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Diferential gene expression and adiposity reduction induced by ascorbic acid supplementation in a cafeteria model of obesity

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Obesity is considered as an inflammatory disease, in which free radical-induced oxidative stress and excessive intake of macronutrients exacerbate their symptoms. In this context, we assessed in rats the possible preventive effect of the supplementation with an antioxidant molecule, ascorbic acid, in order to reduce the adiposity induced by the intake of a high-fat diet. For this purpose, during 56 days, three groups of male Wistar rats were fed on: a) standard pelleted diet, b) Cafeteria diet, c) ascorbate-supplemented (750 mg/kg of body weight) Cafeteria diet. At the end of the experimental period, microarray analysis was used to identify genes transcriptionally induced or repressed by both experimental dietary models (Cafeteria diet supplemented or not with ascorbic acid) in subcutaneous adipose tissue. Dietary ascorbic acid was able to protect against high fat diet effects, reducing the increase of body weight, total body fat and enlargement of different adipose depots induced by the Cafeteria diet without affecting food intake. An association analysis accurately and differentially allowed the detection of gene expression changes related with adiposity and insulin resistance. The genes that more strongly correlated with body fat and HOMA insulin resistance index were involved in adipocyte differentiation, lipid and glucocorticoid metabolism, cell cycle regulation, as well as in several insulin-induced processes. Some other transcripts are regulated by the vitamin C-mediated reduction of adiposity, such as genes that participate in glucocorticoid metabolism, adipogenesis, pentose phosphate pathway, or tricarboxylic acid cycle. This strategy was able to link variations in adipose tissue gene expression with markers of diet-induced obesity in rats, such as insulin resistance and body fat content.

Key words: Vitamin C, Obesity, Cafeteria diet, Microarray, Gene expression.

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Microarray technology is able to assess gene expression changes caused by complex diseases, such as diabetes (16) and atherosclerosis (28) in different tissues. Such approach has been also developed in epididymal adipose tissue (19), skeletal muscle (34), and liver (15) of diet-induced obese rodents. In this sense, Cafeteria diet is a recognized dietary model of animal obesity that shares common Western diet features, such as high-fat intake and hyperphagia, which are thought to drive to hyperinsulinemia, type 2 diabetes, and metabolic syndrome conditions (5, 22, 26).

An enhanced oxidative stress status has been documented in obese patients and animals (12). In addition, the intake of a high-fat diet also increases tissue oxidative stress (32). Likewise, a depletion of the antioxidant mechanisms in excessive weight gain conditions is a common feature (24). In this context, we hypothesized that food supplementation with an antioxidant molecule such as vitamin C, a free-radical scavenger with strong antioxidant properties (11), could reduce fat deposition when conjointly administered within a high fat diet in rats.

The purpose of this work was to find out whether the physiological changes observed in obese animals supplemented or not with vitamin C were mirrored in global gene expression changes, as occurs with fat total content and HOMA insulin resistance index. For this goal, we have applied microarray technology and correlation analysis for identifying genes that are related with adiposity and insulin resistance in subcutaneous white adipose tissue of high-fat fed Wistar rats after vitamin C supplementation.

Materials and Methods

Animals, diets and experimental design.– Male Wistar rats, supplied by the Applied Pharmacobiology Center (CIFA, Pamplona, Spain), were housed at 21-23 ºC with a 12 hours light cycle (8 a.m. to 8 p.m.) and assigned into three different dietary groups. A group of animals (Control group) were fed on standard pelleted diet (Harlan Iberica, Barcelona, Spain) containing 16.6% of energy as protein, 73.1% of energy as carbohydrate and 10.3% of energy as lipid by dry weight. The second group of animals (Cafeteria) were fed on a high-fat diet in order to generate a diet induced obesity model as previously reported (5, 17, 22, 26). High-fat diet components were paté, bacon, chips, cookies, chocolate and chow with proportions 2:1:1:1:1:1, and the diet was given to each rat daily. The composition of this Cafeteria diet was 9.3% of energy as protein, 31.5% of energy as carbohydrates and 59.2% of energy as lipids by dry weight (462 Kcals per 100 g in the Cafeteria diet vs. 349 Kcals per 100 g in the pelleted diet). Cafeteria diet was supplemented in a third group of animals with a daily dose of vitamin C (750 mg/Kg rat) mixed into the food (CafVit group). Chow diet does not contain ascorbic acid and the amount of this vitamin in the Cafeteria diet is 0.86 mg per 100 g. Animals had *ad libitum* access to water and food during the experimental trial. Body weight, food and water intake were recorded daily. At the end of the experimental period (56 days), rats were anaesthetized in the fasted state with Ketamine (50 mg/kg *ip*, Parke-Davis, Madrid, Spain) and Medetomidine (0.025 mg/kg *ip*, Pfizer S.A., Madrid, Spain) for the analysis of the body composition. Then, animals were sacrificed by decapitation and blood and

