Exposure to an Obesity-Inducing Diet Early Affects the Pattern of Expression of Peroxisome Proliferator, Retinoic Acid, and Triiodothyronine Nuclear Receptors in the Rat

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Since evidence has appeared that α and γ isoforms of the peroxisome proliferator receptors (PPARs) are involved in the regulation of triglyceride homeostasis and in the control of the differentiation of adipocytes that is required for the development of obesity, a large number of studies have investigated the physiologic role of nuclear receptors in the control of energy balance. The aim of this study was to determine the early effects of an obesity-inducing diet on the expression of PPARα and γ and other nuclear receptors such as all-*trans* retinoic acid receptor (RAR) and triiodothyronine receptor (TR), which all form functional heterodimers with a common partner, the *9-cis* retinoic acid receptor (RXR). The experiment used a cafeteria diet where 60% of the energy was supplied as lipids. This diet was offered to young rats for 8 and 28 days and the expression of nuclear receptors was determined at the end of each experimental time period (1) in the liver by assaying the binding properties of RAR and TR and by quantifying mRNA levels of RARβ, TR$\alpha_1\beta_1$, RXRα, and PPARα, and (2) in the white adipose tissue (WAT) by quantifying mRNA levels of RARα, RXRα, TR$\alpha_1\beta_1$, and PPARγ_2. After 8 days of cafeteria diet a significant decrease of RAR and TR maximal binding capacity (MBC) was observed in the liver (-20.1% and –35.0%, respectively, $P < .05$) and the level of the mRNA of RARβ was significantly decreased (-17.4%, $P < .05$). After 28 days of cafeteria diet, the level of the mRNA of PPARα and acyl-CoA oxidase (ACOX) was significantly increased (+54.5% and +37.8%, $P < .01$ and $P < .05$, respectively), whereas the MBC of RAR and TR was significantly decreased (-16.0% and –23.4%, $P < .01$), as were the mRNA levels of RARβ and TRα_1 β_1 (-28.5% and –32.0%, $P < .05$). The level of RXRα mRNA was unchanged. In WAT, the mRNA level of PPARγ_2 was significantly increased after 28 days of cafeteria diet (+49.5%, $P < .05$) and the mRNA levels of RARα and TRα_1 β_1 significantly decreased (-22.3% and –31.0%, $P < .05$). These results as a whole showed that a high-fat diet can induce early modifications in the pattern of expression of nuclear receptors in the liver and the WAT. These modifications could be compatible with an early adaptive phenomenon. Further investigations are necessary to better understanding the link between the modifications of the pattern of expression of these receptors and plasticity of adipose tissue leading to the onset of obesity.

THERE IS ACCUMULATING epidemiologic evidence that body weight and the prevalence of obesity are rising rapidly in many countries.[1] A number of studies have suggested that environmental factors, and notably dietary factors, are associated with obesity. Macronutrient energy distribution and particularly calories supply from dietary fat have received attention.[2] Great progress has been made in knowledge of the physiological and molecular mechanisms of the various dietary fatty acids in health and disease in recent years.[3] Fatty acids are now considered as important modulators of gene expression in various tissues, such as liver and adipose tissues, in response to nutritional change. Most of this transcriptional action is mediated by activation of specific nuclear hormone receptor, called peroxysome proliferator-activated receptors (PPARs).[4] Recently, other nuclear modulators such as retinoids have been implicated in energy balance and adipose tissue development through modulating gene expression.[5]

The cellular action of retinoic acid is mediated by 2 types of nuclear receptors retinoic acid receptors (RARs) and retinoid X receptors (RXRs). RXR is the common partner for the formation of RAR, PPAR, and also triiodothyronine receptor (TR) functional heterodimers that are ligand-inducible transcription factors. Thus, the relative availability of RXR in the cell results in a complex balance between RAR/RXR, TR/RXR, and PPAR/RXR heterodimers and subsequently of the corresponding signaling pathways.[6,7]

Recent evidence points out to the fact that obesity is a disease that could be related to gene expression homeostasis disturbance. Particularly, an altered expression of several transcription factors, including PPAR and some others, which could be involved in the development of obesity and associated syndromes in humans.[8] Thus, the white adipocyte differentiation program has been extensively studied and the importance of RAR and PPAR activation in eliciting the acquisition of the adipocyte phenotype has been shown.[9] We suggest that the excess in dietary fat often associated with the onset of obesity could induce in the early stage of overweight development of this disease, a modification in the pattern of expression of these nuclear receptors.

