

Response of ApoA-IV in pigs to long-term increased dietary oil intake and to the degree of unsaturation of the fatty acids

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ApoA-IV is a protein constituent of HDL particles; the gene coding for it is a member of the ApoA-I–ApoC-III–ApoA-IV cluster. To investigate the effects of the quantity and the degree of saturation of dietary lipid on the long-term response of this Apo, and on the hypothetical coordinated regulation of the cluster *in vivo*, pigs were fed isoenergetic, cholesterol-free, low-lipid or lipid-enriched diets (containing either extra olive oil (rich in MUFA) or sunflower oil (rich in *n*-6 PUFA)) for 42 d. In animals fed on the control diet, ApoA-IV was mainly associated with plasma lipoproteins. An increase in plasma ApoA-IV concentration, mainly in the lipoprotein-free fraction, was induced by the lipid-enriched diets, independent of the degree of saturation of the fatty acids involved. The latter diets also led to increases in hepatic ApoA-I, ApoA-IV and ApoC-III mRNA levels, more so with the sunflower oil-rich diet. The present results show that porcine plasma ApoA-IV levels and their association with lipoproteins are very sensitive to increases in dietary lipids, independent of the degree of fatty acid saturation. Furthermore, hepatic expression of RNA appears to be coordinated along with that of the other members of the gene cluster.

Apolipoprotein A-IV: Dietary fat: Pig

Reduced plasma levels of HDL are a well-established risk factor for the development of atherosclerosis, although the mechanisms responsible for this reduction are complex and remain unclear (Assman & Nofer, 2003). Apo components play an important role in HDL metabolism (that played by ApoA-I is the best understood). The influence of ApoA-IV in determining HDL concentrations and modifying the atherogenic process is now receiving much attention. It is already known that the over-expression of human ApoA-IV (Duverger *et al.* 1996) in transgenic mice has a protective effect against the formation of diet-induced aortic lesions.

Human ApoA-IV, which is synthesized in the small intestine and liver, is present in plasma, interstitial fluid and lymphatic fluid as a 46 kDa protein (Karathanasis *et al.* 1986). In plasma, it is associated with HDL; a small amount is bound to chylomicrons (Weisgraber *et al.* 1978; Bisgaier *et al.* 1985; Lagrost *et al.* 1989). The role of ApoA-IV in reverse cholesterol transport, as well as its regulation under different nutritional conditions, is being investigated in a number of mammalian species. Diet-induced hypercholesterolaemia is associated with an increased interstitial fluid ApoA-IV concentration in the

dog (Sloop *et al.* 1983); in the rat, a decrease in serum HDL ApoA-IV levels is accompanied by an increase in ApoA-IV levels (DeLamatre *et al.* 1983). In addition, fatty acid chain-length seems to be involved in postprandial intestinal synthesis of ApoA-IV in rats and pigs (Black *et al.* 1996; Kalogeris *et al.* 1996). Few studies, however, have addressed chronic lipid-induced changes in ApoA-IV levels (Stan *et al.* 2003). Under such conditions, the cholesterol content of the diet seems to be more responsible than the degree of fatty acid saturation for the increased ApoA-IV mRNA expression seen in the rat liver (Osada *et al.* 1994). This is not the case, however, in mice, in which increased plasma ApoA-IV appears to be induced by post-transcriptional mechanisms (Aalto-Setälä *et al.* 1994). In human subjects, plasma ApoA-IV concentrations were greater in subjects that consumed diets enriched in unsaturated fatty acids for 4 weeks, independent of the degree of saturation (Kratz *et al.* 2003).

Together, this information shows that the molecular mechanisms underlying the response of ApoA-IV to dietary lipid intake or nutrient composition have not been completely defined, that a species-specific mechanism of

response may exist, and that studies over periods longer than 1 month (long-term dietary interventions) are required in many species.

The ApoA-IV gene is a member of a closely linked cluster found on human chromosome 11 and pig chromosome 9. This cluster also includes ApoA-I and ApoC-III genes (Karathanasis, 1985; Trieu *et al.* 1993). Two common enhancers have been described that regulate ApoC-III and ApoA-IV gene expression (Vergnes *et al.* 1997). The coordinated regulation of this ApoA-I–ApoC-III–ApoA-IV gene cluster is therefore likely. This hypothesis needs to be tested in different species, tissues and experimental settings.

