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Feeding conditions control the expression of genes involved in sterol metabolism in peripheral blood mononuclear cells of normoweight and diet-induced (cafeteria) obese rats☆

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Abstract

Peripheral blood mononuclear cells (PBMC) are easily obtainable cells from blood whose gene expression profiles have been proven to be highly robust in distinguishing a disease state from healthy state. Sterol metabolism is of physiological importance, and although its nutritional response in liver has been described, it is poorly studied in PBMC. To examine if PBMC sterol metabolism reflects diet-induced physiological responses, we analysed the whole genome gene expression response of PBMC and focused on sterol metabolism-related genes affected by different feeding conditions (ad libitum feeding, fasting, and refeeding) in normoweight (control) rats and in diet-induced (cafeteria) obese rats.

Our results of microarray analysis show that, in control rats, 21 genes involved in sterol metabolism were regulated by the different feeding conditions, whereas in cafeteria-obese rats, only seven genes showed a changed expression. Most of the genes identified were classified into three pathways: sterol biosynthesis, cholesterol transport and uptake and sterol signaling. The expression profile of these genes was similar to that previously described for liver, decreasing in response to fasting conditions and recovering the levels found in fed animals after 6-h-refeeding. In addition, our data and the comparable expression pattern of sterol metabolism-related genes between PBMC and liver suggest similar sterol regulatory element-binding protein-mediated regulatory mechanisms in response to feeding conditions in both tissues.

In conclusion, the expression of genes involved in sterol metabolism is highly controlled by feeding conditions in PBMC of control rats, but this control is impaired in cafeteria-obese animals. The pathophysiological significance of this impairment requires further investigation.

Keywords: PBMC; Microarray; Sterol metabolism; Cafeteria diet; Feeding conditions

1. Introduction

Cholesterol is a major structural component of the plasma membrane of eukaryotes and is required for proper membrane permeability and fluidity, membrane protein function and organelle identity. As membrane cholesterol dynamics participates in key cellular processes, such as intracellular organization and vesicle transport, cell cholesterol needs to be tightly regulated. Cells maintain sterol homeostasis by multiple feedback controls that act through transcriptional and posttranscriptional mechanisms (reviewed in [1,2]). The sterol regulatory element-binding proteins (SREBP) family of transcription factors, which consists of 3 different members (SREBP1a, SREBP1c and SREBP2), are the principal modulators of both sterol synthesis and uptake. These proteins control the expression of more than 30 genes involved in cholesterol, fatty acid, triglyceride and phospholipid metabolism. Subsequently, the modulation of many enzymes involved in different steps of sterol biosynthesis, such as the flux-limiting 3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase, are characterized for their SREBP dependence (reviewed in [1–5]). In mice and rats, the liver is quantitatively the most important site for cholesterol synthesis [6–8] and it has been described that, in this tissue, the expression of some

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important genes involved in sterol metabolism is affected by feeding conditions [9–11]. Several studies performed in humans and in experimental animals have shown that cholesterol is also produced in extrahepatic tissues, including endocrine organs, intestine, muscle and skin [6–8,12,13] as well as in peripheral blood mononuclear cells [14–21]. Peripheral blood mononuclear cells (PBMC) are a subset of white blood cells, consisting of lymphocytes and monocytes/macrophages. They are relatively easily accessible in humans by isolation from blood [22,23] and can be used to assess biological responses as their gene expression profile may reflect the pathological and physiological state of the organism [22–25]. Several studies of PBMC gene expression have shown that the expression of a variety of adipokine and cytokine genes are altered in the obese state [26–31]. Here we studied, by microarray analysis and subsequent confirmation by quantitative reverse transcriptase-polymerase chain reaction (Q-PCR), the response of genes involved in sterol metabolism to different feeding conditions (ad libitum feeding, fasting and refeeding) in PBMC of normoweight (control) and diet-induced (cafeteria) obese rats.

2. Methods and materials

2.1. Animals

The animal protocol followed in this study was reviewed and approved by the Bioethical Committee of our university and guidelines for the use and care of laboratory animals of our University were followed.

Two-month-old male Wistar rats (Charles River Laboratories España, Barcelona, Spain) were distributed into two dietary groups for 4 months: the control group (n=15) was fed with a standard chow diet (Panlab, Barcelona, Spain), whereas the second group (cafeteria group, n=15) was fed with a cafeteria diet in addition to the standard chow. Cafeteria diet is a fat-rich hypercaloric diet containing the following foodstuffs: cookies with liver pate and sobrassada (a typical Majorcan sausage), candies, fresh bacon, biscuits, chocolate, salted peanuts, cheese, milk containing 20% (w/v) sucrose and ensaïmada (a typical Majorcan pastry). The gross composition of the diets was as follows: for the standard chow diet, 60.5% carbohydrate, 2.9% lipid, 15.4% protein, 12% water, 5.3% minerals and 3.9% fiber, and for the cafeteria diet, 35.2% carbohydrate, 23.4% lipid, 11.7% protein, 28.4% water and 1.31% fiber. The animals were single housed at 22°C with a period of light/dark of 12 h and with free access to food and water. When rats were 6 months old, and cafeteria rats had an increase of 29% in body weight, each group of rats were distributed in three subgroups submitted to different feeding conditions (n=5 for each condition): feeding (animals provided with ad libitum access to diet), fasting (animals deprived of food for 14 h) and refeeding (fasted animals with a posterior free access to diet for 6 h). Coprophagy was prevented by changing the cage immediately prior to food deprivation. For PBMC isolation, and prior to the sacrifice of the animals, blood samples (1.5–2.5 ml) were collected from the saphena vein, using heparine in NaCl (0.9%) as anticoagulant; immediately after the blood collection, PBMC were isolated by Ficoll gradient separation, according with the instructions indicated by the manufacturer (GE Healthcare Bio Sciences, Barcelona, Spain).

