HYPERLIPIDIC DIETS INDUCE EARLY ALTERATIONS OF THE VITAMIN A SIGNALLING PATHWAY IN RAT COLONIC MUCOSA

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Objective. Dietary factors can be associated with colorectal cancer. Fatty acids modulate gene expression in various tissues, mediated by activation of the peroxisome proliferator activated receptor: PPAR. Vitamin A signalling is mediated by retinoic acid (RA) receptors (RAR) and retinoid X receptors (RXR). The steroid nuclear receptors PPAR, RAR, RXR, are DNA-binding proteins and they induce gene transcription upon activation by specific ligands and interacting with distinct promoter sequences in the target genes. The aim of this study was to investigate the impact of hyperlipidic diets on the expression of PPARg, RXRa and RARb mRNA in rat colon.

Methods. Rats were fed during 4 weeks with the following diets: a cafeteria diet where 60 % of the energy was supplied as lipids and a high fat diet (HFD) represented by 25 % of a safflower oil (w/w) rich in polyunsaturated fatty acids, mainly n-6. Nuclear receptors mRNA were quantified by real-time RT-PCR with TaqMan probe process or SYBRGreen I chemical.

Results. The cafeteria diet and the HFD induced a significant decrease in RARb mRNA: -36 % ($p<0.02$) and –64 % ($P<0.001$) respectively. Simultaneously, an increased expression of PPARg mRNA was observed for cafeteria diet +35% ($P<0.05$) and for HFD +45 % ($P<0.05$). The level of RXRa mRNA was significantly increased for cafeteria diet: +53% ($P<0.0002$), while no significant difference in RXRa mRNA was observed in colonic mucosa rats whose fed with the 25 % HFD.

Conclusions. These results showed that an hyperlipidic diet could induce early modifications in the pattern of expression of nuclear receptors in rat colon. Many mechanisms could be probably involved but one hypothesis is that a modification of the balance between the nuclear receptors, resulting from an increased expression of PPARg, could induce a decreased expression of RARb in rat colon.

Key words: Rat colon – Real-time RT-PCR – High polyunsaturated fatty acid diet – Nuclear receptors expression

A number of studies suggest that environmental factors, and notably dietary factors, as fat diets, are associated with colorectal cancer (Levi et al. 1999). Concerning lipids, it would seem that n-6 fatty acids and/or saturated fatty acids riched diet might increased the risk of chemically induced colon carcinogenesis (Collett et al. 2001) but the underlying molecular mechanisms by which distinct classes of dietary PUFA exert their effects are still unknown. Fatty acids are now considered as important modulators of gene expression in various tissues, such as liver and adipose tissue, in response to nutritional change (Bonilla et al.2000; Collett et al. 2001; Redonnet et al. 2001). It is actually recognized that most of their transcriptional actions are mediated by activation of specific nuclear hormone receptor, the peroxisome proliferator-activated receptors (PPAR) (Desvergne and Wahli 1999). PPAR are members

of the steroid nuclear receptor superfamily and are activated by a broad range of structurally diverse chemical xenobiotics called peroxisome proliferators, as well as by fatty acids (Kliewer et al. 1997).

Several arguments show that PPARs are a key element of lipid homeostasis and provide molecular link between nutrition and gene regulation. Among them, it is interesting to note that activation of PPARg could promote the development of colon tumors in C57BL/6J-APC^{Min}/+ mice, a relevant model for both human familial adenomatous polyposis and sporadic colon cancer (Lefebvre et al. 1998). Moreover, an overexpression of PPARg (mRNA as well as protein) has been highlighted in rodent colon tumors and in selected human cancer tissue (DuBois et al. 1998).