We recently characterized pig ApoA-IV. This protein shows high sequence identity (75.6%) with the human protein and is mainly associated with HDL (Navarro *et al.* 2004), reinforcing the idea that pigs are one of the best animal models in which to study lipid metabolism (Maeda *et al.* 1988; Black & Rohwer-Nutter, 1991; Moghadasian, 2002). Furthermore, the high sensitivity of these animals to dietary lipids (Mustad *et al.* 1996) may be reflected in responses sufficiently clear to allow the mechanisms involved to be determined. The aims of the present experiment were to determine the influence of the amount of long-term dietary lipid and its degree of saturation (in the absence of cholesterol) on the regulation of ApoA-IV in pigs, and to study Apo regulation.

Material and methods

Animals

The study animals were twenty-nine York × Landrace male pigs weighing 30 kg. They were fed a standard diet until they reached a body weight of 55 kg, before being randomly allocated to receive a control diet, an olive oil-enriched diet or a sunflower oil-enriched diet. All animals were handled in accordance with European Union criteria regarding the care and use of laboratory animal in research. The experimental protocol was approved by the Ethical Committee for Animal Research of the University of Zaragoza.

Experimental diets

After reaching a body weight of 55 kg, the animals received one of three isoenergetic and isonitrogenous cholesterol-free diets. The control diet was a commercial feed containing 20 g lipid/kg; the experimental diets were the same except that the feed was supplemented with 40 g olive oil or sunflower oil/kg. All food was offered *ad libitum*. The control diet provided lower amounts of saturated fatty acids, MUFA and PUFA than did the oil-enriched diets. Although the oil-enriched diets both contained similar amounts of saturated fatty acids, they differed in the degree of saturation of their unsaturated fatty acids. While the olive oil-enriched diet was a better source of MUFA, the sunflower oil-enriched diet had more PUFA, especially linoleic acid. The chemical composition of all three diets (Table 1) was analysed as previously described (Calleja *et al.* 1999). The experimental period lasted 42 d, until the animals reached a mean live weight of 95 kg without differences in average daily gain

Table 1. Chemical composition of the control, olive oil-rich and sunflower oil-rich diets*

Chemical composition (g/kg DM)	Experimental diets		
	Control	Olive oil	Sunflower oil
Metabolizable energy (kJ/kg DM)	12800	13400	13400
Carbohydrate	416	396	392
Protein	170	173	173
Fat	21	61	61
Fatty acids:			
Lauric (12:0)	<0.1	0.6	1.0
Myristic (14:0)	<0.1	0.4	0.3
Palmitic (16:0)	2.7	10.6	8.2
Palmitoleic (16:1)	<0.1	0.6	0.1
Stearic (18:0)	0.3	3.3	5.4
Oleic (18:1)	2.7	17.9	8.4
Linoleic (18:2 <i>n</i> -6)	8.5	14.7	22.0
Linolenic (18:3 <i>n</i> -3)	0.4	1.1	0.9
Arachidic (20:0)	0.3	0.1	0.2
Saturated fatty acids	3.3	15.0	15.1
MUFA	2.8	18.5	8.6
PUFA	8.9	15.8	22.9

* Mean values for other components (g/kg): DM 875; crude fibre 50; ash 100; lysine 8.

(0.98 (SD 0.07) kg/d for the control and olive-oil groups, 1.03 (SD 0.07) kg/d for the sunflower-oil group).

Sample collection

After overnight fasting, the experimental animals were slaughtered and bled at a local slaughterhouse. Blood was collected and samples for lipid and lipoprotein analysis stored at -70°C until use. The liver and duodenum were removed rapidly and samples of these organs stored in liquid N_2 for later total RNA extraction.

Lipid and lipoprotein analysis

Plasma total cholesterol (TC), triacylglycerol (TG) and HDL-cholesterol were quantified by enzymic methods (Sigma, Madrid, Spain). To analyse HDL-cholesterol, particles containing ApoB were precipitated with phosphotungstic acid–magnesium chloride (Roche, Barcelona, Spain). VLDL + LDL-cholesterol were calculated as TC – HDL-cholesterol.

Plasma lipoprotein profiles were determined in 150 μl pooled plasma samples from three animals belonging to each experimental group by fast protein liquid chromatography (FPLC) gel filtration using a Superose 6B column (Amersham-Pharmacia, Barcelona, Spain; see Calleja *et al.* 1999). The fractions were then concentrated by centrifugation in Centricon tubes (pore size 10 K; Amicon Inc., Beverly, MA, USA) and their protein contents determined by the BioRad dye-binding assay (BioRad, Madrid, Spain).