The purpose of the current study was to determine whether the overfeeding of a fat energy-dense diet (cafeteria diet) modifies the nuclear receptor profiles. We investigated the effects of this diet intake on the expression of RAR, RXR, TR, and PPAR in rat liver (the major site of retinoid metabolism, but also the major place of fatty acid and lipoprotein metabolism) and in the white adipose tissue (WAT). Their expression was determined

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Submitted July 27, 2000; accepted April 9, 2001.

Supported in part by the Conseil Régional d'Aquitaine (Fonds Commun de Coopération: Aquitaine [France]-Navarra [Spain]-Euskadi [Spain]).

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0026-0495/01/5010-0021$35.00/0

doi:10.1053/meta.2001.26759

by assaying binding properties and/or mRNA levels of the isoforms that are sensitive to change in nutritional and hormonal status.

MATERIALS AND METHODS

Animals and Diets

Official French regulations for the care and use of laboratory animals were followed. Six-week-old male Wistar rats were obtained from IFFA-CREDO (I'Arbresle, France), and were randomly divided into 4 groups with 8 rats each, according to the type and term of diet given. The animals were housed 2 rats per cage in an air-conditioned room of mean temperature 21 ± 1°C and with a light/dark cycle of 12 hours:12 hours during the experiment. After 10 days of acclimation to the housing conditions, rats (weighing 250 ± 6 g) received either a control diet or a cafeteria diet for 8 or 28 days. The control diet was a standard laboratory chow (A04-type pellets from U.A.R., Villemoisson-sur-Orge, France) . The cafeteria diet was prepared from a variety of highly palatable human foods that induced a voluntary and spontaneous hyperphagia in rats. The animals receiving the cafeteria diet were presented daily with a fresh offering of the following items: pâté, bacon, chocolate, potato chips, biscuits, and a pelleted diet in a proportion of 2:1:1:1:1:1 as previously published.[10] All of these food constituents were weighed before feeding and presented in excess. In this cafeteria diet, energy supplied as lipids represented 60% of the total energy intake and these lipids consisted mainly of saturated (SFA) and monounsaturated fatty acids (MUFA): 45% SFA, 45% MUFA, and 10% polyunsaturated fatty acids (PUFA). The 2 experimental time periods used in this experiment correspond to (1) the time of exposure to the obesity-inductive diet (8 days), and (2) the time necessary in order to observe the beginning of weight gain (28 days). Rats were weighed and their food intake calculated daily.

Rats were killed by decapitation (between 9 and 10 AM) either 8 days or 28 days after the start of dietary treatment. Blood was collected, and the liver and WAT were rapidly excised and washed twice in cold saline (NaCl 0.15 mol/L) solution. Portions of the liver and WAT were immediately frozen in liquid nitrogen and stored at -80°C for subsequent analysis.

Biochemicals Measurements

Serum levels of cholesterol and triacylglycerol were determined using an automatic analyzer (Systemes Synchron CX 5, Beckman, Gagny, France) following routine procedures.

Receptors Preparation and Binding Studies

All tissue fractionations were performed at 4°C. Nuclei were isolated as described by DeGroot and Torresani.[11] All subsequent steps were performed at 0 to 4°C. The binding properties of receptors (maximal binding capacity and dissociation constant [*K*d]) were derived from Scatchard analysis of results obtained by in vitro binding studies.

