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Changes in Plasma Fatty Acid Composition in Hyperlipidemia Dogs

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ABSTRACT

Hyperlipidemia refers to increase of triglyceride (TG) and/or total cholesterol (T-cho) in blood. Fatty Acids (FAs) have important roles in the lipid metabolism. The aim of this study was to determine the FA composition of plasma lipid fractions in dogs with hyperlipidemia and to evaluate the FA composition as a new diagnostic marker for obesity at early stage. Thirty-nine dogs were classified into healthy or hyperlipidemia based on the criteria to diagnose hyperlipidemia. The blood biochemical values, such as TG, T-cho, glucose, insulin, adiponectin and Non-Esterified Fatty Acid (NEFA) were measured. FA composition profile was performed on GC/MS system. The values of plasma TG, insulin and NEFA of the hyperlipidemia group were significantly higher than that of control group. Hyperlipidemia group tended to show lower concentration of adiponectin. It was found that only the levels of TG and NEFA, but not T-cho increased significantly in early stage of hyperlipidemia. In hyperlipidemia group, percentages of myristic acid (C14:0), parmitoleic acid (C16:1) and oleic acid (C18:1) increased in total FAs. And the percentage of C18:1 increased in NEFA. Indeed, the higher level of insulin and lower adiponectin concentration were seen in hyperlipidemia group. These results suggest that appearance of insulin resistance may be the result of increases of certain FAs in early stage of insulin resistance.

Key words: Hyperlipidemia, fatty acid composition, dog, GC/MS, NEFA

INTRODUCTION

Obesity and Diabetes Mellitus (DM) have increased in dogs in recent years (Guptill *et al*., 2003), German (2006) and hyperlipidemia is one of the risk factor for obesity and DM. Hyperlipidemia refers to increase of triglyceride and/or cholesterol in blood (Watson and Barrie, 1993; Ford, 1996). Primary cause of hyperlipidemia in dog is associated with specific breed such as Miniature Schnauzers (Rogers *et al*., 1975; Ford, 1993; Whitney *et al*., 1993). Secondary hyperlipidemia with diseases such as diabetes mellitus and chronic obesity dogs occurrs more often than primary one. Critical hyperlipidemia could induce some complications. For example, endocrine disorder-derived increase of cholesterol can develop atherosclerosis or ocular diseases. In addition, obesity-related hyperlipidemia is closely associated with insulin resistance (Serisier *et al*., 2008; Chiu *et al*., 2009; Xenoulis *et al*., 2011). And severe hypertriglyceridemia is known as a risk factor for pancreatitis in humans (Yadav and Pitchumoni, 2003) and dogs (Bauer, 1995; Bauer, 2004).

On the other hand, Fatty Acids (FAs) have important roles in the lipid metabolism. The triglycerides in blood, which increases in hyperlipidemia, have different biochemical characteristics with the composition of FAs (Berry, 2009). The balance of these two types of saturated and unsaturated FAs is important because of each of them have various effects on biological function. Whereas the increase of saturated FAs in plasma is associated with the low-grade inflammation in overweight adolescents, polyunsaturated FAs inhibits pro-inflammatory cytokines production in cells and tissues (Jia *et al*., 2008; Fernandes, 1995). Therefore, the unbalance of plasma FA composition might aggravate lipid metabolism abnormality or induce complications.

The aim of this study was to determine the FA composition of plasma lipid fractions in dogs with hyperlipidemia and to evaluate the FA composition as a new diagnostic marker for obesity at early stage.

MATERIALS AND METHODS

Cases: Forty-three dogs referred to the five animal hospitals from march to may in 2012 were examined with in this study. Informed consents were obtained from all owners whose animals were included in this study. Four dogs were excluded due to disorders such as DM. Thirty-nine dogs were classified into healthy and hyperlipidemia based on the new criteria to diagnose hyperlipidemia (Kawasumi *et al*., 2012) any two out of three factors as follows:

- Raised plasma triglyceride (TG) level≥165 mg dL^{-1}
- Raised plasma total-cholesterol (T-cho) level≥200 mg dL^{-1}
- Raised plasma non-esterified fatty acid (NEFA) level≥1.5 mEq L^{-1}

The Body Condition Score (BCS) was assessed and expressed as a five point scale: 1, thin; 2, lean; 3, optimal; 4, obese, 5, gross as described by Laflamme (1997).