Western blot analysis

The distribution of the ApoA-IV fractions was determined by Western blot analysis using concentrated samples separated by FPLC. These samples were loaded onto an

SDS-polyacrylamide gel with a 4–24% gradient. Electrophoresis was performed for 3 h at 25 mA and protein molecules transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Madrid, Spain) using a transblot transfer apparatus (BioRad) at 400 mA for 18 h. Bands were detected using rabbit polyclonal antibodies to human ApoA-IV and a secondary antibody, anti-rabbit IgG peroxidase conjugate (Amersham-Pharmacia). The results were visualized by chemiluminescence (Amersham-Pharmacia). The membranes were exposed to enhanced chemiluminescence (ECL) film (Amersham-Pharmacia) and the intensities quantified using a laser densitometer (LKB 2202; Amersham-Pharmacia). The lipoprotein profile was obtained by concentration using the speed-vac method. No differences were observed between the speed-vac and Centricon tube methods.

Extraction of total RNA

Total RNA was isolated using Trizol reagent (Sigma). Tissue (100 mg) was homogenized with 1 ml reagent. RNA was quantified by absorbances at 260 and 280 nm, the ratio of which was > 1.75. The integrities of the 28S and 18S ribosomal RNA were verified by agarose formaldehyde gel electrophoresis followed by ethidium bromide staining.

Northern blot analysis

Apo mRNA expression was determined by Northern blotting. Total RNA (5 µg) was denatured in formaldehyde (180 g/l)–formamide (700 g/l) at 65°C for 15 min before being run in an agarose (10 g/l) gel containing 2.2 M-formaldehyde. RNA was transferred to a nylon membrane (Hybond-N; Amersham-Pharmacia) by capillary blotting, fixed with 2 kJ UV light/m² in an ultraviolet crosslinker (Hoefer; Amersham-Pharmacia) and hybridized at 42°C for 18 h under standard conditions (Osada & Maeda, 1998). A 330 bp pig ApoA-I probe was generated by PCR using the following oligonucleotides: forward 5'-CAGGAGGAGATGGAGACGT-3' and reverse 5'-GCGTTCAGCTTCTTGGAGG-3'. A 171 bp pig ApoC-III probe was also PCR-generated employing the 5'-GGGGCTGGGTAACCGACAG-3' and 5'-GGTGGGATGGGAGGGCAT-3' oligos (Trieu *et al.* 1993). Both PCR products were verified by DNA sequencing. An *EcoRI/XhoI* 0.8 kb fragment of pig cDNA was used as the probe for ApoA-IV (Navarro *et al.* 2004). A 0.7 kb *BamHI/EcoRI* rat 18S probe was used to normalize the amount of RNA loaded on the gel, and as a housekeeping gene. Probes were labelled using α -³²P-labelled dCTP and Rediprime (Amersham-Pharmacia). Filters were exposed to Biomax film (Kodak; Amersham-Pharmacia) and quantified using a laser densitometer (LKB 2202; Amersham-Pharmacia).

Statistics

Plasma lipid and Northern blot results were examined by one-way ANOVA and multiple comparisons of means (Tukey–Kramer *post hoc* test) using the Instat 3.02 statistics package for Windows (GraphPad Inc., San Diego, CA, USA).

Results

Table 2 shows the concentrations of plasma lipids in the pigs fed the different diets. No statistically significant changes in plasma TC or LDL-cholesterol were observed. Most of the cholesterol (> 50% TC in pigs fed the control diet) was carried by LDL particles. Compared with the control diet, HDL-cholesterol levels decreased significantly when sunflower oil was the main source of dietary lipid ($P < 0.05$). No changes were observed with the olive oil-rich diet. Plasma TG increased significantly ($P < 0.05$) in response to the higher dietary lipid content; no significant differences were seen with regard to the type of lipid consumed.