Extraction of RAR and Binding Assay

Nuclei were washed 3 times in ice-cold HMK buffer (10 mmol/L HEPES, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, pH 7.9) and then submitted to a DNase I digestion for 30 minutes at 25°C, followed by a high-salt extraction (0.5 mol/L NaCl, final concentration). The nuclear extract was then obtained by centrifugation at 10,000 × *g* for 5 minutes as described by Daly et al.[12] Study of RAR binding was performed according to Audouin-Chevallier et al[13] using [^{3}H]CD367 (4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl) benzoic acid), a synthetic analog of retinoic acid supplied by Galderma R.D (Sophia Antipolis, Valbonne, France). Briefly, 96 μL of nuclear extract (containing a≈200 μg proteins) was mixed with 4 μL of increasing concentrations (0.025 to 0.150 μmol/L in dimethyl sulfoxide) of [^{3}H]CD367 (specific activity, 0.116 mCi/μg). After 1 hour of incubation at 4°C, bound and free CD367 were submitted to a high-performance size-exclusion chromatography on TSK (Tosoh Corp) gel G3000SW column (CIL, Cluzeau, Puteaux, France) and eluted with 0.3 mol/L KH_2PO_4, pH 7.8. Fractions were collected and counted in a liquid scintillation counter using Ready Safe Cocktail (Beckman Instruments, Gagny, France) as the scintillation liquid. Nonspecific binding was determined by incubation in the presence of 1,000-fold excess of unlabeled CD367.

Extraction of TR and Binding Assay

TR extraction and binding assay were performed using the method of Torresani and DeGroot.[14] Briefly, nuclear extract was obtained from purified nuclei with a high ionic strength TKEM buffer (400 mmol/L KCl, 2 mmol/L EDTA, 20 mmol/L, 1 mmol/L $MgCl_2$, Tris-HCl, pH 7.9) at 0°C for 30 minutes and by subsequent ultracentrifugation at 100,000 × *g* for 30 minutes. Incubations of nuclear proteins (50 μg) were performed in 0.2 mL TKEM with [^{125}I]triiodothyronine (0.03 to 0.06 nmol/L; specific activity, 3 mCi/μg) for 3 hours at 20°C. The binding reaction was stopped by the addition of 0.8 mL of an ice-cold Dowex 1X8 anion exchange resin (Sigma, St Louis, MO) suspension in TKEM (60 mg/mL) that binds free triiodothyronine (T_3). Bound and free T_3 were separated by centrifugation (1,000 × *g*, 5 minutes), then protein-bound T_3 and free T_3 radioactivity were measured. Nonspecific T_3 binding was determined by incubation in the presence of 1,000-fold excess of unlabeled T_3.

Quantification of mRNA

Total RNA was isolated in the liver by the method of Chomczynski and Sacchi[15] and in WAT using an extraction kit (RNeasy Mini Kit, QIAGEN, Courtaboeuf, France). Aliquots of RNA samples were electrophoresed through a 1% agarose denaturing gel to ensure RNA integrity. The mRNA was quantified, using a semiquantitative method, by reverse transcription and amplification by the polymerase chain reaction (RT-PCR) as described by Noël-Suberville et al[16] using 10 μg of total RNA and 100 U of Superscript II reverse transcriptase (Gibco BRL, Cergy Pontoise, France) for the reverse-transcription step. PCR was performed on 15 μL of the cDNA in a total volume of 180 μL containing 10 mmol/L Tris-HCl (pH 8.5), 2 mmol/L $MgCl_2$, 50 mmol/L KCl, 0.2 mmol/L of each of the dNTPs, 1.85 MBq [α-^{32}P]dCTP, 0.5 U *Taq* polymerase, and 1 μg each of the forward and reverse primers. The PCR reaction was carried out for 33 cycles. Each cycle consisted of 1 minute at 95°C, 1 minute at 60°C, and 2 minutes at 72°C. For quantitative analysis of PCR products, 8 μL PCR reaction mixture was withdrawn after each amplification cycle (from 7th to 24th) and the coamplified fragment were separated by electrophoresis on 10% acrylamide gel. The incorporated radioactivity was visualized by autoradiography, and the bands were excised from gel and quantified by scintillation counting. For example, a semilogarithmic representation of the relative amplification products of β-actin and PPARγ$_2$ genes measured by counting the amount of ^{32}P incorporated is illustrated in Fig 1.