After the experimental feeding period, animals were sacrificed and different white adipose tissue depots — epididymal, inguinal, mesenteric, and retroperitoneal — were removed and weighted. Truncular blood was collected from the neck, stored at room temperature for 1 h and overnight at 4°C and was then centrifuged at 1000 g for 10 min to collect the serum.

2.2. Adiposity index

Adiposity was determined by an adiposity index computed for each rat as the sum of epididymal, inguinal, mesenteric and retroperitoneal white adipose tissue depot weights and expressed as a percentage of total body weight.

2.3. Quantification of circulating insulin, leptin, and glucose levels

Serum insulin and leptin levels were measured using enzyme-linked immunosorbent assay kits (from DRG Instruments, Marburg, Germany, and R&D Systems, Minneapolis, MN, USA), and blood glucose using an Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain).

2.4. Homeostatic model assessment for insulin resistance analysis

Insulin resistance was assessed by the homeostatic model assessment for insulin resistance (HOMA-IR) in rats submitted to overnight (14 h) fasting (n=5 for all groups). HOMA-IR score was calculated from fasting insulin and glucose concentrations using the formula of Matthews et al. [32]. HOMA-IR=fasting glucose (mmol/l)×fasting insulin (mU/l)/22.5.

2.5. Total RNA isolation

Total RNA from PBMC samples was extracted using Tripure Reagent (Roche Diagnostic Barcelona, Spain) and purified with a Quiagen RNeasy Mini Kit spin columns (Izasa, Barcelona, Spain). RNA yield was quantified on a NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and its integrity was measured on an Agilent 2100 Bioanalyzer with RNA 6000 Nano chips (Agilent Technologies, South Queensferry, United Kingdom).

2.6. Microarray processing

For the microarray analysis, PBMC RNA samples of Wistar rats of the control (n=15) and the cafeteria-obese groups (n=15) submitted to different feeding conditions (feeding, fasting, and refeeding) (n=5 animals for each condition) were used. From the 30 PBMC samples, 29 were used for microarray processing. One sample in the control fed group was excluded because of low RNA amount.

For microarray hybridization, 0.5 μg RNA of each sample was reverse transcribed using the Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent), according to the manufacturer's protocol (all materials and reagents are from Agilent Technologies, Palo Alto, CA, USA, unless stated otherwise). Half of the cDNA sample (10 μl) was used for linear RNA amplification and labelling with Cy5 or Cy3. All reactions were performed using half of the amounts indicated by the manufacturer. Briefly, a transcription master mix was prepared (7.65 μl nuclease-free water; 10 μl 4× transcription buffer; 3 μl 0.1 M DTT; 4 μl NTP mix, 3.2 μl 50% PEG; 0.25 μl RNaseOUT; 0.3 μl inorganic pyrophosphatase; 0.4 μl T7 RNA polymerase; 1.2 μl cyanine 3-CTP or cyanine 5-CTP, total volume 30 μl); 30 μl of transcription mastermix was added to 10 μl cDNA. In vitro transcription and labelling were carried out at 40°C for 2 h. The labelled cRNA samples were purified using Qiagen RNeasy mini-spin columns (Qiagen, Venlo, The Netherlands). Dye incorporation and cRNA concentration was measured using the 'micro-array measurement mode' of the Nanodrop spectrophotometer (NanoDrop Technologies). Yield of each individual sample was >825 ng and specific activity >8.0 pmol Cy3 or Cy5 per μg cRNA. All Cy3 cRNAs were pooled to serve as standard reference pool. Hybridization was performed by preparing a 2× cRNA target solution containing 825 ng Cy5-labeled cRNA, 825 ng Cy3-labeled pool cRNA and 11 μl 10× blocking agent in a total volume of 52.8 μl. Then 2.2 μl fragmentation buffer was added and incubated at 60°C for 30 min. Fragmentation was stopped by adding 55 μl 2× GEx hybridization buffer HI-RPM and hybridized on 4× 44K G4131F whole genome Agilent arrays (Agilent Technologies, Santa Clara, CA, USA) for 17 h at 65°C in Agilent hybridization chambers in an Agilent hybridization oven rotating at 10 rpm. After hybridization the arrays were subsequently washed with "GE wash buffer 1" for 1 min at room temperature, "GE wash buffer 2" for 1 min at approximately 37°C, acetonitrile for 1 min at room temperature and 30 s at room temperature with "stabilization and drying solution" according to manufacturers protocol (Agilent Technologies).