Blood sampling and Biochemical analysis: Peripheral blood from cervical vein was collected into heparinized tubes and centrifuged at 1500 g for 10 min, then stored at -30°C. The blood biochemical values, such as TG, T-cho, glucose (GLU), were measured using an auto analyzer (AU680, Beckman Coulter, CA, USA).

Plasma hormone assay: Plasma insulin and NEFA concentrations were measured with commercial ELISA kits, Lbis dog insulin kit (SHIBAYAGI Co., Gunma, Japan) and NEFA-C test Wako (NEFA-C test, Wako Pure Chemical Industries, Inc., Tokyo, Japan).

Derivatization of Fas: Fas were methylated and extracted using the commercial kit. Dried 40 mg of sample was incubated with 0.5 mL of reagent A (toluene 52%, methanol 48%) and 0.5 mL of reagent B (methanol 93%) at 37°C for 60 min. Then the mixture was kept at 37°C for 20 min after added 0.5 mL of reagent C (methanol 30%). After 1.0 mL of extraction reagent (n-hexane 96%) was added to each sample and mixed, the upper layer was collected. Then upper layer was washed with distillated water and injected into a washed silica-gel cartridges. After 3.0 mL of wash solvent (n-hexane 96%) was added, 3.0 mL of an eluting solvent (n-hexane 96%, methyl acetate 2%) was added to elute the FA methyl esters.

Derivatization of NEFAs: Mixture of chloroform-methanol-water (0.5 mL; 1:2.5:1, v/v/v) was added to 50 μL of samples and incubated at 37°C for 60 min with shaking. Subsequently each

sample was centrifuged at g for 3 min and 30 μL of lower layer was transferred to 1.5 mL centrifuge tube. The sample was concentrated to dryness in evaporator for 30 min. Then the dried samples were derivatized by adding 200 μL of 20% methanol hexane and 100 μL of trimethyldiazomethane at room temperature for 30 min.

FA composition with GC/MS analysis: Analysis of FA composition was performed using a Shimadzu QP2010 GC/MS system (Shimadzu, Kyoto, Japan). A DB-5MS capillary column (30 m×0.25 mm×0.25 μm; J and W Scientific, Folsom, CA) was used to analyze derivatized plasma samples. Helium was used as the carrier gas at 1.0 mL min^{-1}. An injection volume of 1 μL was used and the injector and source temperature was 280°C. The column oven temperature was programmed from 40-320 at 6°C min^{-1} and held at 40 and 320°C for 2 and 1 min, respectively. To detect and eliminate retention time shifts, standard alkane series mixture (C-10 to C-40) was injected periodically into the GC/MS systems. Retention time correction of peaks based on retention time of standard alkane series mixture was performed using the AART (Automatic Adjustment of Retention Time) function of the Shimadzu GC/MS solution software. Chromatogram acquisition and compound identification by the mass spectral library search were performed using the Shimadzu GC/MS solution software.

Statistical analysis: Results were expressed as Mean±SD. Total FAs and NEFA were expressed as Mean±SD as a percentage of all measured FAs. Statistical analyses between control and hyperlipidemia group were conducted to assess the normality assumptions using the student t-test. Statistical significance was considered at $p<0.05$.

RESULTS

The Mean±SD age of the 38 dogs was 7.2±4.0 years; they comprised 16 females (12 neutered) and 22 males (13 neutered). The Mean (±SD) body weight was 10.1±6.28 (range, 3.6-29.8) kg.

Table 1 shows sex frequencies, age and metabolic data in control and hyperlipidemia. The mean age (6.3±10.1 years) and BCSs (3.4±3.9) in hyperlipidemia group were significantly higher than those of control group ($p<0.05$). The plasma TG (55.2±129.6 mg dL^{-1}), insulin (1.0±2.4 ng mL^{-1}) and NEFA (0.8±1.4 mEq L^{-1}) values of the hyperlipidemia group were significantly higher than those of the control group. There were no significant differences between groups for GLU and T-cho concentrations. And adiponectin concentration in hyperlipidemia group tended to show lower than control group.