Vitamin A and its derivatives (essentially retinoic acids) are essential for many essential fundamental physiological processes in number of tissues (Mangelsdorf et al. 1994). Retinoid signalling is mediated by two classes of receptors, retinoic acid receptors (RARa, b and g) and retinoid X receptors (RXRa, b and g) respectively activated by all-*trans* retinoic acid and *9-cis* retinoic acid. In the same manner as PPARs, RARs are nuclear receptors and DNA-binding protein. Upon activation by their specific ligands, they induce gene transcription by interacting with distinct promoter sequences (RARE and RXRE) in the target genes (Chambon 1996). *In vivo*, RAR expression is modulated by vitamin A and hormonal status (Garcin and Higueret 1984). RAR and PPAR need RXR to form functional heterodimers which are ligand-inducible transcription factors. Thus, the relative availability of RXR in the cell could result from a complex balance between both RAR/RXR and PPAR/RXR, and subsequently on their corresponding signalling pathways (Juge-Aubry et al. 1995). Indeed, positive gene regulation by PPAR has been shown to involve its heterodimerization with RXR, indicating that retinoid and peroxisome proliferator signalling pathways could converge through the direct interaction of their respective nuclear receptors (Miyata et al. 1994).

Recently, we have shown that hyperlipidic diet enriched in polyunsaturated fatty acids (PUFA (n-6)) induced an increased expression of PPARa and a concomitant decreased expression of RARb in rat liver (Bonilla et al. 2000). Furthermore, our data have shown that PPARg expression increased and RARa expression decreased in rat liver and adipose tissue with an hyperlipidic diet called : cafeteria diet (Redonnet et al. 2001).

Thus, the aim of this investigation was to compare the effects of two distinct hyperlipidic diets on the pattern of expression of the nuclear receptors PPARg, RXRa, RARb, all expressed in colic tissue. Rats were fed one of two qualitatively distinct diets: a High Fat Diet (HFD, 80 % of PUFA) or a cafeteria diet (40 % SFA). The expression of nuclear receptors genes was evaluated by mRNA quantification using a real time PCR method.

Table 1
Composition of safflower diet, values are g/100g diet

Component	Experimental diets	
	CD[1]	HFD[1]
Casein	18	18
Safflower oil	5	25
Mayze starch	70.3	50.3
Cellulose	1.9	1.9
Mineral mix[2]	3.8	3.8
Vitamin mix[3]	1	1

[1]CD: Control Diet for HFD: High Fat Diet
[2]Mineral mix composition (per kg mineral mix): 380 g calcium monohydrogen phosphate, 240 g dipotassium phosphate, 180 g calcium carbonate, 69 g sodium chloride, 20 g magnesium hydroxide, 90 g magnesium sulfate, 8.6 g iron sulfate, 5 g zinc sulfate, 5 g manganese sulfate, 1 g copper sulfate, 20 mg cobalt carbonate, 40 mg potassium iodide, 20 mg ammonium molybdate, 20 mg sodium selenite, 800 mg sodium fluoride, and 500 mg chromium potassium sulfate dodecahydrate.
[3]Vitamin mix composition (mg/kg diet): 1,500 choline concentrate (50 %), 100 vitamin E (500 IU/g), 10 vitamin A acetate (500,000 IU/g), 25 vitamin D3 (100,000 IU/g), 45 niacin, 30 calcium pantothenate, 10 thiamin hydrochloride, 10 riboflavin, 10 pyroxidine hydrochloride, 100 ascorbic acid, 50 *p*-aminobenzoic acid, 2 folic acid, 13.5 vitamin B-12 concentrate (1g/kg), 10 D-biotin, 1 menadione, 50 *meso*-inositol and 8,033.5 sucrose

Materials and Methods

Animals. Official French regulations for the care and the use of laboratory animals were followed. The animals were housed 2 rats per cage with a 12:12-hour light-dark cycle at 50% humidity and 21 ± 1 °C. The animals and their food intake were weighed daily. The animals had free access to drinking water

and diet. Rats were killed by decapitation 28 days after the start of dietary treatment.

1. Cafeteria diet: Five-week-old male Wistar rats were obtained from Iffa-Credo (I'Arbresle, France), and were randomly divided into two groups of 6 rats each, according to the type and term of diet. After 6 days of acclimatization to the housing conditions, the rats (weighing 250 ± 6 g) have received either a standard chow diet (6 % lipids) or a cafeteria diet. Standard laboratory chow (type pellets from U.A.R., Villemoisson-sur-Orge, France) was the control diet for cafeteria diet. 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 in excess 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 (Berraondo et al. 2000). In this cafeteria diet, the lipids consisted of saturated, monounsaturated and polyunsatured fatty acids (45 % SFA, 43 % MUFA and 12 % PUFA). The experimental time period (28 days) used in this experiment correspond to the time necessary in order to observe the beginning of weight gain.