2.7. Microarray data analysis

Arrays were scanned with an Agilent Microarray Scanner (Agilent Technologies). Spot intensities were quantified using Feature extraction 8.5 (Agilent Technologies, Santa Clara, CA, USA). Median density values and background values of each spot were extracted for both the experimental samples (Cy5) and the reference samples (Cy3). Subsequently, quality control was performed for each microarray using both LimmaGUI package in R from Bioconductor and Microsoft Excel. All the arrays passed the quality control based on MA plot and signal intensity distribution [33]. Thereby, the dataset contained 29 arrays in total. Data was exported into GeneMaths XT (Applied Maths, Sint-Martens-Latem, Belgium) for background correction and normalization. We discarded spots with an average intensity, over all arrays, of Cy5 lower than 2-fold above average background. Then, the Cy5 intensities were normalized against the Cy3 reference as described previously [34]. The data have been deposited in NCBIs Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE14497. The statistical significance for the three different feeding conditions (fed, fasting and refeeding) in control or cafeteria-obese rats was assessed by one-way analysis of variance (ANOVA) and least significant difference (LSD) post hoc comparisons. In order to know the degree of the change in the expression and the expression pattern (increase or decrease) of the genes, fold change calculations between two different feeding conditions (fasting vs. feeding; refeeding vs. fasting; refeeding vs. feeding) were performed in Microsoft Excel. Fold change equals ratio fasting/feeding or refeeding/fasting or refeeding/feeding in the case of increase or equals −1/ratio in the case of decrease. One-way ANOVA was performed in GeneMaths XT (Applied Maths, Sint-Martens-Latem, Belgium), the generated p-values were used to obtain insight into significantly affected genes. For one-way ANOVA, we chose a threshold of P<.01. All the genes with a P<.01 were considered as significantly affected by the three different feeding conditions.

To interpret functional changes in the dataset, we applied two pathway analysis programs, Metacore (GeneGo, St. Joseph, MI, USA) and Ermine J [35], with different complementary pathway-classification properties. For MetaCore analysis we used classification based on GO-term and classification based on GeneGo annotation. The GeneGo annotation database is a curated database of gene networks based on several databases (KEGG, GO) and scientific literature. We selected pathways with P<.05 in Metacore. Ermine J analysis is based on GO-term, only GO-processes with 5–500 genes

and with $P<.05$ were taken into account. Agilent gene annotation version 20060331 was used for both programs. Since only about 30% of the genes on the whole genome array were recognized in both pathway programs, we manually supplemented the significantly enriched biological processes with non-annotated genes from the selected gene-set using biological databases (Biocarta, SOURCE, GenMAPP, KEGG) and scientific literature. As processes overlap, we bundled some processes and renamed them.

2.8. Real-time Q-PCR analysis

Q-PCR was used to measure mRNA expression levels of *abcgl*, *acat2*, *dhcr7*, *fdft1*, *lss*, *scmol4*, *sqle*, *srebf2*, *srebf1* and *star* in PBMC samples used for the microarray experiment in order to validate the data analysis. Analyses of all individual samples were performed in duplicate.

Total RNA 0.5 μg (in a final volume of 20 μl) was denatured at 90°C for 1 min and then reverse-transcribed to cDNA using MuLV reverse transcriptase (according to Applied Biosystem's procedure) at 42°C for 1 h, with a final step of 5 min at 99°C in a Perkin Elmer 9700 Thermal Cycler (Norwalk, CT, USA).

Q-PCR was completed using the LightCycler System with SYBR Green I sequence non specific detection (Roche Diagnostics, Barcelona, Spain). Primers for the different genes are described in Table 1. All primers were obtained from Sigma Genosys (Sigma Aldrich Química, Madrid, Spain).

Each PCR was performed in a total volume of 10 μl, made from diluted (1/10) cDNA template (2 μl), forward and reverse primers (1 μM each), and SYBR Green I master mix (1.8 μl, including Taq polymerase, reaction buffer, $MgCl_2$, SYBR Green I dye, and dNTP mix). After an initial Taq activation at 95°C for 10 min, LightCycler PCR was performed using 40–45 cycles with the cycling conditions described in Table 1. In order to verify the purity of the products, a melting curve was produced after each run by increasing the temperature of the reaction mixtures up to 95°C, by 0.1°C/s, starting at 55°C for 10 s. Values for the threshold (Ct) were determined using the LightCycler software.

Relative gene expression numbers were calculated as a percentage of control rats, using the $2^{-\Delta\Delta Ct}$ method [36]. For the genes used to validate the microarrays analysis, data were normalized against the reference genes surfeit 4 (*surf4*) and β-*actin* in control rats and guanosine diphosphate dissociation inhibitor 1 (*gdi1*) and β-*actin* in cafeteria-obese rats. *Surf4* and *gdi1* were chosen because our microarray data showed equal expression for all microarrays in control and cafeteria-obese rats, and β-*actin* was chosen because it is a well accepted reference gene.

