Lipoproteins Abnormalities in Obese Insulin-Resistant Dogs

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Many studies have shown that obesity and low insulin sensitivity are associated with lipoprotein abnormalities, which are risk factors for coronary heart disease. The effects of insulin resistance on lipoprotein metabolism were investigated in hyperenergetic-fed beagle dogs, a new model of insulin resistance. Insulin resistance was assessed by the 3-hour euglycemic-hyperinsulinemic glucose clamp technique. Lipoproteins were separated by fast-protein liquid chromatography (FPLC) and lipid composition of the different lipoproteins was determined by enzymatic methods. Hyperenergetic diet was associated with a 43% ± 5% increase in dog body weight and a reduction in insulin-mediated glucose uptake (28 ± 3 to 16 ± 1 mg · kg^{-1} · min^{-1}, $P < .05$). Low insulin sensitivity associated with obesity was related to an increase in plasma triglyceride (TG) through an increase in very–low-density lipoprotein (VLDL)-TG (0.071 ± 0.020 *v* 0.382 ± 0.242 mmol/L, $P < .05$) and high-density lipoprotein (HDL)-TG (0.025 ± 0.012 *v* 0.242 ± 0.143 mmol/L, $P < .05$). Other lipid abnormalities common in insulin resistant humans were also found: lower plasma HDL-cholesterol (4.690 ± 0.151 *v* 3.937 ± 0.141 mmol/L, $P < .05$) and higher plasma nonesterified fatty acids (NEFA) (0.974 ± 0.094 *v* 1.590 ± 0.127 mmol/L, $P < .05$) levels. These data show that this model of the insulin-resistant obese dog could be useful in studying insulin resistance–associated dyslipidemia.

IN HUMANS, many studies[1,2] have shown that obesity and low insulin sensitivity are associated with dyslipidemia and that abnormalities in lipid and lipoprotein metabolism contribute to the increased risk of coronary heart disease. Hypertriglyceridemia is one of the most common abnormalities reported in insulin-resistant humans as a result of an increased very–low-density lipoprotein triglyceride (VLDL-TG) production.[3,4] This overproduction of VLDL could be due to an increased supply of substrates to the liver, particularly glucose and nonesterified fatty acids (NEFA).[5] Kinetic studies[3] have shown that a decrease in lipoprotein lipase (LPL) activity might account for the increase in VLDL-TG. Moreover, the LPL decrease, which would be responsible for the delayed clearance of VLDL-TG, would also lead to altered high-density lipoprotein total cholesterol (HDL-TC) metabolism.[6] In addition to a reduced LPL activity, an increased activity of hepatic lipase (HL), an enzyme involved in HDL remodeling by stimulating HDL-TG hydrolysis, could contribute to the low HDL-cholesterol level associated with obesity and insulin resistance.[7-9] Another common lipoprotein abnormality in insulin-resistant humans is an increase in plasma NEFA. Indeed, insulin resistance induces excessive adipose tissue lipolysis, which increases the NEFA flux to the liver.[10]

The lipoprotein profile of a dog is quite different from that of humans.[11] In contrast to humans, whose low-density lipoproteins (LDL) are the major lipoprotein, the dog is a species with very few VLDL and predominant HDL.[12] In this species, HDL are the main plasma carriers of cholesterol.

The aim of this study was first to set up insulin resistance associated with obesity in dogs, assessed by the euglycemic hyperinsulimemic clamp technique, and then to characterize abnormalities in lipoprotein associated with this low sensitivity to insulin status.

MATERIALS AND METHODS

Animals and Diet

The adult beagle dogs used in the study were housed according to the regulations for animal welfare of the French Ministry of Agriculture and Fisheries. The experimental protocols adhered to European Union guidelines and were approved by the Animal Use and Care Advisory Committee of the University of Nantes. Only healthy animals were enrolled: hematocrit $>$ 38%, leukocyte count $<$ 18,000/μL, good appetite, no medications, normal stools, and normal body temperature (38.5 to 39.5°C).