2. High Fat Diet: Male Fisher344 rats weighing 50-80 g were obtained from Harlan (Gannat, France). Each diet was obtained as a dry powder from the Institut National de la Recherche Agronomique (Centre de Recherches de Jouy-en-Josas, France). Vitamin and mineral mixes of the diets were used as described by Potier de Courcy et al. (1989). Lipids added to the powder for the control diet (CD) and for the high fat diet (HFD) were respectively 5 g or 25 g of safflower oil per 100 g of diet. The safflower oil (Provence Régime) contains approximatively 77 % of PUFA, mainly linoleic acid (C18:2 n-6) (Table 1).

The fatty acid composition (Table 2) were determined after chloroform:methanol (2/1 V/V) extraction (Chomczynski and Sacchi 1987). After methylation by boron fluoride methanol at 100°C for 10 min, methylated extracts were analyzed by gas-liquid chromatography. The HFD provided 2085 kJ/100 g diet while the CD provided only 1665 kJ/100 g diet. Diets were freshly mixed three times a week. To minimize oxidation, oil was stored in the dark, at 4 °C, under nitrogen and in distinct containers.

Table 2
Composition of fatty acids in different diets (g/kg dry weight)

	CD[1] 5 % Safflower Oil	HFD[1] 25 % Safflower Oil	Standard chow	Cafeteria diet
C12:0	_[2]	_[2]	_[2]	_[2]
C14:0	0.05	0.27	3.50	12.40
C16:0	3.52	17.60	14.10	173.00
C18:0	1.42	7.15	2.50	83.20
SFA[1] total	**4.99**	**25.02**	**20.10**	**268.60**
C16:1 (ω-7)	_[2]	_[2]	0.20	9.10
C18:1 (ω-9)	5		13.10	223.6
C20:1 (ω-9)	_[2]	_[2]	0.30	3.30
MUFA[1] total	**5.00**	**24.95**	**13.60**	**336.00**
C18:3 (ω-6)	**38.85**	**194.25**	**39.80**	**98.80**
C18:2 (ω-6)	**0.05**	**0.25**	**2.70**	**6.50**
Other[3]	1.14	5.08	3.80	40

[1] CD: Control Diet for HFD: High Fat Diet; SFA: Saturated Fatty Acids; MUFA: Mono Unsaturated Fatty Acids.
_[2] Fatty acids no detected
[3] Other includes fatty acids detected at < 0.1 % of total fatty acids weight and fatty acids not identified

Colon samples. The colons were cut from caecum to rectum and washed in cold saline solution (NaCl 0.9 %, diethyl pyrocarbonate : DEPC 1 %, Sigma). The colonic mucosa was rapidly scrapped, immediately frozen in liquid nitrogen and stored at – 80 °C for subsequent analysis.

Nucleic acid extraction. Total RNA was isolated from the colonic mucosa by using an extraction kit (RNAgents® Total RNA Isolation System, Promega, France) according to the method of Chomczynski and Sacchi 1987). Aliquots of RNA samples were separated on a formaldehyde-containing 1 % agarose gel and visualized after ethidium bromid staining to ensure RNA integrity.

cDNA synthesis. 5 µg of total RNA mixed with RNasin (40 U, Life Technology, France) and with DNase (20 U Life Technology, France) were incubated for 15 min at 37 °C in order to denature DNA and to inhibit RNase. Then, RARb and GAPDH (glyceraldehyde (3P) phosphate deshydrogenase) reverse primers, were added for an incubation for 10 min at 70 °C. Then, the solution was mixed with 5X First Strand Buffer (Gibco BRL), 0.1 M dithiothreitol (DTT, Gibco BRL), the dNTPs (10 mM of each one) and 200 U of Superscript II reverse transcriptase