2.9. Statistical analysis

All data are expressed as the mean±S.E.M. The statistical significance of differences in body weight, adiposity index, HOMA-IR, and circulating insulin, leptin, and glucose levels between fed animals of the control and cafeteria-obese groups was assessed by Student's *t* test.

The statistical analysis on the mRNA expression studied by Q-PCR in PBMC in different feeding conditions in control or cafeteria-obese rats was assessed by one-way ANOVA and LSD post hoc comparisons.

All the analyses were performed with SPSS for windows (SPSS, Chicago, IL, USA). Threshold of significance was defined at $P<.05$ and is indicated when different.

The statistical analysis of the microarray data has been indicated in the microarray data analysis section above.

3. Results

3.1. Body weight, adiposity and circulating parameters

As previously described in the same cohort of animals [37], at the end of the experiment, compared with control rats, cafeteria-obese rats presented a 37% increased body weight (652±4 vs. 475±22 g in cafeteria-obese vs. control rats), a 120% higher adiposity index (17.5±0.9 vs. 7.95±0.86%), and were hyperleptinemic (38.0±4.9 vs. 10.7±1.8 μg/l). In the fed state, cafeteria diet-induced obesity resulted in raised blood glucose levels (6.41±0.39 vs. 5.18±0.22 mM) and did not affect circulating insulin levels (3.44±0.98 vs.

Table 1
Nucleotide sequences of primers and cycling conditions used for Q-PCR amplification in PBMC

	Gene forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Cycling conditions	Amplicon size (pb)
Abcg1	CCCTCCTGTTATGTTCTTTGATG	GCACTGGGTTGGTGGATG	95°C for 2 s 60°C for 6 s 72°C for 6 s	132
Acat2	CACAACCTGGGGACAACTG	AAGGCACACGGCTTTTAGG	95°C for 2 s 60°C for 6 s 72°C for 9 s	213
Dhcr7	CTCACTGCCTTCACCAATCA	GGCTCTCAAAATGTCCAGGT	95°C for 2 s 60°C for 6 s 72°C for 9 s	223
Fdft1	ACCAACGCCCTACAACACAT	GTAACTGCTTGCCCCTTCC	95°C for 2 s 60°C for 6 s 72°C for 7 s	179
Lss	GCTGGCTGTCCTGAATGTTT	CGAGTGGCGTAGCAGTAGC	95°C for 2 s 60°C for 6 s 72°C for 7 s	153
Sc4mol	CCTGTTCTCTTTACCTGGCTTT	TGGCATTCTTTCCCAATCAT	95°C for 2 s 60°C for 6 s 72°C for 9 s	211
Sqle	GGCAGAGCCCAATGTAAAGT	TAATGAAGCCAACGAAGTGG	95°C for 2 s 60°C for 6 s 72°C for 9 s	218
Srebf2	GTCCTCACCTTCCTGGGTCT	CAGCAGTAGAGTCGGCATCA	95°C for 2 s 62°C for 6 s 72°C for 7 s	168
Srebf1	CCCACCCCCTTACACACC	GCCTGCGGTCTTCATTGT	95°C for 2 s 60°C for 6 s 72°C for 8 s	198
Star	GGGTGGATGGGTCAGGTC	CTGCTGGCTTTCCTTCTTCC	95°C for 2 s 60°C for 6 s 72°C for 7 s	168
B-actin	TACAGCTTCACCACCACAGC	TCTCCAGGGAGGAAGAGGAT	95°C for 2 s 60°C for 6 s 72°C for 5 s	164
Surf4	CAGCATTTTGTGGGACTTG	TGAACATCAGGACCAGCAG	95°C for 2 s 60°C for 6 s 72°C for 7 s	185
Gdi1	CCGCACAAGGCAAATACATC	GACTCTCTGAACCGTCATCAA	95°C for 2 s 60°C for 6 s 72°C for 7 s	159

Table 2
Detailed classification of genes involved in sterol metabolism processes affected by feeding conditions in control rats