Fig. 1 shows the distribution of cholesterol among the different lipoproteins as revealed by FPLC. The pattern of lipoproteins corroborated the results described earlier. Increases were seen in fractions 2–6, corresponding to the VLDL fractions, in both lipid-enriched diets. This explains the increased plasma TG levels. Reductions in LDL (fractions 7–14) were more pronounced for the olive oil-rich diet. This diet also induced a loss of HDL size uniformity, resulting in two peaks (fraction numbers 15–24), an effect not shared by the sunflower oil-rich diet. This induced a clear reduction in the amount of cholesterol carried by HDL. The amount of cholesterol carried by LDL particles, as determined by FPLC, was 20% greater than that estimated by precipitation methods. This suggests that some large HDL particles may be filtered out with the LDL particles in FPLC. Similar findings have been reported in other animal models (De Silva *et al.* 1994).

Table 3 shows the ApoA-IV concentrations and its distribution among lipoproteins and lipoprotein-free plasma, as determined by Western blotting of the FPLC fractions. The lipid-enriched diets led to a 2.5-fold increase in plasma ApoA-IV concentration independent of the degree of fatty acid saturation. The ApoA-IV carried by VLDL was insignificant (< 0.05%). Low levels of this Apo in LDL were observed; they remained unaffected by the lipid-rich diets. In the animals fed the control diet, the main plasma location of this Apo was the HDL fraction. For the olive oil- and sunflower oil-rich diets, increases in ApoA-IV HDL were noted, reaching 55.0 and 51.0

Table 2. Plasma lipids in pigs fed the control, olive oil-rich and sunflower oil-rich diets*
(Mean values and standard deviations)

Plasma lipids (mmol/l)	Diets					
	Control (n 11)		Olive oil (n 9)		Sunflower oil (n 9)	
	Mean	SD	Mean	SD	Mean	SD
Total cholesterol	2.1	0.4	2.5	0.6	2.1	0.5
HDL-cholesterol	1.0 ^a	0.2	0.8 ^{ab}	0.2	0.7 ^b	0.2
VLDL + LDL-cholesterol	1.1	0.4	1.7	0.7	1.3	0.6
Triacylglycerol	0.5 ^a	0.1	0.8 ^b	0.2	0.7 ^b	0.1

^{a,b} Mean values within the same row with unlike superscript letters were significantly different ($P < 0.05$).

* For details of diets and procedures, see Table 1 and p. 764.