Seven adult beagle dogs, males (initial body weight [BW], 12.2 ± 1.0 kg) were studied before and after an overfeeding period. Before the initiation of the study, dogs consumed in a single meal a dry commercial dog food (containing corn meal, poultry meal, gluten, animal fat, corn, corn flakes, rice, beet pulp, green beans, carrots, minerals, and vitamins and consisting of 27% crude protein, 13% ether extract, and 3,730 kcal metabolizable energy/kg, on a dry matter basis). Dogs were fed according to the National Research Council (NRC) recommendation (132 kcal metabolizable energy/kg BW$^{0.75}$). In order to develop obesity and insulin resistance, dogs were given a hyperenergetic diet fed ad libitum, with 75% of the energy allowance from a dry diet (containing poultry meal and byproducts, corn meal, animal fat, vegetable oil, beet pulp, poultry liver meal, fish, psyllium, brewer's yeast, fish oil, minerals, DL-methione, L-carnitine, and vitamins, and consisting of 34% crude protein, 32.6% ether extract, and 4,790 kcal metabolizable energy/kg, on a dry matter basis) and 25% from a canned food (containing meat and meat byproducts, cereals, minerals, sugars, animal fat, and vegetables, and consisting of 35% crude protein, 20% ether extract, and 3,860 kcal metabolizable energy/kg, on a dry matter basis). Intake of each food was recorded daily.

Insulin sensitivity was assessed before weight gain and when BW gain had been at least 20% of initial BW for at least 5 weeks. Lipoprotein profile and composition were assessed when insulin sensitivity was at least 28 % lower than initial value.

Assessment of Insulin Sensitivity

In vivo insulin sensitivity was assessed using the euglycemic glucose clamp technique[13] in conscious dogs. Three catheters were inserted in 20-hour food-deprived dogs; the first (20G, 1 in, Vasocan Braunüle, Melsungen, Germany) in the cephalic vein for infusion of insulin, the

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second one in the contralateral forelimb for glucose infusion, and the third one (20G, 8 cm, Vygon, Ecouen, France) in the jugular vein for blood sample collection.

After baseline plasma glucose had been assessed, a bolus of insulin (Actrapid, human insulin 40 IU/mL, Novo Nordisk, Bagsvaerd, Danemark) was injected (8 mU · kg^{-1}), immediately followed by a continuous infusion of insulin (2 mU · kg^{-1} · min^{-1}). Four minutes after the primed insulin infusion, a glucose (Glucose 20%, Laboratoire Aguettant, Lyon, France) infusion was performed in order to maintain euglycemia. Blood samples were taken at 5-minute intervals for 1 hour and then at 10-minute intervals for rapid determination of blood glucose concentration. Plasma glucose was maintained at baseline level by adjustment of the glucose infusion rate. The amount of glucose infused is a measure of tissue sensitivity to insulin.

Because serum potassium levels tend to fall during this procedure, phosphate dipotassic (K 6 mmol/L, HPO_4 3 mmol/L, Laboratoire Aguettant) had been added to the glucose solute. The clamp procedure was continued for 180 minutes. Blood samples of 2 to 3 mL were drawn from the jugular vein for determination of insulin concentration. These samples were placed in heparinized (150 U/tube) tubes. Blood was immediately refrigerated at 4°C and centrifuged at 5,000 rpm for 10 minutes. Plasma was frozen at −20°C for determination of insulin concentration and the first blood sample was used for the determination of baseline plasma glucose and for lipoproteins separation.

Suppression of hepatic glucose production was validated during the clamp using a primed-constant [6,6-D_2]glucose infusion.[14]

Lipoprotein Separation

The fast-protein liquid chromatography (FPLC) system (Pharmacia Biotech, Orsay, France), consisted of 2 Superose 6HR 10/30 columns in quick succession, equilibrated in saline buffer (0.15 mol/L NaCl, 1 mmol/L EDTA, 0.02% NaN_3, pH 8.2). A multisample injector adapted with a 200-μL sample loop was available for sample loading, and the flow rate was maintained constant at 0.2 mL/min over 270 minutes. The sample was injected 2 minutes after the beginning of the procedure. The fractions were collected from 62 to 180 minutes and a 90-minute wash was included before each sample injection. The absorbance of the eluent was monitored continuously at 280 nm using a UV monitor. FPLC was programmed to collect elution volume from 14 mL to 38 mL, which represented 80 fractions of 300 μL and resolved 3 lipoprotein fractions, VLDL (fractions 7 to 17, elution volume 14.5 to 17.5 ml), to LDL (fractions 25 to 45, elution volume 19.9 to 25.9 ml), and to HDL (fractions 49 to 77, elution volume 27.1 to 35.5 ml). Those fractions were subsequently analyzed for TG, TC, unesterified cholesterol (UC), cholesteryl ester (CE), phospholipids (PL), and NEFA. Peaks were identified as described by Kieft et al.[15]