Table 1: Population characteristics and blood biochemical analysis

Characteristics	Control	Hyperlipidemia	p-value
Sex, No. M:F (%)	18:11 (62:38)	4:5 (44:56)	
Age (years)	6.3±3.84	10.1±3.41	<0.05
BCS	3.4±0.56	3.9±0.60	<0.05
GLU (mg dL^{-1})	103.1±18.51	106.7±13.39	NS
Insulin (ng mL^{-1})	1.0±0.71	2.4±3.13	<0.05
TG (mg dL^{-1})	55.2±34.6	129.6±91.97	<0.05
T-cho (mg dL^{-1})	212.4±75.28	246.9±35.43	ns
Adiponectin (mL^{-1})	28.3±16.83	15.2±21.33	ns
NEFA (mEq L^{-1})	0.8±0.47	1.4±0.81	<0.05

All values are Mean±SD, ns: Not significant, M: Male, F: Female, BCS: Body condition score, GLU: Glucose, TG: Triglyceride, T-cho: Total-cholesterol, NEFA: Non-esterified fatty acid

Table 2: Fatty acid fractions in total fatty acids

Characteristic	Control (%)	Hyperlipidemia (%)	p-value
C14:0	0.23±0.06	0.32±0.16	<0.05
C15:0	0.08±0.02	0.08±0.02	ns
C16:0	15.42±1.32	15.34±2.61	ns
C17:0	0.33±0.07	0.32±0.07	ns
C18:0	24.54±2.04	23.36±2.00	ns
C16:1	0.61±0.23	1.24±0.96	<0.05
C17:1	0.08±0.02	0.10±0.04	ns
C18:1	15.62±2.00	19.10±3.70	<0.05
C18:2n-6	23.64±3.47	21.93±3.19	ns
C20:3n-6	0.85±0.34	0.92±0.38	ns
C20:4n-6	14.26±2.35	14.18±3.89	ns
C22:4n-6	0.33±0.12	0.32±0.17	ns
C20:5n-3	2.20±2.93	1.07±0.73	ns
C22:5n-3	1.05±0.39	1.13±0.80	ns
C22:6n-3	0.78±0.78	0.59±0.54	ns
SCD-1 index	0.64±0.12	0.83±0.22	<0.05

All values are Mean±SD, ns: Not significant, Stearoyl coenzyme A desaturase (SCD-1) index = C18:1/C18:0

Table 3: Fatty acid fractions in NEFA

Characteristic	Control (%)	Hyperlipidemia (%)	p-value
C14:0	0.12±0.08	0.23±0.19	ns
C15:0	0.02±0.02	0.04±0.04	ns
C16:0	3.15±1.65	4.34±2.20	ns
C18:0	3.37±1.48	4.27±0.86	ns
C16:1	0.02±0.03	0.04±0.03	ns
C18:1	0.50±0.32	1.10±0.82	<0.05
C18:2n-6	1.64±0.99	2.89±2.21	ns
C20:4n-6	0.51±0.27	0.57±0.42	ns
C22:5n-3	0.03±0.04	0.02±0.04	ns
C22:6n-3	0.05±0.04	0.03±0.02	ns

All values are Mean±SD, ns: Not significant, NEFA: Non-esterified fatty acid

The changes in composition of plasma FAs between the control and hyperlipidemia groups are shown in Table 2. When compared to the control group, percentage of C14:0 (0.23±0.32%), C18:0 (24.54±23.36%), C18:1 (15.62±2.00%) in total FAs were significantly higher in hyperlipidemia group ($p<0.05$), while other FAs were not changed. The indexes of desaturase activity were reflected by the C18:1/C18:0 ratios. It was increased in hyperlipidemia group ($p<0.05$).

Table 3 shows the FA composition of plasma NEFA in dogs. Hyperlipidemia group had significant higher percentage of C18:1 than in control group (15.62±19.10%).

