Obesity induced during sexual maturation is linked to LDL-triacylglycerols in Yucatan miniature swine

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The incidence of childhood obesity is rising dramatically throughout industrialised countries. To evaluate and study the impact of childhood obesity on lipoprotein metabolism, we developed a new animal model of premature obesity. Yucatan mini-pigs aged 4 months were studied over a 12-month period from childhood to adulthood. Animals were divided into two groups: the first group were overfed a Western misbalanced diet; the second group were normally fed a recommended human-type diet. Cholesterol and triacylglycerol concentrations in VLDL-, LDL- and HDL-lipoproteins were followed from baseline to adulthood by fast protein liquid chromatography. At 10 (the end of sexual maturation) and 16 months old (adulthood), liver, visceral and subcutaneous adipose tissues were sampled. Real-time RT-PCR was performed in order to compare apo AI, apo B, apo C-III, PPAR-α, insulin receptor and lipoprotein lipase gene expression between groups and ages. Differences between groups were observed only after sexual maturity. Adult overfed mini-pigs had a higher LDL-cholesterol:HDL-cholesterol ratio (P<0.05; 0.55 (SE 0.06) for overfed v. 0.42 (SE 0.04) for normally fed pigs at the tenth month of the study). In both groups, VLDL-triacylglycerol decreased (P<0.05). VLDL-triacylglycerol evolution in the overfed group was associated with an increase in LDL-triacylglycerol plasma concentrations (P<0.05) after sexual maturation. LDL-triacylglycerol concentration in overfed mini-pigs went from an average of 0.28 mmol/l before sexual maturation to reach an average concentration of 0.56 mmol/l afterwards. This phenomenon has never been observed in similar studies when obesity is induced in adult mini-pigs and may represent a specific hallmark of an obesity induced during sexual maturity.

Obesity: Childhood: Adulthood: Lipoproteins: Mini-pigs

Epidemiological studies of children and adolescents have revealed a constant increase in the prevalence of overweight and obesity in industrial (Rolland-Cachera et al. 2002; Jolliffe, 2004) and in developing (Yajnik, 2004) countries. It is, however, too early to measure the long-term consequences of obesity acquired during development and puberty on individuals’ health.

Adult obesity, especially central obesity (Klesges et al. 1992; Maffeis et al. 2002), is universally recognised as an independent risk factor for CVD (Han et al. 1997). A cluster of modifications in lipid metabolism associated with obesity are linked to CVD risk (Ginsberg, 2000). The quantity and the quality of plasma lipoprotein in relation to triacylglycerols and cholesterol ester distribution in VLDL, LDL and HDL could reveal early events in the deregulation of lipid metabolism. Several pathways are involved in lipid synthesis and transport, including liver PPAR-α and insulin signalling pathways, lipoprotein lipase activity and also modifications of specific lipid transfer proteins: cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (Ren et al. 1996; Desvergne et al. 1998; Fruchtman, 2001). Both cellular and plasma effector are involved in the control of lipoprotein synthesis, lipid release and uptake through apo and lipogenic enzyme synthesis and are known to be modified by the Western lifestyle (a high-fat, high glycaemic index diet, low physical activity, etc.) and obesity (Vu-Dac et al. 1994).

As in adulthood, the Western lifestyle during childhood can promote lipid disorders (Franklin et al. 1998). Compared with their normal-weight counterparts, obese children and adolescents frequently have higher LDL-cholesterol concentrations and lower HDL-cholesterol plasma levels (Franklin et al. 1998, Plourde, 2002), and are at risk of developing triacylglycerolaemia (Haya-shibe et al. 1997). Moreover, some data from statistical analyses suggest that early obesity could have an impact on adulthood obesity and increase the risk of developing a metabolic syndrome leading to CVD in adulthood (Dietz, 1998; Freedman et al. 1999). Continuous obesity can serve as a generator for prolonged insulin resistance (Vanhala et al. 1998). Other authors have, however, reported conflicting results and suggest that childhood obesity does not have an adverse effect on adult health (Wright et al. 2001; Maffeis et al. 2002). Children and adolescents are, by definition, not mature. Interestingly, as with the case of adult obesity, the hormonal changes that occur during puberty are associated with natural modifications of lipid metabolism (Kouda et al. 2001).
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and insulin sensitivity (Moran et al. 1999). Nevertheless, very little has until now been known about the cumulative effects of obesity, a Western lifestyle and puberty on lipid metabolism.

