plasma significantly decreased for animals under Treatment A compared to Control A (decrease of 28.7%), but in animals under Treatment B no difference was found when compared with Control B. This result could be explained by the fact that under a minimal feeding regime all free fatty acids are used to generate ketone bodies and there are almost no free fatty acids left to form triacylglycerols that can be transported through the blood. Others also reported such a decrease in fasting animals: Lee et al. [7] demonstrated a fall of 32% in plasma triacylglycerols in wild type of mice. Moreover, these authors found a 38% increase of total cholesterol in the wild type mice and a 30% decrease of total cholesterol in the PPARα- (peroxisome proliferators-activated receptor) deficient mice after starvation [7]. In their study, it was demonstrated that PPARα is the major factor required for production of ketone bodies during starvation [7]. We found a decrease in total cholesterol of 27.7% in the mice under treatment A (fasting). This decrease resembles the findings in the PPARα- deficient mice, thus leading to the conclusion that PPARα does not seem to play a role in maintaining plasma triacylglycerol levels, but may play an important role in regulation of plasma cholesterol homeostasis during energy deprivation or starvation. Under Treatment B, no change is probably detected, as there is an elevated flux through the system, undetectable at plasma level.

We found no significant differences of plasma insulin and free fatty acids between Treatment A and Control group A. This corresponds to the results of earlier studies in rodents [5,8]. Rodents apparently have an efficient free fatty acids metabolism as under these test conditions the liver is still able to take up all free fatty acids from the blood stream. In humans, free fatty acids are important mediators of liver lipotoxicity [9] and their levels have been shown to correlate with disease severity [10].

In the animals under Treatment B, we found a 4.4 fold increase of insulin in plasma. This is one of the major outcomes of this study, which points into the direction of insulin insensitivity of the mice after high-fat diet feeding. The mice on high-fat diet feeding (Treatment B) need much more insulin to generate the same effect as mice from Control group B. This effect is keeping the plasma glucose levels in this study at an elevated level of ±9.1 mol/l, a sign of developing insulin-resistance.

It is known that elevated levels of insulin suppress the secretion of VLDL particles by the liver [11]. This could explain that no significant increase of VLDL in blood plasma was found in mice under Treatment B. However, LDL increased and an increase of HDL was observed in this group. One of the major differences between human and mouse metabolism is the expression of plasma cholesteryl ester transfer protein (CETP), which does not happen in rodents but does in humans. This enzyme transfers cholesterol esters from HDL to VLDL and LDL. A low CETP activity is connected with high HDL-C levels [12-19]. Consequently, rodents tend to have more HDL than humans and humans tend to have more LDL [6,20-29], which explains the higher amount of HDL in the blood plasma of rodents under Treatment B.

From this study, we can conclude that a period of 24 hours fasting has no major impact on the lipid composition of the blood plasma while nearly all lipid compounds in blood plasma are affected by a period of fatty diet. Apparently, the high-energy content of lipids makes these compounds an attractive form of fuel storage.

Although it is always difficult to implicate results from a mouse model directly on humans, we think that especially the latter result can be important. It demonstrates that the effects of a fat diet on the blood plasma composition cannot be neutralized by a single period of fasting.

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