Table 3
Primers and probes for real time PCR

primers		5' → 3'	Sites	Amplicons	Tm
GAPDH*	Forward	GAACATCATCCCTGCATCCA	1455-1474	78 pb	59-61°C
	Reverse	CCAGTGAGCTTCCCGTTCA	1514-1532		
Probe	FAM–GCTGCCAAGGCTGTGGGCAAG-TAMRA		1480-1500		66-68°C
RARβ**	Forward	CTTGGGCCTCTGGGACAAAT	642-661	67 pb	59°C
	Reverse	TGGCGAACTCCACGATCTTAAT	688-709		60°C
Probe	FAM-CAGTGAGCTGGCCACCAAGTGCA-TAMRA		663-685		68°C
RXRα #	Forward	GCTGGTGTCGAAGATGCGTGAC	1238-1259	171 pb	59°C
	Reverse	GGGTACTTGTGTTTGCAGTACG	1388-1367		59°C
PPARγ ##	Forward	GCCATCTTCACGATGCTGTCC	1078-1099	146 pb	60°C
	Reverse	GCGAAGTCAAACTTGGGTTCC	1225-1204		60°C

* From Medhurst et al. (2000)
** Determined by Primer Express Software (version I, PE Applied Biosystem)
(Zelent et al. 1989)
Determined from cDNA of RXRa of rat (Gearing et al. 1993)
Determined from cDNA of PPARg of rat (Miyakita et al. 1998)
FAM : 6-carboxy-fluorescein
TAMRA : 6-carboxy-tetramethyl-rhodamin

(Life Technology, France) for 1 hour at 42 °C. The total volume was 40µl.

Quantification of PPARgmRNA or RXRamRNA by SYBR green I chemical RT-PCR. The real time PCR assay involving LightCycler™ technology associates rapid thermocycling with on-line fluorescence detection of the PCR products. PCR reactions were performed in a volume of 20µl containing oligonucleotide primers (5 µM of each), $MgCl_2$ (5 mM) and DNA Master SYBR green (Roche Molecular Biochemicals) containing Taq DNA polymerase, reaction buffer, dNTP and the double stranded DNA – specific fluorescent dye SYBR green I. Amplification occurred in two-steps procedure: denaturation at 95 °C for 10 min and 30 cycles with denaturation at 95 °C for 8 s, annealing at 60 °C for 6 s and extension at 72 °C for 10 s. Acquisition of the fluorescent signal from the samples was carried out at the end of elongation step. The PCR products were subjected to analysis by electrophoresis on a 1.5 % agarose gel.

Quantification of RARb mRNA by TaqMan RT-PCR. The TaqMan RT-PCR, recently developed technique, is able to the measurement of an accumulating PCR product in real time by using a dual-labelled TaqMan fluorogenic probe (Freeman et al. 1999). A reporter fluorophore emission is suppressed by a quencher fluorophore. During PCR, the probe anneals to the DNA template and the 5'-3'-nuclease activity of Taq polymerase releases the reporter from the vicinity of the quencher dye resulting in increased reporter fluorescence. Fluorescence intensity is directly related to the target DNA amount. The fluorescent signal is captured using a LightCycler® (Roche). The reaction was performed in a volume of 20 µl containing oligonucleotide primers (5 µM of each), TaqMan probe (5 µM), $MgCl_2$ (5 mM) and LightCycler DNA Master hybridisation Probes 10X (Roche Molecular Biochemicals) containing FastStart Taq DNA polymerase, reaction buffer, dNTP (with dUTP instead of dTTP). Amplification needs 45 cycles (denaturation at 95 °C for 5 s, annealing at 60 °C for 30 s) of PCR.

Primers and fluorogenic probes. Probe and primer sequences (Table 3) were designed using Primer Express software (Version I, PE Applied Biosystems). Primers were purchased from Oligogenset and probes from PE Applied Biosystems and each probe was synthesised with the fluorescent reporter dye FAM (6-carboxy-fluorescein) attached to the 5'-end and a quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine) to the 3'-end.

Standard curve generation. Standard curves were generated using serial dilutions of known quantities of cDNA of our samples in triplicate (50; 10; 2; 0.4; 0.08 ng diluted in water PCR grade).

Statistical procedure. Experimental values are expressed as the mean ± SE. The statistical signification of oil level and oil type effects and of their interaction was calculated by a 2-way ANOVA. Then, the differences between values obtained in rats fed 5 % oil (CD) or 25 % (HFD) of the same oil were analysed according to the Tukey's multiple post hoc test.