Studies of the evolution of body weight during obesity and lipoprotein profiles (quantity and quality) during and after sexual maturation would be helpful to better understand childhood obesity and its long-term consequences. This implies a need for longitudinal and interventional studies with a strict control of food intake from childhood to adulthood. To provide an alternative to such long and invasive experiments, we have developed a mini-pig model with obesity induced before sexual maturation and until adulthood.

Adult mini-pigs have already been used to study insulin resistance and lipid metabolism changes in relation to the Western lifestyle (Phillips et al. 1982; Gerrity et al. 2001). Even if pigs and mini-pigs, compared with man, have a very low activity of CETP and present low triacylglycerol plasma concentrations (Larsen et al. 2002; Olsen et al. 2002), compared with mice and rats, pigs and mini-pigs represent one of the species most relevant for modelling lipoprotein metabolism in man. Indeed, pigs are, like man, recognised as an LDL species (Olsen et al. 2002), and they transport a large part of their esterified cholesterol in this lipoprotein. Pigs are also omnivorous (Phillips et al. 1982), their cardiovascular system is quite similar to that of man (Dixon et al. 1999), and their human-like physiology (Moughan et al. 1992) and physiopathology related to obesity, type 2 diabetes and CVD (Dixon et al. 1999), as well as their large size, are very useful for mimicking the situation of obese people. The objective of the present study was to observe possible specific lipid–lipoprotein modulation in relation to the development of obesity induced during sexual maturation in a Yucatan mini-pig model of childhood obesity.

Material and methods

Animals

Non-castrated male Yucatan mini-pigs (n 10; Yucatan micropig; IFFA-Credo-Charles River, Dardilly, France) aged 4 months were used in this study. Before the experiments, the mini-pigs were acclimated for 2 weeks to their local environment: natural light/dark cycle, a temperature-controlled room (20–24°C), individual feeding boxes and free access to water. All experimental treatments were in accordance with French legislation on animal experimentation (Decree 87–848 of the French Penal Code, 1987).

Experimental design

The mini-pigs were divided into two identical groups of five animals: the first group was overfed (OF; 1·5 times the recommended energy intake for mini-pigs; Bollen et al. 1999) a Western-type diet, and the second one was normally fed (NF) a recommended human diet (Table 1). This study was conducted from mini-pig ‘childhood’ (4 months old ± 1 week) to adulthood (16 months old ± 1 week), the first 6-month period studied corresponding to the mini-pigs’ sexual maturation (sexual maturity being between 7 and 10 months of age). The mini-pigs were fed twice per day. Both diets had identical energy densities of 18 MJ/kg DM. The OF diet was designed to provide 1818 kJ/body weight0·75 per 24 h, and the NF diet 1212 kJ/body weight0·75 per 24 h. During sexual maturation, the amount of food distributed to the groups was adjusted each week. After sexual maturity (10 months old), the amount of food distributed to pigs was then maintained at a constant level. Throughout the entire study, food distribution for OF pigs was calculated on the basis of the mean metabolic weight (body weight0·75) of NF pigs. Food distribution to the NF group was calculated on the basis of their individual body weights.