Chemical Analysis

Blood glucose concentration was determined with a glucose oxydase method. Insulin was measured by radioimmunoassay on each blood sample (RIA Insik-5, Sorin Biomedica, Sorin, Italy). UC, CE, TG, and PL were analyzed in the different lipoproteins, and NEFA in plasma, using enzymatic methods (Cholesterol RTU, BioMérieux, Marcy-l'Etoile, France; "Cholestérol libre enzymatique" Color, Biotrol Diagnostic, Lognes, France; "Triglycérides and Phospholipides enzymatiques" PAP 150, BioMérieux, Marcy-l'Etoile, France; NEFA C, WAKO, Oxoid, Dardilly, France).

Statistical Analysis

Data were reported as means ± SEM. Statistical analysis using the Instat Software package (GraphPad, San Diego, CA) was performed with the Wilcoxon paired test to determine significant differences between parameters in normal and obese dogs. A 2-sided P value less than .05 was considered statistically significant.

Table 1. Summary of the Effect of the Hyperenergetic Diet on Weight, Basal Glycemia, Basal Insulinemia, Plateau of Insulin Levels, and Glucose Infusion Rate During the Euglycemic Hyperinsulinemic Clamp

Parameter	Normal	Obese
Weight (kg)	12.2 ± 1.0	17.5 ± 1.7*
Basal glycemia (mg/dL)	83 ± 3	88 ± 2
Basal insulinemia (μU/mL)	10 ± 1	24 ± 1*
Plateau of insulin level (μU/mL)	140 ± 10	150 ± 8
Glucose infusion rate (mg · kg^{-1} · min^{-1})	28 ± 3	16 ± 1*

*$P < .05$.

RESULTS

Body Weight and Insulin Sensitivity

BW increased by 43% ± 5 %, from 12.2 ± 1.0 to 17.5 ± 1.7 kg ($P < .05$) (Table 1) over 198 ± 18 days. Mean energy intake was 1.88 ± 0.08 times the NRC recommendation (132 kcal metabolizable energy/kg $BW^{0.75}$) on the basis of initial BW. The effects of BW increase on baseline glycemia and insulinemia, plateau of insulinemia, and glucose infusion rate during the euglycemic hyperinsulinemic clamp are shown in Table 1. Baseline glucose concentrations did not differ in normal and obese dogs (83 ± 3 mg/dL and 88 ± 2 mg/dL, respectively). Baseline insulin concentrations were significantly higher in obese dogs than in normal ones (24 ± 1 μU/mL and 10 ± 1 μU/mL, respectively; $P < .05$). The insulin infusion elevated the plasma insulin value to a steady-state plateau (150 ± 8 μU/mL) during the last 2 hours. Hepatic glucose production was suppressed during the hyperinsulinemic clamp (results not shown). Glucose infusion rate increased during the first hour of the clamp, then remained constant. The glucose infusion rate needed to maintain euglycemia was significantly higher in normal than in obese dogs (28 ± 3 v 16 ± 1 mg · kg^{-1} · min^{-1}, respectively; $P < .05$).

Lipoproteins Profiles

Figure 1 illustrates representative FPLC elution profile for plasma obtained from a dog when it was normal and when it was obese. The proteic absorbance profiles at 280 nm are shown in Fig 1A. While the LDL and HDL peaks were seen in all cases, the VLDL peak was sometimes very small, almost not detectable. The proteic absorbance profiles did not differ between normal and obese dogs except for an increase of VLDL peak. Lipids profiles of VLDL, LDL, and HDL are shown for TG, UC, CE, and PL content in Fig 1B through E. TG, UC, CE, and PL concentrations in these lipoproteins are shown in Table 2. The recovery of each lipid constituent in the 80 fractions collected was comparable to what was applied to the column. Losses were not significant (3% for TC, 4% for CE, 6% for TG, 4% for PL, and 2% for NEFA, $P < .05$). Most plasma TG were found in VLDL and LDL in normal dogs. VLDL-TG concen-