tissue samples were immediately collected and weighed. All the procedures were performed according to national and institutional guidelines of the Animal Care and Use Committee at the University of Navarra. Body composition was assessed at the end of the experimental period in overnight fasted animals under anesthesia, by using a non-invasive electromagnetic apparatus devised for rodents (EM-SCAN model SA-2, Springfield, IL, USA) as described elsewhere (6). After the sacrifice of the animals, epididymal, retroperitoneal and subcutaneous white adipose tissue were carefully excised, isolated, weighed, and stored at –80 ºC.

Serum measurements.– Glucose was measured with the HK-CP kit (ABX diagnostic, Montpellier, France) adapted for a COBAS MIRA (Roche, Basel, Switzerland) equipment. Serum leptin and insulin were assayed by radioimmunoassay, as described by the supplier (Linco Research, Missouri, USA). The homeostatic model assessment (HOMA) as an insulin resistance index was calculated using the following formula: (fasting plasma insulin x plasma glucose)/22.5.

Microarray analysis.– Total RNA was extracted for microarray analysis from frozen subcutaneous adipose tissue of selected animals from the experimental trial of 56 days (n = 4, for Control group; n = 4 for Cafeteria group and n=4 for Cafvit group) using sequentially Trizol (Invitrogen, CA, USA) and RNeasy Mini Kit (QIAGEN Inc., CA, USA) according to the manufacturer's instructions. Total RNA (5 µg) was used for synthesizing cDNA using One-Cycle cDNA synthesis kit (Affymetrix, CA, USA) following the Expression Analysis Technical Manual (Affymetrix). The purified cDNA was used as a template for *in vitro* transcription reaction for the synthesis of biotinylated cRNA using the IVT labelling kit (Affymetrix) and purified using GeneChip Sample Cleanup Module (Affymetrix). Finally, cRNA was fragmented and hybridized in the Rat genome RAE230 2.0 array (Affymetrix). For the processing of the chips and the results, GeneChip Operating Software (GCOS 1.2) was applied and GeneSpring v7.2 (Agilent), dChip (18) and Affy/AffyPLM (Bioconductor) software were used to perform gene expression analysis. After global normalization (RMA (4) and median normalization), a Pearson correlation analysis was performed comparing gene expression values with total fat content (g) and HOMA insulin resistance index. For the selection of the fat- and HOMA-correlated genes, Pearson Coefficient ≥ 0.7 was considered as cut-off criterion. Finally, after gene filtering for each experimental measurement, significant genes were calculated applying a parametric test assuming not equal variances (ANOVA or Welch´s approximate *t* test for two groups). Fold changes (ratio of the average of signals of the three different groups) for Cafeteria/Control and Cafvit/Cafeteria group were calculated and analyzed. The final criterion of selection of the differentially fat- and HOMA-correlated genes was a p-value <0.1.

Statistical analysis.– All results are expressed as mean ± standard error of the mean (SEM). The differences between the groups were evaluated by one-way ANOVA, and Tukey post-hoc test was applied when suited (SPSS 13.0 packages for Windows, Chicago, IL, USA). A level of probability up at $p<0.05$ was set up as statistically significant. Correlation analysis was performed with the Pearson corre-

lation coefficient (R), being 1 for a perfect correlation, 0 for no relationship what-soever. Only genes with a Pearson R of 0.7 or higher have been included in the analysis.

Results

Body weight, adipose tissue weights and food intake.– Rats fed on Cafeteria diet, as expected, weighed more and showed more adiposity than their control counterparts (Fig. 1). Vitamin C supplementation reduced body weight and total fat content, and the subcutaneous and retroperitoneal fat depots tended to decrease in comparison with those of non-supplemented Cafeteria fed rats (Fig. 1).

Biochemical serum values.– Feeding on Cafeteria diet for 56 days induced hyperglycemia, hyperinsulinemia, and hyperleptinemia in male Wistar rats, as well as insulin resistance (Fig. 2). Vitamin C supplementation was able to reduce the levels of circulating leptin (as it had done with the adiposity), without significantly affecting the other serum markers.