Lipid measurements

Blood was taken via the anterior vena cava from overnight-fasted pigs at baseline and after 4, 6, 8, 10 and 12 months of treatment. For lipoprotein cholesterol and triacylglycerol levels, plasma samples (200 μl) were analysed by fast protein liquid chromatography (Amersham Pharmacia Biotech Inc., Orsay, France) on two Superose 6HR 10/30 columns (Amersham Pharmacia Biotech Inc.) and eluted with a saline buffer (0·15 mol/l NaCl, 1 mmol/l EDTA, 0·02 % Na3EDTA, pH 8·2). The absorbance of the eluent was continuously monitored at 280 nm using a UV monitor. Fast protein liquid chromatography was programmed to collect elution volumes ranging from 11·40 ml to 35·70 ml, which represented eighty-one fractions of 300 μl and included the three lipoprotein fractions: VLDL (elution volume 12·9–15·9 ml), LDL (elution volume 18·9–24·0 ml) and HDL (elution volume 26·4–32·4 ml). All the fractions were assayed for cholesterol and triacylglycerol concentrations determined by enzymatic procedures (cholesterol: RTU; triacylglycerol: PAP 1000; BioMerieux, Lyon, France).

Gene expression

Tissues samplings were made under general anaesthesia with a gaseous mix of O2, nitrogen protoxyde and isoflurane (Centravet;
La Millière, Plancoët, France). At sexual maturity and adulthood, liver, subcutaneous and visceral adipose tissues were sampled. Every sample was taken under RNase-free conditions for quantitative RT-PCR. Total RNA was extracted from samples according to the Chomczynski and Sacchi method (Chomczynski & Sacchi, 1987) by using TRIzol reagent (Invitrogen, Cergy Pontoise, France). DNase treatments (RQ1 Dnase; Promega, Charbonnière, France) were performed on each extract to eliminate traces of DNA, and reverse transcription was made with 2 μg total RNA with the superscript II RT (Invitrogen). Quantitative gene expression was measured with a quantitative thermocycler (BioRad, Marnes-La-Coquette, France) in which complementary DNA, and reverse transcription was made with 2 μg total DNA, and reverse transcription was made with 2 μg total DNA. Gene expressions of apo B, apo A-I, apo C-III, PPAR-α and insulin receptor were measured in liver. In adipose tissues, the expression of the gene encoding for lipoprotein lipase (LPL) was quantified. Quantitative gene expressions were expressed as relative values, taking the mean expression of a gene at sexual maturity in the NF pig group as a reference value (1·00) for each gene. To compare gene expression in visceral and subcutaneous adipose tissues, the mean NF pig group value of each gene in the visceral adipose tissue at sexual maturity was used as a reference.

Statistical analysis

Data were recorded as mean with their standard errors. Statistical analyses were made with Statview software (SAS Institute, Boston, MA, USA) in order to perform Student’s t tests, P<0·05 representing a significant difference.

Results

Body weight evolutions

Figure 1 presents the evolution in body weight of the mini-pigs during the 12-month study period. In both groups, average body weight increased (P<0·01). Average NF body weights after 6, 10 and 12 months of the experiment were 3·0, 4·4 and 4·6 times higher than initial NF body weights, respectively. In OF mini-pigs, at the same points in time, body weights were 5·5, 8·4 and 9·0 times higher than baseline body weights, respectively. After 4 months of feeding treatments (8 months old), NF mini-pigs were approximately twice as lean as the OF animals (P<0·01).

Cholesterol measurements

Total cholesterol, VLDL-cholesterol, LDL-cholesterol and HDL-cholesterol plasma concentrations throughout the study are shown in Table 2. In both groups, cholesterol concentrations doubled (P<0·01) during the first 4 months of the study and then remained stable until the end of the study. In NF mini-pigs, VLDL-cholesterol plasma concentrations were unchanged from baseline to the tenth month of the study. The VLDL-cholesterol concentration in NF animals after 12 months of study was lower than baseline concentration (P<0·05). In the NF group, LDL-cholesterol plasma concentrations increased from baseline to the fourth month of the study (P<0·05), but the subsequent measurements did not show any significant differences compared with baseline concentrations. The increase in total cholesterol in NF pigs was characterised by the significant increase in HDL-cholesterol from the fourth month (P<0·05). In OF mini-pigs, changes in cholesterol profile were characterised by a decrease in VLDL-cholesterol after the sixth month of the study (10 months old) (P<0·05), a higher LDL-cholesterol level at 4, 6 and 10 months of study compared with baseline (P<0·05) and an increase in HDL-cholesterol from the fourth month of the study (P<0·05). We did not observe any significant differences related to cholesterol concentration between groups. Only the LDL-cholesterol:HDL-cholesterol ratio (Fig. 2) differed between NF and OF animals, with a decrease of this ratio in NF mini-pigs from the eighth month until the end of the study (P<0·05), whereas this ratio remained stable and significantly higher in the OF animals than in the NF animals (P<0·05).