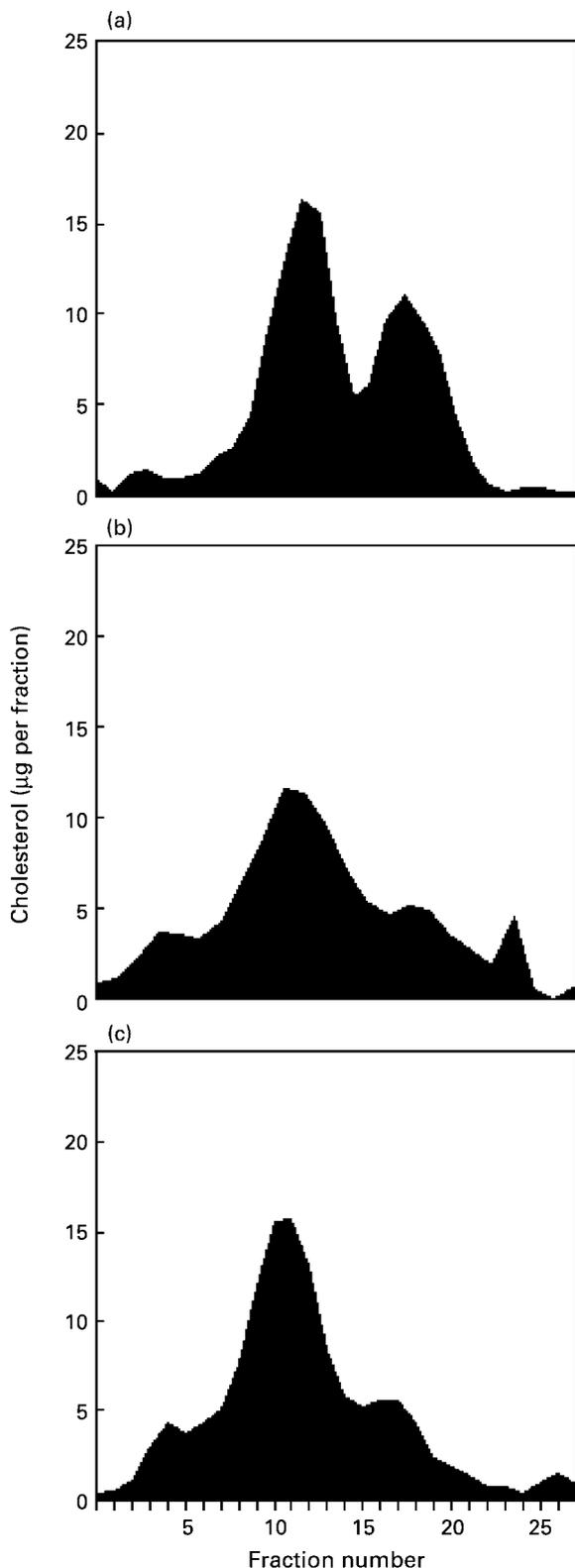


Fig. 1. Plasma lipoprotein distribution in pigs subjected to different experimental conditions (representative profiles from pooled plasma samples of three animals for each experimental group). Fraction numbers 2–6, 7–14 and 15–22 correspond to lipoproteins VLDL, LDL and HDL respectively. (a), Control; (b), olive oil-rich diet; (c), sunflower oil-rich diet. For details of diets and procedures, see Table 1 and p. 764.

Table 3. ApoA-IV concentrations and distributions in different plasma lipid fractions in pigs fed the control, olive oil-rich and sunflower oil-rich diets*

(Mean values and standard deviations)

	Diets					
	Control (n 11)		Olive oil (n 9)		Sunflower oil (n 9)	
	Mean	SD	Mean	SD	Mean	SD
Total ApoA-IV (arbitrary units)	45 ^a	19	104 ^b	12	121 ^b	38
Plasma distribution of ApoA-IV (%)						
LDL	4.3	1.5	2.0	1.7	3.0	1.5
HDL	94.0 ^a	2.0	54.0 ^b	11.0	42.0 ^b	8.0
Lipoprotein-free	1.3 ^a	1.8	43.0 ^b	10.0	57.0 ^b	5.0

^{a,b}Mean values within the same row with unlike superscript letters were significantly different ($P < 0.001$).

* For details of diets and procedures, see Table 1 and p. 764.

absolute arbitrary units (compared with the control diet value of 42.0; calculated from results shown in Table 3). The main increase in ApoA-IV, however, was due to the contribution of the lipoprotein-free fraction, which rose from 0.6 arbitrary units in the control diet to 45.0 and 69.0 arbitrary units in the olive oil- and sunflower oil-rich diets respectively.

Fig. 2 shows the regulation of ApoA-I, ApoC-III and the ApoA-IV gene cluster in terms of mRNA expression in the intestine and liver. The intestinal expression of the ApoA-I and ApoC-III genes were significantly increased with the lipid-enriched diets, more so in the animals receiving extra olive oil. The expression of intestinal ApoA-IV, however, showed a different pattern; only the diet enriched in PUFA (the sunflower oil-rich diet) induced its expression. Hepatic expressions of the three genes were significantly increased in animals fed the lipid-enriched diets. Except for ApoA-I, the sunflower oil-rich diet induced further significant increase in their expression compared with the olive oil-rich diet. These results are consistent with a more coordinated regulation of ApoA-IV in the liver, and with a specialized regulation in the intestine, compared with the other members of the gene cluster. Further, ApoA-IV expression appears to be highly sensitive to the long-term intake of the sunflower oil-enriched diet in this animal, irrespective of the organ studied.

Discussion

The results show that: (1) plasma levels of lipoprotein-bound ApoA-IV depend on the amount of dietary lipid consumed independent of the degree of saturation of its inherent fatty or of dietary cholesterol; (2) this association, at least in pigs, is an extraordinarily sensitive mechanism observed after only small additions of dietary lipids; (3) changes in plasma ApoA-IV (measured in fasting animals) induced by long-term dietary schedules may be due to increased expression of its mRNA in both the intestine and liver; (4) hepatic mRNA levels of this Apo are closely related to those of the other members of the ApoA-I–ApoC-III–ApoA-IV gene cluster in the present experimental setting.