Sterol metabolism	Gene name	Gene symbol	Sequence ID	Fasting vs. feeding	Refeeding vs. fasting
Biosynthesis	7-dehydrocholesterol reductase	*Dhcr7*	NM_022389	−1.30*	+1.49*
	Farnesyl diphosphate farnesyl transferase 1	*Fdft1*	NM_019238	−1.42*	+1.44*
	Hydroxysteroid (17-beta) dehydrogenase 8	*Hsd17b8*	NM_212529	−1.03	+1.21*
	Lanosterol synthase	*Lss*	NM_031049	−1.20*	+1.31*
	Phenylalkylamine Ca2+ antagonist (emopamil) binding protein	*Ebp*	NM_057137	−1.15*	+1.28*
	Squalene epoxidase	*Sqle*	NM_017136	−1.42*	+1.50*
	Sterol-C5-desaturase -like	*Sc5dl*	NM_053642	−1.13	+1.29*
	Sterol-C4-methyl oxidase-like	*Sc4mol*	NM_080886	−1.55*	+1.64*
Transport and cholesterol uptake	Acetyl-Coenzyme A acetyltransferase 2	*Acat2*	NM_001006995	−1.40*	+1.63*
	ATP-binding cassette, sub-family G (white), member 1	*Abcg1*	NM_053502	−1.29*	+1.48*
	Low density lipoprotein receptor-related protein 4	*Lrp4*	NM_031322	+1.08	−1.65*
	Low density lipoprotein-related protein 12 (predicted)	*Lrp12*	XM_235261	+1.24	−2.04*
	Steroidogenic acute regulatory protein	*Star*	NM_031558	+1.25*	−1.25*
Signaling	Membrane-bound transcription factor peptidase, site 1	*Mbtps1*	NM_053569	−1.06	+1.23*
	SNF1-like kinase	*Snf1lk*	NM_021693	+2.43*	−1.93*
	Sp1 transcription factor	*Sp1*	NM_012655	−1.05	+1.13*
	Sterol regulatory element binding factor 2	*Srebf2*	NM_001033694	−1.30*	+1.53*
	Sterol regulatory element-binding protein 1	*Srebf1*	XM_213329	−1.41*	+1.49*
	YY1 transcription factor	*Yy1*	NM_173290	−1.09	+1.23*
Others	Lysosomal acid lipase 1	*Lip1*	NM_012732	−1.50*	+1.82*
	Oxysterol binding protein-like 1A	*Osbpl1a*	NM_172023	+1.19	−2.16*

The expression of all genes changed significantly when considering feeding/fasting/refeeding conditions (one-way ANOVA and LSD *post hoc* comparisons $P<.01$). Ratios indicating fold changes in two different conditions (*fasting* vs. feeding and refeeding vs. fasting) are presented. + indicates up-regulation; − , down-regulation. Red/green colors (and asterisk) indicated that the up-/down-regulation have a $P<.05$. Specific P values and data of refeeding vs. feeding are presented in the supplementary Table 1.

3.23±0.46 μg/l). The HOMA-IR analysis indicated reduced insulin sensitivity in cafeteria-obese rats, although the difference did not reach statistical significance owing to inter-individual variability (11.0±4.9% vs. 5.25±1.52%).

3.2. Sterol metabolism-related gene expression induced by feeding conditions

Of 44,290 tested probe sets on the microarray, 21,466 probes had an expression value of two times above background. The pathway analysis of the affected processes showed that, in control rats, 21 of the genes whose expression was significantly affected by feeding conditions were involved in sterol metabolism (Table 2), whereas only seven sterol metabolism genes were significantly regulated in cafeteria-obese rats ($P<.01$, one-way ANOVA and LSD post hoc comparisons) (Table 3). In both control and cafeteria-obese rats, genes involved in sterol homeostasis were distributed into four sterol metabolism pathways: sterol biosynthesis, cholesterol transport and uptake, sterol signaling, and other processes related to sterol metabolism (Tables 2 and 3). To understand how these genes respond to feeding, fasting, and refeeding, we calculated fold changes between two different feeding conditions (fasting vs. feeding; refeeding vs. fasting; refeeding vs. feeding). Ratios indicating fold changes between fasting vs. feeding and refeeding vs. fasting are shown in Tables 2 and 3. Most of the genes showed the same expression pattern, decreasing their mRNA levels after 14 h of fasting and recovering the levels found in ad libitum fed conditions after 6 hours of refeeding.

The specific P values of all LSD post hoc comparisons (fasting vs. feeding; refeeding vs. fasting; refeeding vs. feeding) and the ratios of refeeding vs. feeding in control and cafeteria rats are presented in Supplementary Tables 1 and 2.

3.3. Confirmation of array results by Q-PCR

Q-PCR analysis were performed on total RNA from PBMC samples in order to confirm the data found with microarray analysis and to observe whether the changes were consistent. In control rats, we selected 8 genes from sterol homeostasis related processes: adenosine triphosphate (ATP)-binding cassette, subfamily G (white), member 1 (*abcg1*), acetyl-Coenzyme A acetyltransferase 2 (*acat2*), lanosterol synthase (*lss*), sterol-C4-methyl oxidase-like (*sc4mol*), squalene epoxidase (*sqle*), sterol regulatory element binding factor 2 (*srebf2*), sterol regulatory element-binding protein 1 (*srebf1*), and steroidogenic acute regulatory protein (*star*). In cafeteria-obese rats, we selected 3 genes: 7-dehydrocholesterol reductase (*dhcr7*), farnesyl diphosphate farnesyl transferase 1 (*fdft1*), and steroidogenic acute regulatory protein (*star*), but *dhcr7* and *fdft1* could not be assessed since the mRNA levels were too low to obtain meaningful results by Q-PCR. Q-PCR confirmed the microarray data for all genes, except for the *star* gene, which did not show changes in response to feeding conditions neither in control nor in cafeteria-obese rats (Table 4).