The values of mRNA were obtained by comparison with the level of an internal standard, β-actin, that was simultaneously reverse-transcribed and amplified in the same test tube. For TR, the chosen primers corresponded to the 2 isoforms α_1 and β_1 that encode for functional receptors, and for RAR, the chosen primer corresponded to RARβ isoform, which is a subtype abundant in rat liver and subject to nutritional regulation. The other primers chosen corresponded to the mRNA isoforms that were mainly represented in rat liver and WAT. RARβ, TR $\alpha_1\beta_1$ and β-actin primers were designed by Noël-Suberville et al.[16] Amplification of RARα, RXRα, PPARα and γ$_2$ subtypes and acyl-CoA oxidase (ACOX) was performed with the following sets of primers: forward 5′GCTGGGCAAGTACACTACGAAC3′ and re-

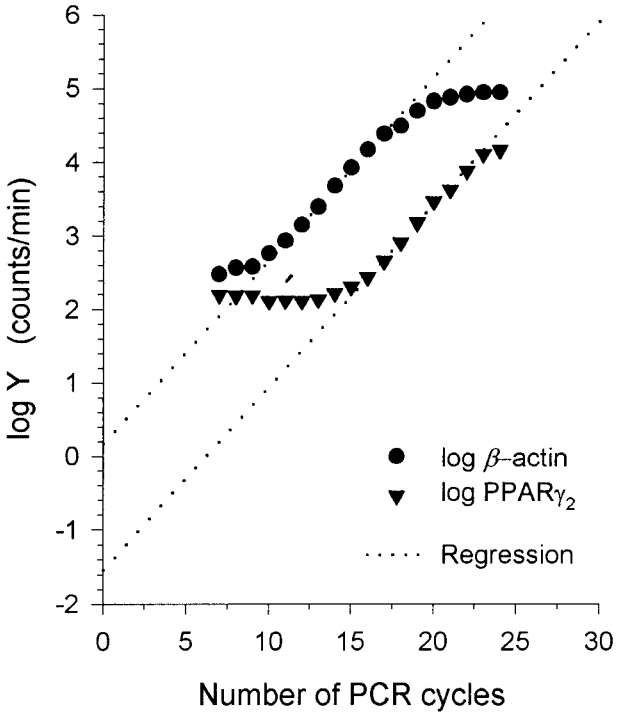

Fig 1. Relative quantification of PPARγ_2 mRNA by RT-PCR in cafeteria-fed rat WAT. The products of RT-PCR were resolved on 10% acrylamide gel electrophoresis and the amount of [α-^{32}P]dCTP incorporated was counted, followed by a semilogarithmic of the relative amplification Y. The PCR efficiency was 78%.

verse 5′GGCGAACTCCACAGTCTTAATG3′, forward 5′GCTGGTGTCGAAGATGCGTGAC3′ and reverse 5′GGGTACTTGTGTTTGCAGTACG3′, forward 5′GCCATCTTCACGATGCTGTCC3′ TTGGTGACTTTATGGAGC3′ and reverse 5′CCTCGATGGGCTTCACGTTCAG3′, forward 5′GGTGCGGTCGGGGAAGTTGGTG3′ and reverse 5′CCTCCAGGCTGTTGATGTCCAC3′, respectively. All primers were purchased from GENSET (Paris, France).

Statistical Procedure

For each particular group of 8 rats, data are expressed as the mean ± SEM of four independent measures performed from 4 pools of 2 rats. All measures were conducted in duplicate. Results were compared using Student's *t* test and differences between mean values were considered significant for $P < .05$.

RESULTS

Animals and Metabolic Data

Rats fed the standard diet (control rats) consumed approximately 26 g/d of chow, while rats fed on the cafeteria diet (cafeteria rats) consumed 29 g/d. Energy intake in cafeteria rats was greater than in control rats (480 KJ *v* 377 KJ). The serum cholesterol concentration was significantly increased as early as 8 days of cafeteria diet (+58%) reaching +138% after 28 days of feeding while serum triglyceride concentration was unchanged.