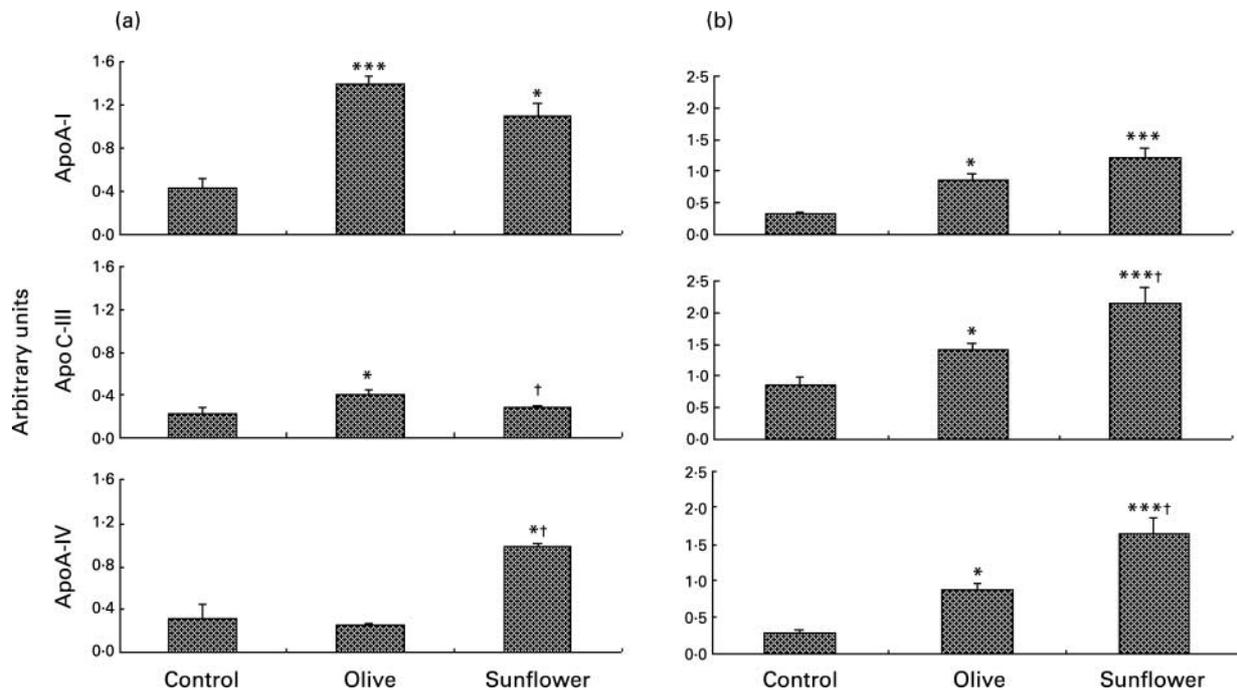


Fig. 2. Effects of quantity of dietary lipid and fatty acid saturation on porcine ApoA-I–ApoC-III–ApoA-IV mRNA gene cluster expression. (a) Intestine; (b) liver. For details of diets and procedures, see Table 1 and p. 764. Values (obtained by Northern blot analysis) are reported as relative arbitrary units for each mRNA normalized to the expression of 18S RNA (used as a housekeeping gene). Values are means with their standard errors shown by vertical bars. Mean values were significantly different from those of the control group: * $P < 0.05$, *** $P < 0.001$. Mean values were significantly different from those of the olive oil-rich diet group: † $P < 0.05$.

Plasma lipids and lipoproteins

The present results suggest that the modulation of plasma TG was due to the addition of dietary lipid rather than to the degree of saturation of its inherent fatty acids. This seems to be peculiar to pigs; it is a characteristic not shared with other species such as the rat, in which the degree of saturation is more important than the amount of dietary lipid consumed (Calleja *et al.* 2000). The HDL-cholesterol concentrations of pigs fed the control diet and the olive oil-enriched diet were similar. This agrees with studies performed in human subjects (Mattson & Grundy, 1985; Mata *et al.* 1992), primates (Rudel *et al.* 1990; Brousseau *et al.* 1993) and rats (Calleja *et al.* 2000). MUFA-enriched diets do not decrease HDL-cholesterol, as do diets containing PUFA. The similar response of HDL-cholesterol to the degree of saturation of the fatty acids observed in the present experiment (in which >50% of plasma cholesterol was carried by LDL), plus the low levels of cholesteryl ester transfer protein observed by Pussinen *et al.* (1997), shows the pig to be a particularly interesting animal in which to study HDL regulation.