Table 3
Detailed classification of genes involved in sterol metabolism processes affected by feeding conditions in cafeteria rats

Sterol metabolism	Gene name	Gene symbol	Sequence ID	Fasting vs. feeding	Refeeding vs. fasting
Biosynthesis	7-dehydrocholesterol reductase	*Dhcr7*	NM_022389	−1.32*	+1.14
	Farnesyl diphosphate farnesyl transferase 1	*Fdft1*	NM_019238	−1.47*	+1.17
	Isopentenyl-diphosphate delta isomerase	*Idi1*	NM_053539	−1.06	−1.23*
Transport and cholesterol uptake	StAR-related lipid transfer (START) domain containing 5	*Stard5*	XM_577816	−1.36*	+1.22*
	Steroidogenic acute regulatory protein	*Star*	NM_031558	+1.30*	−1.19*
Signaling	Upstream binding protein 1 (predicted)	*Ubp1*	AI556563	−1.50*	+1.43*
Others	Lysosomal acid lipase 1	*Lip1*	NM_012732	−1.44*	+1.59*

The expression of all genes changed significantly when considering feeding/fasting/refeeding conditions (one-way ANOVA and LSD post hoc comparisons $P<.01$). Ratios indicating fold changes in two different conditions (fasting vs. feeding and refeeding vs. fasting) are presented. + indicates up-regulation; −, down-regulation. Red/green colors (and asterisk) indicated that the up-/down-regulation have a P value <.05. Specific P values and data of refeeding vs. feeding are presented in the supplementary Table 2.

Table 4
Q-PCR confirmation of microarray data

	Gene name	Gene Symbol	Sequence ID	Microarray			Q-PCR		
				*P**	Fasting vs. feeding	Refeeding vs. fasting	*P***	Fasting vs. feeding	Refeeding vs. fasting
Lean rats	Acetyl-Coenzyme A acetyltransferase 2	*Acat2*	NM_001006995	0.0046	−1.40	+1.63	0.0027	−1.23	+1.42
	ATP-binding cassette, sub-family G (white), member 1	*Abcg1*	NM_053502	0.0039	−1.29	+1.48	0.0081	−1.27	+1.45
	Lanosterol synthase	*Lss*	NM_031049	0.0030	−1.20	+1.31	0.0146	−1.11	+1.40
	Squalene epoxidase	*Sqle*	NM_017136	0.0010	−1.42	+1.50	0.0022	−1.56	+1.64
	Steroidogenic acute regulatory protein	*Star*	NM_031558	0.004	+1.25	−1.25	0.3214	+1.05	+1.10
	Sterol-C4-methyl oxidase-like	*Sc4mol*	NM_080886	0.0029	−1.55	+1.64	0.0045	−1.64	+2.01
	Sterol regulatory element binding factor 2	*Srebf2*	NM_001033694	0.0018	−1.30	+1.53	0.0013	−1.14	+1.30
	Sterol regulatory element-binding protein 1	*Srebf1*	XM_213329	0.0007	−1.41	+1.49	0.0003	−1.29	+1.51
Cafeteria rats	Steroidogenic acute regulatory protein	*Star*	NM_031558	0.0089	+1.30	−1.19	0.5489	−1.00	+1.14

**P* value of microarray data and ***P* value of Q-PCR data when considering feeding/fasting/refeeding conditions are given (one-way ANOVA). Threshold of significance was defined at $P<.01$ and $P<.05$ for microarray and Q-PCR data. Ratios indicating fold changes in two different conditions (fasting vs. feeding and refeeding vs. fasting) are presented for microarray and Q-PCR data. + indicates up-regulation; −, down-regulation.

The *P* value for one-way ANOVA in the Q-PCR analysis reached significance ($P<.05$) for seven out of eight genes. The *star* gene expression had a *P* value of >.05.

4. Discussion

PBMC are being used in gene expression studies because they can be easily and repeatedly collected from blood in sufficient quantities and can reflect the pathological and physiological state of a person or animal [22–25]. Different studies have described that blood cells are able to synthesize cholesterol [14–21]; however, up to now, there are only scarce data available in PBMC on how different feeding conditions, ad libitum feeding, fasting, and refeeding, affect the expression of genes related with sterol metabolism and how a dietary induced obesity affects the nutritional modulation of these genes. Our data indicate that in response to different feeding conditions, there are important changes in the expression of key genes involved in sterol metabolism in control rats and that these changes are attenuated in cafeteria-obese rats. An impairment of the nutritional control in the overweight or obese state has been well described for some genes involved in energy homeostasis in studies performed with tissues related with energy maintenance such as adipose tissue [38–40]. Our data give evidence that this impairment also occurs in cafeteria-obese rats with genes involved in sterol metabolism and in a tissue which is not pivotally related with energy homeostasis, such as blood cells. In principle, this alteration in the response to feeding conditions could be related to an impaired insulin response in the cafeteria-obese animals, as they have a higher HOMA-IR, and moreover, it has been described an alteration in binding or binding affinity for the insulin receptor in mononuclear cells in the obese state [41]. However, further studies are required to clarify other potential pathophysiological implications.