Microarray analysis.– After microarray data analysis of the mRNA gene expression from subcutaneous adipose tissue of the three experimental groups, 6800 transcripts and variants were detected (23% of the total). After applying the criterion of selection in order to obtain fat- and HOMA-correlated genes (> 0.7 Pearson

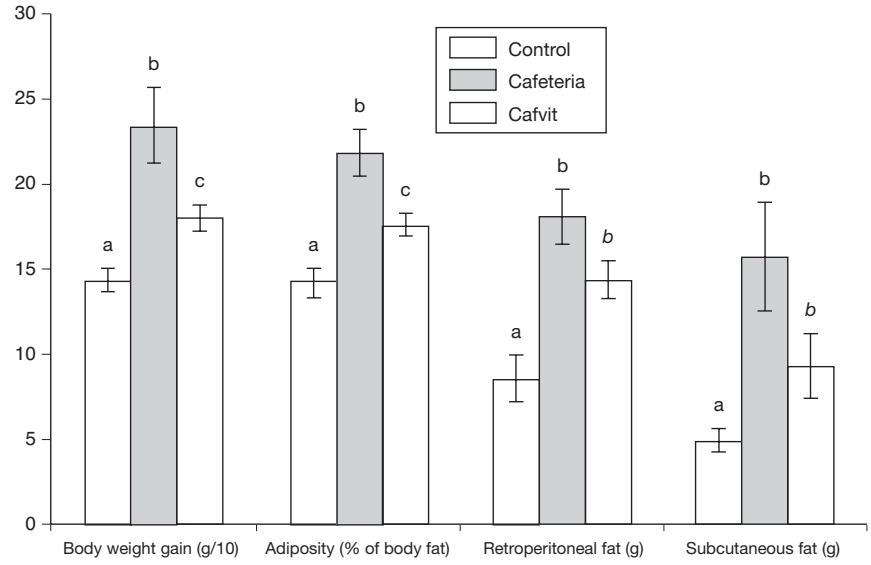

Fig. 1. *Variations in body weight gain, total fat content and two analyzed fat depots (retroperitoneal and subcutaneous) from rats fed for 56 days with pelleted diet (Control), high fat diet (Cafeteria) or high fat diet supplemented with ascorbic acid (Cafvit).*

Statistical analysis was performed using Student's *t* test, and groups sharing the same superscript are not significantly different, meanwhile groups with different superscript are significantly different, with, at least, $p<0.05$ (italic letter means marginal differences).

correlation test, considered as strong relationship), 191 (98 positively correlated/93 negatively correlated) transcripts were detected as fat-correlated, and 224 (+101/-123) as HOMA-correlated when comparing Control and Cafeteria groups. Regarding the Cafeteria-Vitamin C comparison, 1338 (+1157/-181) transcripts were detected as fat-correlated, and 1182 (+573/-609) as HOMA-correlated.

In order to choose only those transcripts that were differentially regulated by the diet, a criterion of $p<0.1$ for the comparison Control-Cafeteria and Cafeteria-vitamin C (Student's *t* test for the fold change of induction/repression) was applied. Thus, 182/172 transcripts (Control-Cafefeteria/Cafeteria-Vitamin C) were differentially expressed by the diet and strongly correlated with body fat, and 138/50 transcripts with HOMA. A list of selected genes playing a possible role in the development of obesity induced by the high-fat diet is reported in Table I. Also, after applying the criteria of selection for fat content, HOMA, or both, the genes regulated by the adiposity reduction mediated by vitamin C supplementation are presented in Table II.

Association analysis. - Genes that positively associated with body fat (Table I) are related with adipocyte differentiation and lipid metabolism. Upon including also those HOMA-correlated transcripts, the involved metabolic pathways also encompasses glucocorticoid metabolism, unsaturated fatty acid biosynthesis, and cell cycle regulation, among others. On the contrary, pathways that are negatively correlated with HOMA and body fat include β-oxidation and several insulin-induced genes (table I).