Rats fed the highly palatable diet for 8 days did not exhibit a significant increase in body weight (300 ± 3 g *v* 303 ± 4 g) above chow-fed controls (Table 1). Twenty-eight days of cafeteria diet feeding resulted in a slight increase in weight gain (404 ± 7 g *v* 385 ± 5 g, $P = .06$). After 1 week, the fat mass of cafeteria diet-fed rats had significantly increased in weight compared with controls and the same increase in fat mass was observed after 4 weeks of cafeteria diet. However, control animals had an increase (≈3 times) in WAT between 8 and 28 days similar to cafeteria diet-feeding animals (Table 1).

Effect of 8 Days Dieting Treatment on Nuclear Receptors Expression

MBC and *K*d were determined by Scatchard analysis (Fig 2). The effects of 8 days of cafeteria diet on RAR and TR binding properties in the liver and on several hepatic nuclear receptor mRNA levels are reported in Table 2. After 1 week on the cafeteria diet, the MBC of liver RAR and TR significantly decreased by 20.1% and 35.0%, respectively ($P < .05$). At 1 week, only a significant decrease in the abundance of RARβ mRNA was observed (-17.4%, $P < .05$) in the liver of cafeteria rats (Table 2).

Determination of the expression of PPARα (measured by PPARα and ACOX mRNA levels in rat liver) showed no significant changes after 8 days of feeding (Table 2).

Effect of 28 Days Dieting Treatment on Nuclear Receptor Expression

Most of the parameters measured in the liver of rats fed the cafeteria diet for 4 weeks were affected (Table 2 and Fig 2). The RAR and TR MBC were significantly lower (-16.0% and –23.4%, respectively, $P < .01$), as well as the abundance of the mRNA of these receptors (-28.5% and –32.7%, respectively,

Table 1. Changes in Body Weight, Tissue Mass, and Blood Analyses in Animals Fed the Cafeteria Diet for 8 and 28 Days Versus Chow-Fed Controls

	Rats Fed 8 Days		Rats Fed 28 Days	
	Controls	Cafeteria	Controls	Cafeteria
Body weight (g)	303 ± 4	300 ± 3	385 ± 5	404 ± 7
Liver/rat weight (%)	2.90 ± 0.10	3.20 ± 0.10*	2.90 ± 0.10	3.16 ± 0.06*
WAT (g)	5.3 ± 0.6	8.7 ± 0.5*	16.6 ± 1.3	27.5 ± 1.8*
Serum cholesterol (mmol/L)	0.93 ± 0.05	1.47 ± 0.08*	0.57 ± 0.02	1.36 ± 0.10*
Serum triacylglycerol (mmol/L)	0.70 ± 0.04	0.68 ± 0.06	1.13 ± 0.11	1.08 ± 0.11

NOTE. Data are the mean values ± SEM from 8 rats.

*Significantly different from control value using the Student's *t* test ($P < .05$).