Our results show that most of the genes involved in sterol metabolism whose expression changed in response to different feeding conditions, both in control and cafeteria-obese animals, showed the same pattern of expression, decreasing their mRNA levels after 14 h of fasting and recovering the levels found in *ad libitum* fed conditions after 6 hours of refeeding. Interestingly, this agrees with the nutritional modulation of several genes involved in sterol homeostasis described in the liver [9–11], and suggests similar regulatory mechanisms in response to different feeding conditions in both PBMC and liver. Although the number of sterol metabolism-related genes altered was higher in control than in cafeteria-obese rats, in both rat models genes involved in sterol biosynthesis, cholesterol transport and uptake, and sterol signaling were found to respond to fasting and refeeding.

4.1. Sterol biosynthesis

Cholesterol synthesis is mediated by an extensive series of biochemical reactions which can be considered in four big steps: (1) synthesis of mevalonate from acetyl-CoA; (2) conversion of mevalonate to squalene; (3) cyclization scheme of squalene to lanosterol and (4) cholesterol synthesis from lanosterol (Ref. [42] and reviewed in Refs. [1,43,44]). In our microarray analysis of control rats we found that, except farnesyl-diphosphate farnesyltransferase (*fdft1*) — also known as squalene synthase (*sqs*) — a gene that codifies for a key enzyme involved in the last step of squalene synthesis, most of the genes regulated by feeding conditions were operating in the two last steps of cholesterol biosynthesis. Thus, squalene epoxidase (*sqle*) and lanosterol synthase (*lss*) have a role in lanosterol synthesis, whereas sterol-C-5 desaturase-like (*sc5dl*) — also known as lathosterol oxidase — sterol-C-4-methyl oxidase-like (*sc4mol*), phenilalkylamine Ca2+ antagonist (emopamil) binding protein (*ebp*), and 7-dehydrocholesterol reductase (*dhcr7*) participate in the last step of cholesterol synthesis, which consist in 19 consecutive reactions. Furthermore, our results showed that hydroxysteroid (17-beta) dehydrogenase 8 (*hsd17b8*), a gene involved in steroid hormone metabolism, was also regulated in response to the different feeding conditions. Interestingly, except the *hsd17b8* gene, all of these genes were down-regulated in response to fasting and their mRNA levels recovered after a 6-h refeeding in comparison with ad libitum fed animals. Several studies performed years ago suggested that fasting markedly reduces the rate of cholesterol synthesis in liver [45,46] through a severe depression of the activity of HMG-CoA reductase, a key enzyme that participates in the first step of sterol biosynthesis, controlling the conversion of HMG-CoA to mevalonate. Moreover, another study demonstrated that, in liver, this enzyme and others involved in the first steps of cholesterol synthesis are very sensitive to the nutritional state of the animal, declining rapidly on fasting and returning to normal of above normal levels during refeeding [46]. However, in our study, no significant changes in the expression of the gene that codifies for HMG-CoA reductase were found in PBMC in response to different feeding conditions, differently to what previously observed in liver. On the contrary, our results indicate that genes involved in the two last steps of sterol biosynthesis seem to be more sensitive to nutritional control than those involved in the first steps, at least in PBMC. Related to this, it has been shown that the conversion of squalene to sterols (the two last steps in sterol biosynthesis) was also reduced in the liver of starved rats and suggested that cholesterol biosynthesis may be regulated in two critical points: between mevalonate and squalene and between squalene and cholesterol, by changes in enzyme activities [47,48]. In our study, six out of the eight genes involved in sterol biosynthesis altered in response to feeding conditions were involved in the

conversion of squalene to cholesterol whereas only one (*fdft1*) participated in the synthesis of squalene. Thus, while PBMC and liver have a similar expression profile, some different sensitivity to nutritional modulation exists between genes expressed in both tissues. It is of note that the number of genes affected by feeding conditions in cafeteria-obese animals was highly reduced, with only two of the above commented genes changing their expression (*dhcr7* and *fdft1*).

4.2. Cholesterol transport and uptake

Fasting and refeeding also affected the expression of genes involved in cholesterol transport and uptake. Here, we report that 5 of these genes were regulated in response to different feeding conditions in control rats, whereas only 1 was found to be affected in cafeteria-obese rats.