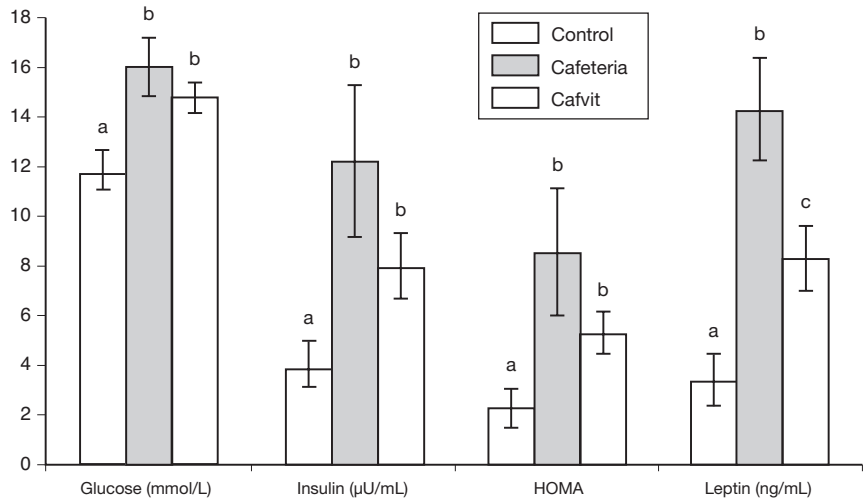

Fig. 2. *Variations in several serum measurements (glucose, insulin, HOMA, and leptin) from rats fed for 56 days with pelleted diet (Control), high fat diet (Cafeteria) or high fat diet supplemented with ascorbic acid (Cafvit).* Statistical analysis was performed using Student's *t* test, and groups sharing the same superscript are not significantly different, meanwhile groups with different superscript are significantly different, with, at least, $p<0.05$.

Table I. *List of selected genes, including Entrez Gene ID, playing a possible role in development of obesity induced by the diet and fulfilling the criterion of selection (> 0.7 for Pearson correlation factor for fat content, HOMA or both) and Student't test p < 0.1 for fold changes (Control vs Cafeteria group).*

Genes positively correlated *(Upregulated by Cafeteria diet)*		Genes negatively correlated *(Repressed by Cafeteria diet)*	
	Fat		
140868	fatty acid binding protein 5, epidermal	259247	alpha-2u globulin PGL4
286939	adipocyte-specific adhesion molecule	29740	Dodecenoyl-coenzyme A delta isomerase
25317	Fibroblast growth factor 1		
79131	fatty acid binding protein 3		
89784	isopentenyl-diphosphate delta isomerase		
	HOMA		
79431	Basic helix-loop-helix domain containing, B2	24484	insulin-like growth factor binding protein 3
		295691	frizzled-related protein (predicted)
		25086	cytochrome P450, family 2, subfamile e, pp
	Fat and HOMA		
25342	oxytocin receptor	89813	pyruvate dehydrogenase kinase, isoenzyme 4
303565	glucose 6 phosphatase, catalytic, 3	25256	flavin containing monooxygenase 1
25341	TNF receptor superfamily, 11b (osteoprotegerin)	65038	inositol polyphosphate phosphatase-like 1
25117	hyroxysteroid 11-beta dehydrogenase 2		
246074	stearoyl-Coenzyme A desaturase		
25557	steroidogenic acute regulatory protein		
64532	adenylate cyclase 5		
246060	cyclin-dependent kinase inhibitor 1C (P57)		

Many more genes are regulated by vitamin C-mediated adiposity reduction (Table II). For instance, genes participating in glucocorticoid metabolism, adipogenesis, pentose phosphate pathway, or tricarboxilic acid cycle are inhibited by vitamin C and positively correlated with body fat, whereas the insulin-dependent GLUT4 facilitated glucose carrier or the LDL pro-oxidative paraoxonase 3 gene, are negatively correlated with fat content.

Discussion

Multiple cellular processes have been implicated in obesity development, such as oxidative stress (22), inflammation (7), alterations of lipid and carbohydrate metabolism, effects of hormones, or transcription regulation (23). Nevertheless, some of the physiopathological factors participating in these processes remain unclear (21). There are many reasons why understanding the pathogenesis of obesity is complicated. The extent and complexity of the disease, the affected number of genes, tissues, metabolic pathways involved, the different dietary models studied, genetic backgrounds, and stages of the pathogenic process are just few reasons to mention. We have addressed the problems of the high complexity and overlapping criteria by using a strategy combining gene microarray technology, that facilitates the simultaneous measurement of the expression of thousands of genes (36), with statistical correlation analyses (25). This strategy allows the linking of variations in adipose tissue gene