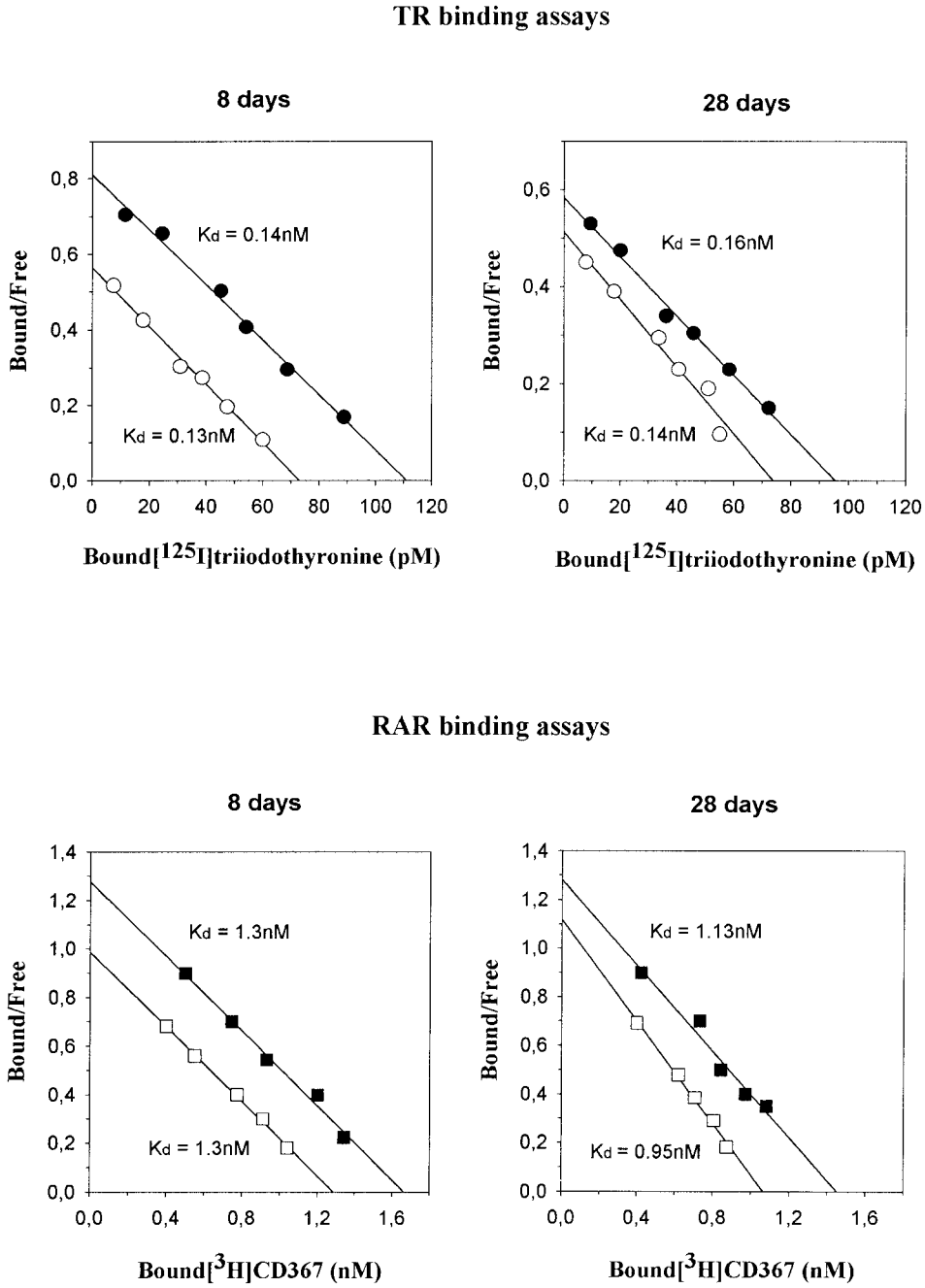

Fig 2. Representative Scatchard analysis of ligand specific binding to rat liver nuclear proteins. Hepatic nuclear fractions were obtained from rats fed on control diet for 8 days (●) or for 28 days (■), and rats fed on cafeteria diet for 8 days (○) or for 28 days (□). Experimental conditions did not affect *K*d values. Each plot represents the mean of data from 4 separate experiments performed in duplicate.

$P < .05$). On the other hand, the amounts of PPARα and ACOX mRNA were increased by 54.5% ($P < .01$) and 37.8% ($P < .05$), respectively (Table 2). However, the amount of RXR a mRNA was not affected

Results of nuclear receptors mRNA levels in WAT are reported in Fig 3. We observed a significant increase in PPARγ_2 mRNA (+49.5% ± 6.2%, $P < .05$) and a significant decrease in TR$\alpha_1\beta_1$ and RARα mRNA (-31.0% ± 2.3% and -22.3% ± 2.1%, $P < .05$) in cafeteria rats versus controls rats. As in the liver, the amount of RXRα mRNA was not affected in the WAT.

DISCUSSION

In this study, we showed that early exposure to an obesity-inducing diet modified the pattern of expression of nuclear receptors in liver and WAT.