Acetyl-CoA acetyltransferase 2 (ACAT2) is an enzyme mainly found in the intestine and liver that catalyzes the formation of cholesteryl esters, playing an important role in lipoprotein metabolism in these tissues [49–51]. Moreover, this enzyme has been related with atherosclerosis by participating in accumulating cholesterol esters in macrophages and vascular tissue [52]. Our microarray data show that the gene that codifies for ACAT2, *acat2*, is expressed in PBMC and indicate for the first time that the expression of this gene is strongly modulated by feeding conditions in these cells, decreasing their mRNA levels after 14-h fasting and increasing after 6-h refeeding, in control rats but not in cafeteria-obese rats. Furthermore, we showed that *abcg1*, a gene that codes for the ATP-binding cassette, subfamily G (white), member 1 (ABCG1), a protein that is involved in macrophage cholesterol and phospholipid transport [53,54], had a similar expression pattern as the *acat2* gene, with its nutritional modulation being also impaired in cafeteria-obese rats. The strong nutritional modulation of these 2 important genes, *acat2* and *abcg1*, observed in our study, suggest that cholesterol transport in PBMC is acutely controlled by feeding/fasting fluctuations in control rats; the impairment of this process in cafeteria-obese rats could be related to pathophysiological complications. Moreover, although not confirmed by Q-PCR, our results indicate that the *star* gene, which codifies for the steroidogenic acute regulatory protein (StAR), a transport protein that modulates cholesterol transfer within the mitochondria for the production of steroid hormones in adrenal cortex, gonads, brain and placenta [55–58], is also expressed in PBMC and is affected by different feeding conditions in both control and cafeteria-obese rats. The low-density lipoprotein (LDL) receptor (LDLR) is a cell-surface receptor that mediates the endocytosis of cholesterol-rich LDL, chylomicron remnants, and very LDL remnants (IDL) [59–61]. Here, we show that *lrp4* and *lrp12* genes, which codify for 2 members of LDLR superfamily, low density lipoprotein-receptor related protein 4 and 12, are expressed in PBMC and, contrary to what observed for most of the genes regulated by feeding conditions found in our study, their expression was not significantly affected by fasting but was strongly down-regulated by refeeding conditions in control rats. Although several studies have demonstrated that LDLR protein levels do not change during fasting or refeeding [62,63], it is well established that insulin increases the *ldlr* transcription via SREBP [64,65]. Thus, the nutritionally modulated expression pattern observed for *lrp4* and *lrp12* genes in comparison with the expression pattern described for the *ldlr* gene reinforces the hypothesis that these two genes are more involved in other processes such as signal transduction [66–68] than in cholesterol homeostasis.

4.3. Sterol signaling

Interestingly, our results indicate that, in PBMC of control rats, important genes involved in the regulation of sterol synthesis and cholesterol uptake are also under a significant nutritional modulation and that this modulation is altered in cafeteria-obese rats. Several studies have demonstrated that, in the liver, the expression of the genes that codify for the sterol regulatory element binding proteins SREBP-1 and -2, *srebf1* and *srebf2*, are controlled by fasting/refeeding fluctuations [9–11], and it is known that the effect of feeding conditions is stronger and more dramatic on srebf1 than on srebf2 mRNA levels [11]. In our study, we describe that *srebf1* and *srebf2* are also regulated by feeding conditions in PBMC of control rats, showing a similar expression pattern to that described in the liver, decreasing their mRNA levels after 14-h fasting and with restored levels after 6-h refeeding. Moreover, also as occurs in the liver, our data indicate that *srebf1* expression was more significantly affected by fasting conditions than *srebf2*, although the increase of their mRNA levels in response to refeeding was significant and changed comparable in both genes. At high levels of expression, all SREBP isoforms can activate genes involved in sterol and fatty acid metabolism, but under normal conditions, SREBP-1c mainly regulates the lipogenesis pathway, whereas SREBP-2 is the predominant isoform affecting cholesterol homeostasis (reviewed in Refs. [1–5]). It is suggestive that five genes involved in cholesterol synthesis that have shown expression changes in our study in response to fasting and refeeding conditions (*dhcr7*, *fdft1*, *lss*, *sqle*, and *sc5dl*) are known to be regulated by SREBP-2 (reviewed in ([3,5]) and all of these genes present the same nutritional expression pattern than *srebf2*. Moreover, we found that *mtbps1*, a gene that codifies for the Site-1 protease (S1P), which catalyzes the first step in the proteolytic activation of SREBPs (revised in Refs. [1–5]), as well as *sp1* and *yy1* genes, which codify for two transcription factors, Sp1 and YY1, required for the transcriptional control of several SREBPs-sterol regulated genes such as *abcg1* and *star* [69,70] were also affected by feeding conditions in PBMC of control rats. All in all, these data indicate that cholesterol homeostasis is highly regulated in PBMC in response to different feeding conditions in control rats and that this is most likely mediated by SREBPs.

In summary, here, we report that in PBMC of control rats many genes involved in sterol metabolism are under an acute nutritional regulation and that this regulation is impaired in cafeteria-obese rats, with unknown pathophysiological implications. Moreover, the expression pattern of these genes in response to fasting/refeeding fluctuations observed in PBMC is, to an important extend, comparable to that previously described for liver, which indicates that similar modulatory mechanism in sterol metabolism in response to feeding conditions are operating in both tissues. The observed response to feeding conditions in control vs. cafeteria-obese rats, of the PBMC gene expression related with cholesterol metabolism, supports its use to characterize potential aspects of cholesterol metabolism in physiology or pathology.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2009.10.001.

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