Triacylglycerol measurements

The evolution of fasting total triacylglycerol concentrations and distributions between fast protein liquid chromatography lipoproteins are presented in Table 3. Compared with baseline values, fasting triacylglycerol concentrations remained statistically unchanged in the NF group. In this group, compared with total and VLDL-triacylglycerol concentrations measured after 6 months’ treatment, we measured significant decreases in these triacylglycerol determinants after 8, 10 and 12 months of study (P<0·05). LDL- and HDL-triacylglycerol were unchanged throughout the study in NF animals. In OF mini-pigs, baseline total and VLDL-triacylglycerol were significantly higher than the concentrations measured at 4, 8, 10 and 12 months of study (P<0·05). LDL- and HDL-triacylglycerol were unchanged throughout the study in NF animals. In OF mini-pigs, baseline total and VLDL-triacylglycerol were significantly higher than the concentrations measured at 4, 8, 10 and 12 months of study (P<0·05). In OF animals, LDL-triacylglycerol concentrations remained stable during sexual maturation (i.e. from baseline to the sixth month of the study) and increased thereafter (P<0·05). In OF mini-pigs, LDL-triacylglycerol plasma concentration presented a tendency to increase after sexual maturity without presenting significant differences compared with baseline HDL-triacylglycerol concentrations.
Few significant differences have been observed between groups for triacylglycerol measurements. VLDL-triacylglycerol at 4 months of the study was higher in NF than in OF pigs ($P<0.05$), and HDL-triacylglycerol at 6 months of the study was higher in NF than OF animals ($P<0.05$). These differences were selective changes, the only significant differences that lasted between groups being observed for LDL-triacylglycerol concentration. LDL-triacylglycerol plasma concentrations were about twice as low in the NF group as in the OF group after 8 months of the study ($P<0.05$). These differences were selective changes, the only significant differences that lasted between groups being observed for LDL-triacylglycerol concentration. LDL-triacylglycerol plasma concentrations were about twice as low in the NF group as in the OF group after 8 months of the study ($P<0.05$). Between the sixth and twelfth months of the study, LDL gene expression in subcutaneous adipose tissue decreased ($P<0.05$) compared with the end of sexual maturation. In OF adult mini-pigs, only PPAR-α and insulin receptor gene expressions decreased between 6 (sexual maturity) and 12 months of study (adulthood; $P<0.05$). Apo C-III, apo B and apo A-I remained stable from 6 months of study to the end. After 12 months of study, apo B gene expression measured in NF adult mini-pigs was significantly lower than in OF mini-pigs ($P<0.05$).

**Liver gene expression**

Liver gene expressions measured at the end of sexual maturation and adulthood are shown in Table 4. At the end of sexual maturation (10 months old, 6 months of study), NF mini-pigs had higher apo A-I gene expression than OF animals ($P<0.05$). The other gene expressions measured at that time did not differ between groups. After 12 months of study (adulthood), all liver gene expressions measured in NF mini-pigs decreased ($P<0.05$) compared with the end of sexual maturation. In OF adult mini-pigs, only PPAR-α and insulin receptor gene expressions decreased between 6 (sexual maturity) and 12 months of study (adulthood; $P<0.05$). Apo C-III, apo B and apo A-I remained stable from 6 months of study to the end. After 12 months of study, apo B gene expression measured in NF adult mini-pigs was significantly lower than in OF mini-pigs ($P<0.05$).