Table II. *List of selected genes, including Entrez Gene ID, playing a possible role in prevention by Vitamin C of obesity induced by the diet and fulfilling the criterion of selection (>0.7 for Pearson correlation factor for fat content, HOMA or both) and Student't test p < 0.1 for fold changes (Cafeteria* vs *Cafvit group).*

Genes positively correlated *(Repressed by Vitamin C)*		Genes negatively correlated *(Upregulated by Vitamin C)*	
	Fat		
25557	steroidogenic acute regulatory protein	25139	insulin-dependent glucose transporter GLUT4
89784	isopentenyl-disphosphatase Δ isomerase	312086	paraoxonase 3
24377	glucose-6-phosphate dehydrogenase	298085	glyoxylate reductase/hydroxypyruvate reductase (p)
25104	Pyruvate carboxylase	192272	Mitochondrial acyl-CoA thioesterase 1
171516	20 alpha-hydroxysteroid dehydrogenase	259247	alpha-2u globulin PGCL4
84006	Protein kinase C, lambda		
78968	sterol regulatory element binding factor 1		
24413	Glucocorticoid receptor		
	HOMA		
309918	elongation factor RNA polymerase II 2 (p)	25560	ATP-binding cassette, sub-family C (CFTR/MRP), m9
		497758	lecythin-retinol acyltransferase
	Fat and HOMA		
85249	peroxisomal biogenesis factor 11A	83514	delta sleep inducing peptide, immunoreactor
360716	ATPase, H^+transporting V1 subunit A, is 1	81526	nephroblastoma overexpressed gene
25357	thyroid hormone responsive protein		
29740	Dodecenoyl-coenzyme A delta isomerase		
360785	adaptor protein complex AP-1, sigma 1 (p)		
64532	Adenylate cyclase 5		
25541	sterol carrier protein 2		

expression to pathological markers of diet-induced obesity in rats, such as insulin resistance and body fat content. This approach is similar to that applied by other authors to study differences related to adipocyte size and insulin signalling (3).

Thus, the current work provides new information about transcriptional regulation of a number of genes, some of them identified in subcutaneous fat for the first time, and some others with unknown function at the moment. Moreover, the real interest of this paper's approach is the application of these data for studying the molecular mechanisms regulating gene expression in white adipose tissue of diet-induced obese animals, as well as the molecular effects of antioxidant (ascorbic acid) supplementation in obesity. In fact, antioxidant supplementation has been previously applied in other metabolic alterations, such as type 2 diabetes (30) and endothelial dysfunction (33). Thus, ascorbic acid supplementation of obese diabetic rodents is able to inhibit protein glycation (8), ameliorate hyperinsulinemia, and improve glucose homeostasis (1).

Searching for genes that could be considered as good markers and that are involved, as cause or as consequence, in obesity development, we have identified several transcripts strongly and positively correlated with adiposity. Among them, genes belonging to adipocyte differentiation and fatty acid transport pathways are upregulated in obese animals, as was sim-

ilarly observed in visceral fat (19, 20), but also genes playing a role in isoprenoid and unsaturated fatty acid biosynthesis. Genes participating in steroid metabolism, such as steroidogenic acute regulatory protein and hydroxysteroid 11- β dehydrogenase 2, are also altered by diet-induced obesity, as suggested by other authors (35).

On the other hand, some genes participating in fatty acid β-oxidation, such as dodecenoyl-coenzyme A delta isomerase, are repressed by Cafeteria diet and correlate negatively with body fat and insulin resistance. Similar pattern has been observed for other genes partaking in xenobiotics oxidation, *i.e.* flavin containing monooxygenase 1, and insulin-mediated mitogenesis, *i.e.* inositol polyphosphate phosphatase-like 1. Similar findings have been reported by other authors when comparing adipose tissue of lean and different strains of obese mice (25).

Interestingly, when Cafeteria diet was supplemented with ascorbic acid, a decrease in animal body weight and fat content was observed, suggesting that this compound exerts protective effects on obesity development, perhaps through its antioxidant properties. This work is apparently the first one that applies microarray technology to the study of ascorbic acid effects on adipose tissue, although other reports have used this methodology in the study of the effects of this compound on nervous system (37) and human breast cancer (14). The preventive effect of ascorbic acid on adiposity is accompanied in subcutaneous adipose tissue by a repression of mRNAs encoding proteins participating in the pentose phosphate cycle, tricarboxilic acid cycle, progesterone and isoprenoid biosynthesis, and adipogenesis. The expression of genes belonging to these metabolic pathways appears strongly correlated with body fat content. More particularly, the data support the hypothesis that vitamin C presents a general inhibitory effect on steroidogenesis (27) and could affect the release of glucocorticoids from adrenal gland in rodents (9). In addition, genes of other metabolic pathways correlate not only with fat percent, but also with insulin resistance, such as unsaturated fatty acid β-oxidation, cholesterol transport, and ATPase function.