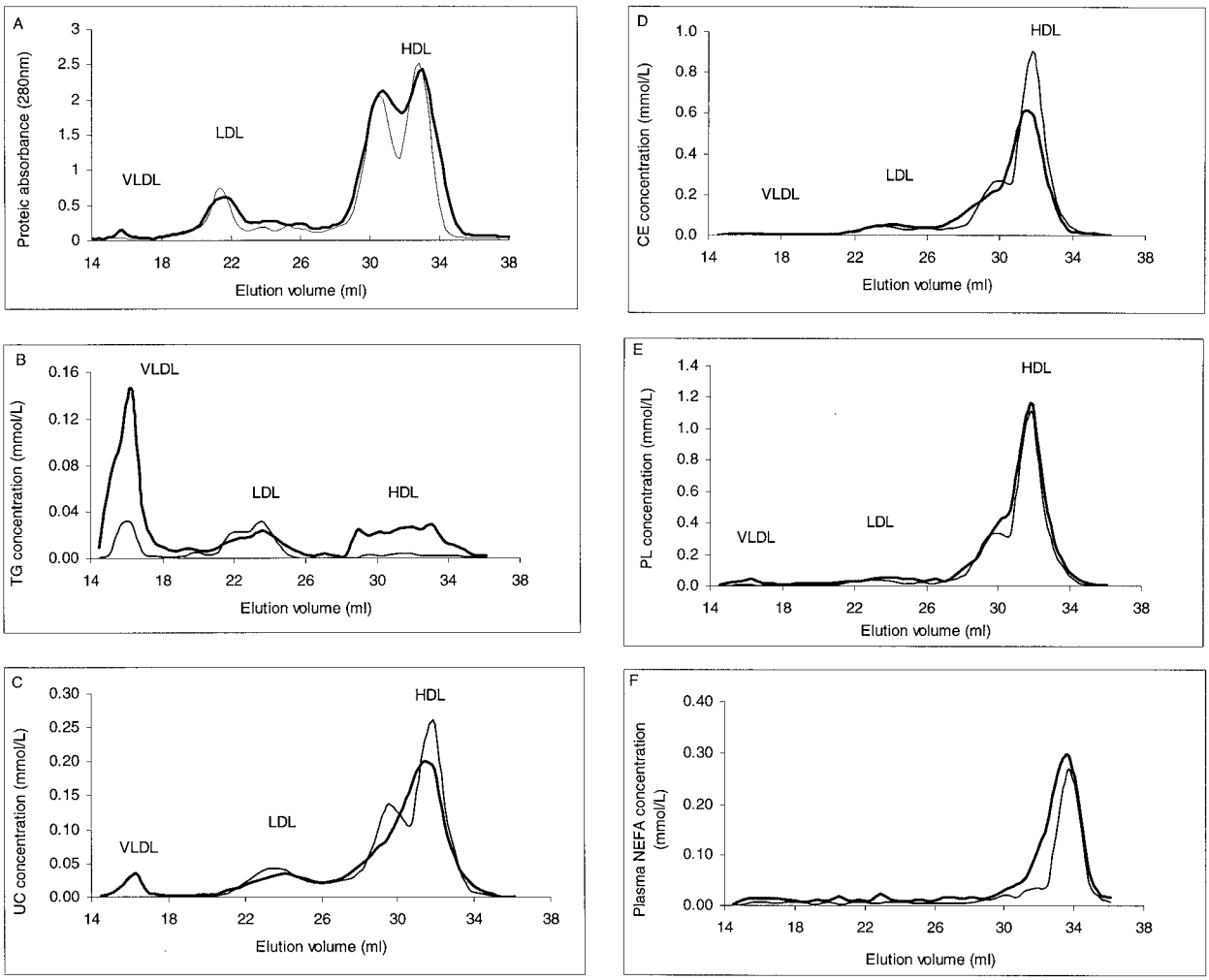

Fig 1. FPLC elution profiles for a representative plasma sample from a dog when it was normal (-) and when it was obese (·) as assessed by proteic absorbance at 280 nm (A), TG (B), UC (C), CE (D), PL (E), and NEFA (F).