Diet treatment rapidly affected lipid status since an increased serum cholesterol concentration was observed as early as 8 days of cafeteria diet feeding. These results are in agreement with previous studies showing that serum cholesterol is increased by saturated fatty acids resulting from the consumption of an excess of dietary fat.[17] A greater increase

Table 2. RAR and TR Expression and Abundance of RXRα, PPARα, and ACOX mRNA in the Liver of Cafeteria-Fed Rats Versus Chow-Fed Controls

	Nuclear Receptor MBC Bound Ligand (fmol/mg nuclear protein)		mRNA Abundance (% β-actin mRNA)				
Diet	RAR	TR	RARβ	TRα_1,β_1	RXRα	PPARα	ACOX
8 days							
Control	529 ± 11	446 ± 15	4.2 ± 0.2	4.0 ± 0.2	23.5 ± 1.3	4.1 ± 0.2	22.2 ± 2.0
Cafeteria	422 ± 10*	291 ± 12*	3.5 ± 0.2*	3.6 ± 0.2	24.3 ± 2.0	4.5 ± 0.3	22.9 ± 2.2
28 days							
Control	560 ± 11	394 ± 8	5.0 ± 0.4	4.6 ± 0.5	22.9 ± 1.2	4.8 ± 0.3	30.1 ± 2.8
Cafeteria	472 ± 6†	302 ± 6†	3.6 ± 0.3*	3.1 ± 0.2*	23.2 ± 1.5	7.4 ± 0.3†	41.5 ± 2.1*

NOTE. Data represent the mean ± SEM of measures from 4 independent pools of 2 rats.
Significantly different from control value using the Student's *t* test, *$P < .05$, †$P < .01$.

in WAT mass and body weight were observed later. Between 8 and 28 days of feeding, control rats and cafeteria diet-fed rats experienced a similar increase in WAT. Cafeteria diet-fed rats experienced a greater development of WAT only after 60 days (data not shown). At this time, the cafeteria diet-fed rats showed a greater weight gain compared to control rats.

Proenza et al[18] noted a moderate increase in weight after 1 month and a high weight gain only after 3 months of cafeteria diet. Moreover, Rothwell and Stock[19] suggested that animals overeating palatable food mount an early adaptive response using thermogenic mechanisms to limit excess energy storage.

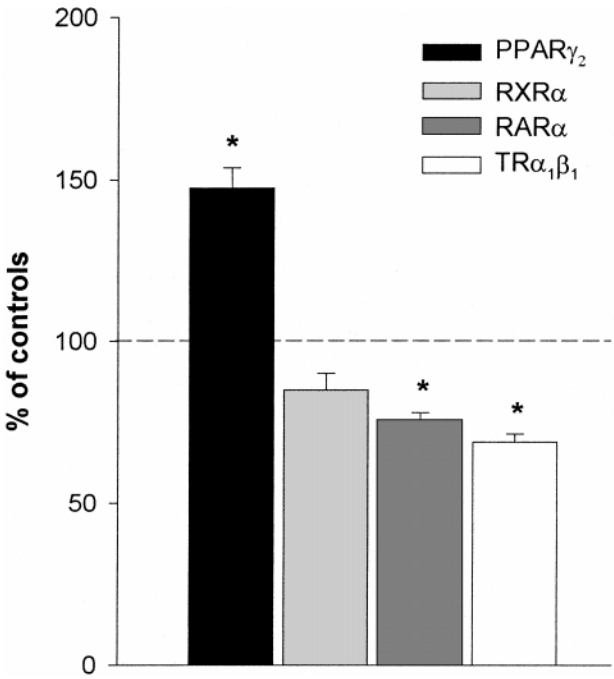

Fig 3. Levels of TR$\alpha_1\beta_1$, RARα, PPARγ_2, and RXRα mRNA in the rat WAT. Data represent the mean ± SEM of measures from 4 independent pools of 2 rats and are represented in % of control data. *Values significantly different from control values using the Student's *t* test ($P < .05$). These data were obtained from WAT of rats fed a control or cafeteria diet for 28 days.

This is consistent with an hypertrophy of brown adipose tissue. The adaptive thermogenic response is thought to account for the greater capacity of young rats to resist the onset of obesity.[20]

The size of the adipose tissue can be modulated by the formation of new adipocytes from precursor cells (adipocyte differentiation) and/or increase in adipocyte size (adipocyte hypertrophy). Faust et al[21] showed that a significant growth of adipose tissue in rats fed a high-fat diet was the result of an increased number of fat cells. The modulation of adipose tissue cellularity by a high-fat diet involved regulation of the genetic development program. Among the adipogenic factors that are required to trigger the adipocyte differentiation, transcription factors such as nuclear receptors probably play an important role. The results of the present study argue in favor of their involvement.