Results

Status of rats. 1. Cafeteria diet: Rats fed the standard chow diet (control rats) have consumed approximatively 26 ± 3.9 g/d, while rats fed the cafeteria diet (cafeteria rats) have consumed 29 ± 0.6 g/d. Energy intake of cafeteria rats was greater than that of their control rats (480 ± 10.0 kJ/d vs 377.0 ± 56.8 kJ/d). Indeed, lipids represent only 8 % of the total energy of standard chow diet but 60 % of the total energy of cafeteria diet. Rats feeding the highly palatable cafeteria diet for 28 days exhibited a slight increase in weight gain (404 ± 7 g vs 385 ± 5 g, P=0.06).

2. High fat diet (HFD): Rats fed the HFD exhibited a dietary consumption slightly lower than their control rats (16.6 ± 2.8 g/d vs 18.1 ± 2.8 g/d). However, the daily energy intakes were similar in the both groups of rats (334.0 ± 45.4 kJ/d vs 295.0 ± 44.8 kJ/d) since the excess of energy provided by the HFD (2070 kJ/ 100g vs 1650 kJ/ 100g) was compensated by the slight decrease of consumption. The body weight of HFD rats was not modified related to control (231 ± 16 g vs 211 ± 19 g). Such a result was already described by Gaiva Gomes da Silva (1996).

Effect of 28 days of dietary treatment on nuclear receptor expression. (Table 4) The relative colonic abundance of PPARg mRNA was significantly increased in rats fed HFD or cafeteria diet than in rats fed control diet (+35% and +45% respectively, P<0.05). As regards to the retinoic acid receptors, RARb and RXRa mRNA expressions are submitted to different variations: indeed, although the amount of RARb mRNA was decreased in rats fed HFD or cafeteria diet compared to control (-36%, P<0.02 and 64%, P<0.001 respectively), the amount of RXRa mRNA was only significantly modified by the cafeteria diet. In this case, the RXRa expression was increased by 53% related to control (P<0.0002). In HFD rats the abundance of RXRa mRNA was no significantly modified.

Discussion

Association between fat diet, high body weight and colorectal cancer has been already highlighted. Fatty acids, key element of high-fat Western-type diet are known to be important modulators of gene expression in various tissues, such as hepatic and adipose tissues, in response to nutritional change. Indeed, fatty acids are now recognized as activators of specific nuclear receptors called peroxisome proliferator-activated receptors (PPARs). PPARs are transcription factors, first implicated in lipid homeostasis, and recently involved in the modulation of expression of a very large range of genes. So, it seems possible that nuclear receptor expression alterations highlighted in hyperlipidic diet fed rats, could provide a better un-

Table 4
Effect of the dietary lipids on the quantification of mRNA of RXRa, PPARg and RARb in rat colon

Diets	mRNA abundance (% GAPDH mRNA) [1]		
	RXRa	PPARg	RARb
Cafeteria diet			
standard chow	2.03 (SE 0.06)	4.60 (SE 0.46)	0.01235 (SE 0.0006)
cafeteria	3.1 (SE 0.13)*	6.21 (SE 0.46)**	0.00800 (SE 0.0012) #
Safflower oil			
CD[2]	2.25 (SE 0.27)	1.35 (SE 0.06)	0.0650 (SE 0.0033)
HFD[2]	2.43 (SE 0.36)	1.95 (SE 0.23)**	0.0235 (SE 0.0017)##

[1] Data represent the mean ± SEM of measures from 6 rats

[2] CD: control diet for HFD: high fat diet

* p<0.0002, ** p<0.05, # p<0.001, ## p<0.02, significantly different from value obtained in rats receiving the control diet respectively for the corresponding high fat diet (Tukey's test)

derstanding of early events constituting a background for carcinogenesis to occur.