The biphasic distribution of cholesterol observed in terms of HDL size (see Fig. 1) in the pigs receiving the olive oil-enriched diet indicates either an activation of hepatic lipase or phospholipid transfer protein. Dreon *et al.* (1998) found that fat intake modulated hepatic lipase activity in human subjects, and that it may induce changes in HDL size. Activation of hepatic lipase would reduce HDL size and facilitate the uptake of cholesterol by SR-B1; polyunsaturated fat provided

by maize or sunflower oil can cause this (Spady *et al.* 1999; van Tol *et al.* 1999). However, for the olive oil-rich diet, the previous mechanism does not satisfactorily explain the reduction in the amount of cholesterol-associated HDL nor the appearance of the second, small HDL peak. If there were an activation of hepatic lipase, no efficient removal of hepatic lipase-metabolized HDL by SR-B1 would occur that might cause the second FPLC peak. However, higher phospholipid transfer protein activity might also explain the presence of this second peak. Phospholipid transfer protein has been shown to break the uniformity of pig HDL, favouring the appearance of a second, smaller peak and indicating the regulation of both proteins by saturation in this animal (Pussinen *et al.* 1997). More research is required to determine which hypothesis is correct. Further support for the activation of HDL catabolism in olive oil-fed pigs comes from the enhanced ApoA-I mRNA expressions in both the organs that synthesize it (Fig. 2)

With regard to the distribution of ApoA-IV, the present results indicate that lipoprotein-bound ApoA-IV levels are influenced by dietary supplementation, but not by the degree of saturation of the fatty acids consumed. The high-lipid diets increased plasma ApoA-IV levels in both the HDL and lipoprotein-free fractions, the latter being more pronounced. A similar observation, particularly with regard to the lipoprotein-free fraction, was noted when cholesterol was present in high-lipid diets fed to rats (DeLamatre *et al.* 1983). However, in the present experiment, no cholesterol was added, indicating that in pigs a high-lipid diet is enough to sustain the mechanism involved in ApoA-IV distribution.

ApoA-I, ApoC-III and ApoA-IV gene cluster expression

The increase in plasma ApoA-IV caused by the high-lipid diets was independent of the saturation of the inherent fatty acids. This agrees with results reported in human subjects (Kratz *et al.* 2003). The present results also indicate that changes in plasma ApoA-IV levels following the consumption of high-lipid diets may be due its increased expression in the intestine and liver. In mice, an increase in liver ApoA-IV mRNA levels was seen after providing a high-fat, high-cholesterol diet (LeBoeuf *et al.* 1994). When studying the effect of dietary fat saturation and cholesterol on hepatic Apo gene expression in rats, Osada *et al.* (1994) observed the increase in ApoA-IV mRNA to be due to cholesterol, and that this was independent of the degree of dietary lipid saturation. Moreover, no coordinated regulation of the gene cluster was observed. However, in pigs, intestinal coordination was seen for ApoA-I and ApoC-III, while ApoA-IV had a different pattern, being sensitive only to high-PUFA diets (Fig. 2). The present results corroborate the observation of a coordinated regulation of duodenal ApoA-I and ApoC-III in a postprandial regimen in newborn pigs (Black *et al.* 1996; Wang *et al.* 1996; Wang *et al.* 1998) and in the long-term administration of lipid-rich diets to transgenic mice (Baroukh *et al.* 2001). This indicates that this intestinal coordination in long-term diets is preserved in adult animals only for the ApoA-I and ApoC-III genes. Of interest in the present study are the changes in liver ApoA-IV mRNA expression in response to dietary lipid quantities and fatty acid saturation, and the expression of this gene being closely coordinated along with the ApoC-III and ApoA-I genes. This extends the observation of gene cluster coordination to the liver in adult animals subjected to long-term dietary schedules. With regard to the lipid-rich diets in the present study, the findings reported can be attributed to a greater contribution by linoleic acid to the increase in ApoA-IV mRNA levels. In fact, mean hepatic ApoA-IV expressions were well correlated with the intake of this fatty acid ($r=0.99$, $P<0.02$). Overall, the present results suggest that the hepatic gene cluster in this animal is highly sensitive to linoleic acid, and that hepatic regulation of ApoA-IV plays an important role in chronic adaptation to dietary response.

In conclusion, the hepatic expression of ApoA-IV is coordinated jointly with the other members of the gene cluster in response to the long-term administration of lipids, and dependent of the degree of saturation of their inherent fatty acids. This is apparently a mechanism peculiar for this animal. Changes in mRNA expression may be responsible for the plasma changes of ApoA-IV. Although packaged with HDL, ApoA-IV mostly remains unassociated with lipids as a nutritional response to higher lipid content diets. These issues are worthy of further research in this animal model.

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