Indeed, the data obtained for WAT and liver of rats fed a cafeteria diet for 28 days showed significant modifications in the expression in the majority of the receptors studied: a decreased expression of TR and RAR and an increased expression of PPAR. On the other hand, the expression of RXR was unchanged, in agreement with Takase et al.[22] Figure 4 gives a representation of the modification in the pattern of receptors expression in liver and WAT induced by the cafeteria diet.

Moreover, the results obtained in the liver of rats after 8 days of cafeteria diet indicate that modifications in the expression of receptor are not simultaneous in that decreased RAR and TR expression was observed before an increased in PPAR expression.

These modifications in nuclear receptor expression could be related to adipose tissue development. Indeed, the decreased expression of RAR and TR (which play an important role in mediating the effects of retinoic acid [RA] and triiodothyronine) may favor adipocyte proliferation in the first stage of adipogenesis (RA was reported as a potent blocker of white adipocyte differentiation),[23] while the increased expression of PPARγ favor lipid accumulation in differentiated adipocytes.[4]

The biomolecular links between diet and cellular processes are numerous and not all are understood, but the results, obtained in animals fed a weight gain-inducing diet, showed

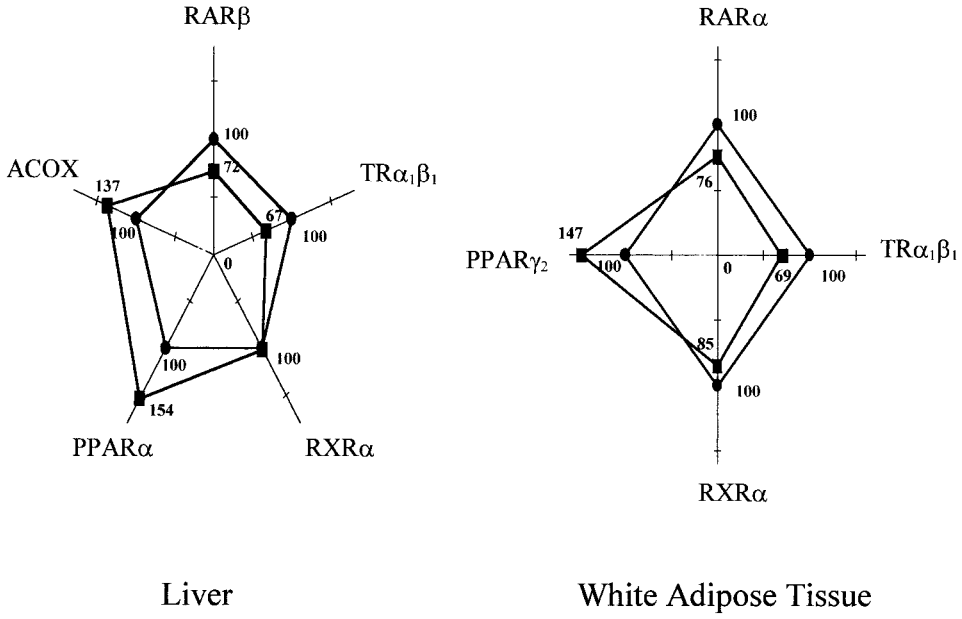

Fig 4. Patterns of expression of nuclear receptors in liver and WAT of rats fed a cafeteria diet for 28 days (●, controls rats; ■, cafeteria rats).

modifications in the pattern of expression of a set of master regulators (RAR, TR, PPAR) of differentiation/proliferation processes. These modifications are in agreement with the 2 successive steps involved in the biological phenomenon induced by a disturbance in the energy balance (ie, adipocytes differentiation and, then, lipid accumulation in these adipocytes). Thus it could be hypothesized that such nuclear receptor modifications play an important role in the adaptive mechanism induced by a high-fat diet.

Further investigations are needed to (1) analyze the mechanisms linking high-fat diet and changes in the nuclear receptor expression, and (2) assess the role played by these modifications in the activity and plasticity of adipose tissues involved in the onset of obesity.

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