It is well recognized that fatty acids from dietary consumption have impact on cell membrane lipid composition and subsequently on signalling pathways (Hwang and Rhee 1999). Our study was interested in the modulation of few nuclear receptors expression by fatty acids from diet. Indeed, PPAR, RAR and RXR, governed in part by the disponibility of their ligands, are involved in numerous biological processes. Retinoic acid and RAR contribute to cell differentiation and antiproliferative process (Li and Wan 1998). Moreover, the expression of RARb, putative tumour suppressor gene, is reduced in various human cancers (Sirchia et al. 2002). The mechanism by which RARb expression is inhibited remains unclear even if involvement of histone acetylase is evoked (Demary et al. 2001). The role of PPARg is more controversial because of divergent results obtained by *in vitro* (Shimada et al. 2002) and *in vivo* experiments. Indeed, PPARg has been shown to inhibit the growth and maintain differentiation of breast, prostate, colon cancer cells, but its activation could increase the number of colonic tumours in APC-*min* mice (Bruce et al. 2000). However, PPARg implication in the balance between apoptosis and mitosis has been already demonstrated.

So, we were interested in the impact of high fat diets on the level of expression of RARb and PPARg. Our study was based on the fact that we do not know yet what exactly in diets may have an impact on colon carcinogenesis onset: excess of energy intake or n-3/n-6 low ratio or both? Animals were fed during four weeks diets exhibiting different compositions of lipids and different lipid enrichments: a safflower oil riched diet (HFD) with a n-3/n-6 low ratio and an obesity-inducer cafeteria diet in which lipids represented respectively 40% and 60 % of the total energy intake. The lipidic status of the rats fed one of the both hyperlipidic diets could present some similitudes with the human dietary status in western countries.

According to Proenza et al. (1992), after 28 days of consumption, cafeteria diet only induced a slight increased final body weight (around +5 %). According to Gaiva Gomes da Silva et al. (1996) and Bonilla et al. (2000), the body weight of rats fed the HFD was not significantly different than the body weight of rats fed the control diet because of a slight decrease of diet consumption attributed to the non-appetizing side of this diet.

The consumption of lipid enriched diets lead to an over-expression of PPARg. This result is in agreement with the fact that both diets contain either PPARg ligands or precursors of ligands later modified by cyclooxygenase pathway (Clay et al. 2000). PPARg acts as a positive regulator of its own gene so, upon activation by specific ligands, leads to an increase of PPARg mRNA in colonic mucosa. Dietary restriction seems able to reduce cell proliferation (Hursting and Fari 1999) and consequently the onset of carcinogenesis process. So, the increase of energy intake and/or the kind of food which provides energy could promote carcinogenesis by favouring proliferation versus differentiation or apoptosis. The way by which lipid enriched diet could promote proliferation is unclear but could pass through an upregulation of PPARg and its target genes. PPARg could be a mediator of the growth promoting role of high fat diet.

Moreover, we have found a decrease in the expression of RARb whatever the type of consumed lipid. This result agrees with a disturbing of vitamin A pathway observed in cancer cells (Swisshelm et al. 1994) and in tumours (Gebert et al. 1991). If high fat diet can modify the expression of RARb, the promotion of proliferation induced by lipids, could pass through the disruption of vitamin A action. A vitamin A signalling pathway disturbing should be confirmed by the analyse of vitamin A target gene expression (as AP1 genes).

The observation of RXRa expression level has shown an increase of mRNA expression with cafeteria diet and no modification of mRNA level with safflower oil rich diet. These results are in agreement with those observed on liver (Bonilla et al. 2000) and adipose tissue (Redonnet et al. 2001).

The increase of PPARg mRNA expression and the decrease of RARb mRNA expression lead us to hypothezise that high fat diet could promote proliferation by favouring the upregulation of PPARg to the detriment of vitamin A pathway. This imbalance might involve RXRa biodisponibility. Indeed, RXRa is the common partner for the essential heterodimerization of PPARg and RARb. In this way, an activation of PPARg by fatty acids could modify RARb expression by competing for the heterodimerization with RXRa.

To our knowledge, it is the first time that a possible link between the expression of PPARg and RARb in rat colonic mucosa was evidenced in a nutritional experiment. Further investigations are necessary to better understand the link between fat diet, nuclear receptor expressions and colon carcinogenesis. The involvement of another PPAR isoform (Park et al. 2001), PPARd, need to be explored in our nutritional experiment, i) for its transcription factor function, ii) because it is a Wnt pathway target gene.

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