**Lipoprotein lipase gene expression**

Comparisons of visceral and subcutaneous adipose tissue LPL gene expression are presented in Fig. 3. No difference was observed between groups. After 6 months of study, mini-pig LPL gene expression was higher in visceral adipose tissue than in subcutaneous tissue ($P<0.05$). Between the sixth and twelfth months of the study, LPL gene expression in subcutaneous adipose tissues increased significantly in both groups ($P<0.05$) and reached the same level as visceral LPL gene expression.

**Discussion**

The objective of the present study was to characterise possible changes in lipoprotein profile during the development of obesity induced during childhood. For this purpose, immature male Yucatan miniature pigs were overfed a Western-type diet enriched with saturated fat and high glycaemic index carbohydrates. These were compared with control animals that were fed a balanced human-type diet providing an adequate daily energy intake for these animals. The tracking of obesity until adulthood allowed us to model a putative impact of the development of an obesity model. For details of diets and procedures, see p. 282.
Nevertheless, even if total cholesterol increased in a similar way in both groups, the NF diet enriched with monounsaturated fats, as has been observed in man (Luscombe et al. 1999; Gill et al. 2003), may have induced the fall in the HDL-cholesterol/LDL-cholesterol ratio (Luscombe et al. 1999).

It is interesting to note that both groups shared common features. In fact, we observed similar changes in cholesterol, PPAR-α and apo A-1 gene expressions and in total and VLDL-triacylglycerol concentrations measured by fast protein liquid chromatography in normally fed (NF) and overfed (OF) mini-pigs.
Indeed, LDL-triacylglycerol is associated with small and dense LDL (Ginsberg, 2000). This LDL sub-fraction has a lower affinity for the LDL receptor (Galeano et al. 1998) and presents a higher oxidability (Guerin et al. 2001). Both of these factors are involved in the development of atherosclerosis.

An increase in LDL-triacylglycerol concentrations related to similar high-fat feeding was never observed in studies carried out on adult mini-pig models (Dixon et al. 2002). This observation suggests that the increase in LDL-triacylglycerol observed in the present study may represent a specific phenomenon that occurs during growth. As frequently observed in obese adolescents, OF mini-pigs were taller than NF mini-pigs (data not shown), supporting the fact that obesity induced before sexual maturity could have stimulated growth factors. It is interesting to note that patients with growth defects such as acromegaly or growth hormone deficiency have high risks of CVD associated with modifications of LDL sub-fractions (Tan et al. 1997; Carrilho et al. 2001). Little is known about the effect of growth hormone and/or insulin-like growth factor-1 on LDL metabolism. Few studies have shown interactions between growth factors and the LDL receptor (Machado et al. 2003), CETP (Carrilho et al. 2001) or IL-6 (De Benedetti et al. 2002). According to the links existing between growth defects and LDL metabolism, we may hypothesise that obesity induced during sexual maturation was able to induce growth factors (growth hormone and/or insulin-like growth factor-1) modifications, which could in turn increase the increase in LDL-triacylglycerol. Furthermore, obese adolescents are frequently taller than normal-weight adolescents (Heude et al. 2003; Freedman et al. 2004) and insulin-like growth factor-1 plasma concentrations are higher in obese adolescents (Wabitsch et al. 1996).

In conclusion, the present study investigated the impact of the development of obesity during sexual maturation on lipid metabolism in a Yucatan mini-pig model. Very small changes in lipoprotein profile and gene expressions were observed in this study. First, lipid metabolism was not modified until the end of puberty in our mini-pigs. Nevertheless, after sexual maturation, we detected a possible specific benchmark of this form of obesity. In obese miniature swine, such an increase in LDL-triacylglycerol concentrations had never been observed in mini-pigs in which obesity was induced in adulthood. Moreover, observations of acromegalic and growth hormone-deficient patients illustrate possible relationships between growth and LDL metabolism. To our knowledge, the present study, on Yucatan mini-pigs, is the first investigation of changes in lipid metabolism during obesity induced during growth. It has been performed on a small number of immature male Yucatan mini-pigs and needs to be confirmed on female and other strains of mini-pig. It suggests, however, that childhood obesity could specifically modify the metabolism of LDL.

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