tration increased significantly in obese dogs (0.071 ± 0.020 mmol/L *v* 0.382 ± 0.242 mmol/L in normal and obese dogs, respectively; $P < .05$) and in HDL (0.025 ± 0.012 *v* 0.242 ± 0.143 mmol/L, $P < .05$). No significant difference was observed in LDL. The greatest quantity of plasma TC was found in the HDL fraction in both normal and obese dogs and its concentration was lower in obese dogs (4.690 ± 0.151 mmol/L *v* 3.937 ± 0.141 mmol/L, $P < .05$), as well as that of CE (3.426 ± 0.191 mmol/L *v* 2.822 ± 0.162 mmol/L, $P < .05$). LDL contained UC and CE at approximately the same concentration. VLDL-TC was significantly higher in obese dogs (0.002 ± 0.001 *v* 0.096 ± 0.055 mmol/L, $P < .05$). PL were

Table 2. Lipid Composition of Lipoproteins by FPLC of Plasma Obtained From Normal Versus Obese Dogs

Parameter (mmol/L)	VLDL		LDL		HDL	
	Normal	Obese	Normal	Obese	Normal	Obese
TG	0.071 ± 0.020	0.382 ± 0.242*	0.141 ± 0.013	0.141 ± 0.032	0.025 ± 0.012	0.242 ± 0.143*
TC	0.002 ± 0.001	0.096 ± 0.055*	0.504 ± 0.095	0.555 ± 0.145	4.690 ± 0.151	3.937 ± 0.141*
UC	0.001 ± 0.001	0.076 ± 0.058*	0.246 ± 0.045	0.226 ± 0.062	1.264 ± 0.028	1.115 ± 0.051
CE	0.001 ± 0.001	0.020 ± 0.006*	0.258 ± 0.053	0.329 ± 0.086	3.426 ± 0.191	2.822 ± 0.162*
PL	0.023 ± 0.014	0.139 ± 0.057*	0.222 ± 0.059	0.376 ± 0.106	4.235 ± 0.278	4.899 ± 0.355

*$P < .05$.

mainly found in HDL and there was no difference between normal and obese dogs except for an increase in VLDL (0.023 ± 0.014 mmol/L *v* 0.139 ± 0.057 mmol/L, $P < .05$). Whereas CE/TG ratio increased significantly in VLDL with obesity (0.02 ± 0.01 and 0.24 ± 0.09 in normal and in obese dogs, respectively; $P < .05$), this ratio decreased significantly in HDL (274 ± 125 and 48 ± 24 in normal and in obese dogs, respectively ($P < .05$)). CE/TG ratio increased in LDL with obesity but not significantly (1.80 ± 0.27 and 2.42 ± 1.80 in normal and in obese dogs, respectively). NEFA concentrations in plasma were higher in obese than in normal dogs (0.974 ± 0.094 *v* 1.590 ± 0.127 mmol/L, $P < .05$) (Fig 1F).

DISCUSSION

In this study, we induced a marked BW increase (43% ± 5%) in dogs by a hyperenergetic high-fat diet in order to lower insulin sensitivity and to characterize related modifications in lipoprotein profile and composition.

First, we induced obesity in dogs in order to set up insulin resistance. In previous studies,[16,17] dogs were made obese using a hyperenergetic high-fat diet that led in a few weeks to hyperinsulinemia, reduced insulin sensitivity, and hypertension, which was their main concern. In our study, the energy allowance was less than in previous study in order to study long-term rather than short-term effects of decreased insulin sensitivity on lipid metabolism, as they can occur in spontaneous conditions in humans. Sensitivity to insulin, usually defined in relation to insulin action on glucose metabolism, was assessed using the euglycemic-hyperinsulinemic glucose clamp technique.[13,18] Basal insulin concentration (24 ± 1 μU/mL and 10 ± 1 μU/mL in obese and normal dogs, respectively) showed hyperinsulinemia in obese animals. The glucose infusion rate needed to maintain euglycemia reflects the whole-body glucose uptake under hyperinsulinemic conditions. It was diminished by 40% in obese compared to healthy state (16 ± 1 $mg \cdot kg^{-1} \cdot min^{-1}$ and 28 ± 2 $mg \cdot kg^{-1} \cdot min^{-1}$ respectively; $P < .05$). Because basal glycemia did not differ and glucose concentration was similar during clamp periods, this difference revealed insulin resistance[19] in overweight dogs without diabetes set-up. Thus, obese dogs were insulin-resistant.