DISCUSSION

Changes in lipid profile: In general, plasma TG and/or T-cho concentrations increase in dog with hyperlipidemia. It was found that only TG and NEFA, but not T-cho increased significantly at early stage of hyperlipidemia in dogs. The increase of FAs may be an important sign of early stage of lipid metabolic disorder. Actually, saturated FAs could induce increase of circulating insulin, T-cho and TG levels in rats (Tranchida *et al.*, 2012). On the other hand, polyunsaturated FAs have been

reported to decrease plasma TG and increase High Density Lipoprotein (HDL)-cholesterol concentrations (Mori *et al.*, 2000). Thus, changes in plasma FA composition could contribute to the exacerbation of abnormality in lipid metabolism.

Increase of FA fractions in total Fas: Plasma FAs are comprised of plasma phospholipid, cholesteryl ester, TG and NEFA. Profiles of these total FAs and NEFAs at early stage of hyperlipidemia were investigated. The percentages of myristic acid (C14:0), which is one of saturated FAs and monounsaturated FAs like parmitoleic acid (C16:1) and oleic acid (C18:1) increased in hyperlipidemia group. C14:0 and C16:1 were related to markers of insulin resistance (Lovejoy *et al.*, 2001). Also, C14:0 is thought to be the most hypercholesterolemic saturated FA (Mensink *et al.*, 1994). Therefore, increased plasma C14:0 and C16:0 levels would induce the exacerbation of insulin resistance and cholesterol concentrations in the future. Stearoyl coenzame A desaturase (SCD-1) converts saturated FA to monounsaturated FA. SCD-1 index is estimated by the plasma C16:1/C16:0 or C18:1/C18:0 ratio (Petersson *et al.*, 2010). In this study, percentage of C18:1 and SCD-1 index increased in hyperlipidemia group. SCD-1 activity has reported to be required for the development of diet-induced hepatic insulin resistance (Gutierrez-Juarez *et al.*, 2006). Together, these results indicate that changes in FA composition could increase insulin resistance.

Increase of FA fraction in NEFA: Importantly, the percentage of C18:1 increased in NEFA. C18:1 is the most abundant NEFA in the plasma (De Almeida *et al.*, 2002; Bysted *et al.*, 2005). NEFA may contribute to the pathogenesis of insulin resistance and inflammation by directly activating plasma membrane receptors, such as toll-like receptor 4 (Subbaramaiah *et al.*, 2011; Reyna *et al.*, 2008). Peculiarly, C18:1 in NEFA could induce insulin resistance through p38 mitogen-activated protein kinase (Liu *et al.*, 2007). Therefore, it was suggested that increased C18:1 percentage in NEFA may also promote insulin resistance.

Changes in value of insulin and adiponectin: The value of insulin was significantly higher and adiponectin concentration was tended to be less in hyperlipidemia group. Elevated fasting plasma insulin level associates with impaired insulin signaling (Korsheninnikova *et al.*, 2002). Adiponectin increases FA oxidation (Yoon *et al.*, 2006) and has potent insulin-sensitizing effects (Kadowaki *et al.*, 2006; Lihn *et al.*, 2005; Hulthe *et al.*, 2003). So, low plasma adiponectin contributes to the pathogenesis of insulin resistance (Singhal *et al.*, 2005; Spranger *et al.*, 2003; Yamauchi *et al.*, 2001). Taken together, increased insulin and decreased adiponectin levels in plasma suggested insulin resistance at early stage of hyperlipidemia in dogs.

Limitation: The limitations of this study include its relatively small sample size. As this was a retrospective study using the clinical case, Insulin resistance could not be measured with more correct index such as Homeostasis Model Assessment ratio (HOMA).

CONCLUSION

In conclusion, increased percentage of C14:0, C16:1 and C18:1 in total FAs and C18:1 in NEFA are thought to play an important role in the pathogenesis of insulin resistance in dogs. Indeed, the higher level of insulin and lower adiponectin concentration were seen in hyperlipidemia group.

These results suggest that appearance of insulin resistance may be the result of increases of certain FAs in early stage of insulin resistance. However, the molecular mechanisms of this FA-induced insulin resistance remain to be determined in future studies.

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