On the other hand, other pathways were upregulated by vitamin C supplementation, being thus negatively correlated with fat content. This is the case of the insulin-dependent glucose transporter GLUT4, also a transporter of dehydroascorbic acid (31), and several genes that play a role in metabolism, i.e., acyl-coenzyme A thioesterase 1, an enzyme that promotes medium- and long-chain acyl-CoAs oxidation (13), glyoxiylate reductase/hydroxypyruvate reductase, which detoxifies glycine-derived glyoxylate into glycolate (2), and the secreted antioxidant glycoprotein paraoxonase 3, that is located on the HDL fraction and protects against LDL oxidation (10), thus preventing atherosclerosis (29).

In conclusion, adiposity reduction mediated by vitamin C intake inhibited adipogenesis in subcutaneous fat pad, enhanced glucose utilization and fatty acid oxidation, and ameliorated blood lipid profile. However, it is difficult to assess whether the expression changes of this group of genes are directly induced by ascorbic acid or they are a consequence of the vitamin C-mediated weight reduction. This article provides new light concerning adipose tissue regulation in situations of weight gain and insulin resistance, relating obesity with variations in the expression of genes in this tissue. Some of the described genes appear likely to be

directly regulated by body fat content, other genes could be dependent on insulin resistance, whereas other differentially regulated transcripts could be related with the amount or type of dietary fat or by the antioxidant status. The specific study of these genes could offer new insights into the knowledge of obesity development, and even some of them could be considered as predictors or early markers of adiposity or insulin resistance.

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J. CAMPIÓN, F. I. MILAGRO, D. FERNÁNDEZ y J. A. MARTÍNEZ. *Efecto de la suplementación con ácido ascórbico sobre la expresión génica diferencial y la adiposidad en un modelo de obesidad inducida por la dieta.* J. Physiol. Biochem., **62** (2), 71-80, 2006.

La obesidad está considerada como una enfermedad inflamatoria, en la que el estrés oxidativo, inducido por los radicales libres, y la excesiva ingesta de macronutrientes incrementan sus síntomas. En este trabajo se estudia el posible efecto preventivo de la suplementación con la molécula antioxidante ácido ascórbico, para reducir la adiposidad inducida por la ingesta de una dieta rica en grasas en rata. Para este propósito, tres grupos de ratas macho de estirpe Wistar se alimentaron durante 56 días con: a) dieta estándar, b) dieta de cafetería, c) dieta de cafetería suplementada con ácido ascórbico (750 mg/kg de peso corporal). Al final del período experimental, se aplicó un análisis por microarray en el tejido adiposo subcutáneo para identificar los genes inducidos o reprimidos por ambos modelos dietéticos experimentales. El ácido ascórbico pudo proteger contra los efectos proadipogénicos de la dieta, previniendo del aumento en el peso corporal, del contenido total de grasa corporal y del peso de los diversos depósitos adiposos inducidos por la dieta de cafetería. Un análisis de asociación permitió además la detección de los cambios en la expresión de los genes más relacionados con la resistencia a insulina y la adiposidad. Los genes que correlacionaron más fuertemente con el porcentaje de grasa corporal y con el índice HOMA están implicados en la diferenciación adipocitaria, el metabolismo de lípidos y de glucocorticoides y la regulación del ciclo celular, así como en varios procesos regulados por la insulina. Otros genes que se mostraron relacionados con la reducción de la adiposidad debida al ácido ascórbico participan en el metabolismo de los glucocorticoides, la adipogénesis, la vía de las pentosas fosfato o el ciclo de los ácidos tricarboxílicos. En suma, la estrategia utilizada permite identificar variaciones en la expresión diferencial de genes del tejido adiposo blanco y asociarlos con marcadores de obesidad inducida por la dieta en ratas tales como la resistencia a la insulina y el porcentaje de grasa corporal.

Palabras clave: Vitamina C, Obesidad, Dieta de cafetería, Microarray, Expresión génica.

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