Then, we characterized lipoproteins abnormalities in obese insulin-resistant dogs. Because in dogs, some HDL have the same density as LDL,[11,12] classic methods for isolating lipoproteins could not have been used. That is the reason why lipoproteins were isolated by FPLC. Whereas the normal dog is a species with few VLDL, HDL are the main lipoproteins in the plasma of dogs as previously shown.[11,12,15,20] They transport as much as 90% of the total circulating lipids and are the main plasma carriers of PL, CE, and UC, in that order (Table 2). Insulin resistance associated with obesity resulted in some important changes in lipoprotein components: an increase in plasma NEFA and triglyceridemia through an increase in VLDL and in HDL, and a decrease in HDL-TC concentration. These modifications are also the main changes that can be observed in insulin-resistant humans.[21] We observed an increase in plasma NEFA of insulin-resistant obese dogs that could be either a cause or a consequence of insulin resistance.[22] A lot of studies have shown that basal NEFA levels are increased in obese diabetic patients,[23-26] whereas Baldeweg et al. showed that association between insulin resistance and NEFA was directly related to obesity.[27] An increase in plasma NEFA has been shown in humans to be related to the hormone-sensitive lipase responsible for excessive adipose tissue lipolysis in the insulin-resistant state.[28] This leads to an increase in NEFA flux to the liver.[10] As NEFA are important regulators of VLDL production both in vitro[29,30] and in vivo,[4] an increased supply of these substrates to the liver could result in enhancement of hepatic TG synthesis and incorporation in VLDL,[31] leading to VLDL overproduction.[5] Indeed, we observed an increase in VLDL-TG concentration in insulin-resistant obese dogs. This increase in VLDL could also be related to the activity of LPL, the enzyme that hydrolyzes the TG components of VLDL and leads to LDL production. Indeed, many studies have shown that a low LPL activity could be an important factor in the development of hypertriglyceridemia by decreasing VLDL lipolysis in diabetic humans.[3,6,32] We showed lower HDL-cholesterol concentration in insulin-resistant obese than in normal dogs as a consequence of lower HDL-CE concentration. Low HDL-CE has often been associated with obesity and insulin resistance in humans.[7,8,33,34] This could be explained by a low HDL concentration due to a low level of LPL activity, which promoted incorporation of lipoprotein surface constituents in HDL_2 in human by hydrolyzing TG-rich lipoproteins.[35] This could also be a consequence of a change in hepatic lipase (HL) activity, which increased in the insulin-resistant state.[9] This enzyme is involved in HDL remodeling by stimulating HDL-TG hydrolysis and is thought to enhance hepatic HDL-cholesterol uptake.[36] Riemens et al showed that HDL-CE was inversely correlated with HL activity.[37] HL activity could have affected HDL core lipid metabolism. Lastly, low HDL-CE concentrations could also be due to the increase of cholesteryl ester transfer protein (CETP) activity, which impoverished HDL in CE. CETP catalyzes CE transfer from HDL to VLDL with exchange of TG. The dog is a species that normally has no significant CETP activity,[38-41] but as we have shown, in insulin-resistant obese dogs, VLDL-cholesterol level as HDL-TG level were slightly elevated. Thus, whereas the CE/TG ratio increased in VLDL with insulin resistance, it decreased in HDL, suggesting an activity of CETP. Such an increase of CETP activity has also been observed in insulin-resistant humans.[9,42,43] Thus, whereas in normal dogs CETP could be not expressed much, insulin-resistant obese dogs could have activated CETP, explaining the TG increase in HDL and cholesterol increase in VLDL.

LDL lipids concentrations in insulin-resistant animals were similar those in their respective controls. It has been observed that there is no relationship between insulin sensitivity and LDL-cholesterol levels in humans.[7,33,44]

In conclusion, the dog is a species whose nutritional factors can be easily disturbed in order to induce insulin resistance. We showed that insulin-resistant obese dogs have high plasma TG levels, as well as low HDL-cholesterol levels, which are the most common abnormalities in insulin-resistant humans.

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