Hepatic Steatosis with Relation to Increased Expression of Peroxisome Proliferator-Activated Receptor- γ in Insulin Resistant Mice

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We have isolated insulin resistant mice (ddY-H mice) which are spontaneously induced even if fed with the standard chow pellets. Since marked accumulation of triglycerides (TG) in liver was observed, the present study investigated causes of hepatic TG accumulation in ddY-H mice fed with the standard chow pellets. In ddY-H mice, hepatic TG content increased from seven-weeks of age, and further marked accumulation of TG was observed at 20-weeks of age. Histologically, fat droplets appeared in pericentral parenchymal cells of the liver from nine-weeks of age, and the size and number of droplets were increased in hepatic lobules at 15-weeks of age, suggesting hepatic steatosis was spontaneously induced. Although secretion of TG from liver to blood in ddY-H mice was not increased, fat absorption from the digestive tract was significantly enhanced. The mRNA expressions of peroxisome proliferator-activated receptor γ (PPAR γ) involved in fat accumulation and fatty acid translocase (CD36) involved in the transportation of fatty acid into the liver were markedly increased. However, gene expressions of factors involved in lipogenesis, β -oxidation of fatty acid and lipoprotein secretion were not changed. Pioglitazone (9 mg/kg), the PPAR γ agonist, administered for six weeks deteriorated hepatic steatosis in ddY-H mice. Although pioglitazone did not affect gene expressions of PPAR γ in the liver, CD36 and fat-specific protein 27 (fsp27), targets of PPAR γ , were markedly elevated. These results suggest that, in the livers of ddY-H mice, hepatic steatosis is induced by increased incorporation of fatty acid into the liver *via* increased PPAR γ expression.

Key words hepatic steatosis; peroxisome proliferator-activated receptor γ ; fat absorption

Non-alcoholic hepatic steatosis, or non-alcoholic fatty liver disease (NAFLD), is the abnormal accumulation of triglycerides (TG) in the cytoplasm of hepatocytes¹⁾ and is considered as the hepatic manifestation of the metabolic syndrome and insulin resistance is regarded as its key pathological hallmark.^{2–5)} NAFLD may progress through non-alcoholic steatohepatitis (NASH) to cirrhosis and eventually hepatocellular carcinoma. However, risk factors for NAFLD are still unrecognized.

Peroxisome proliferator-activated receptor γ (PPAR γ) is a nuclear receptor that activates genes involved in lipid storage and metabolism. It is noteworthy that PPAR γ is expressed at the elevated levels in the liver of murine models of diabetes or obesity, including aP2/DTA mice,⁶⁾ A-Zip/F1 mice,⁷⁾ ob/ob mice,^{8,9)} db/db mice⁸⁾ and KKAY mice.¹⁰⁾ On the other hand, ob/ob mice with a liver-specific disruption of PPAR γ exhibited a dramatic improvement in fatty liver but had exacerbated hyperglycemia and insulin resistance.¹¹⁾ Also, deletion of PPAR γ in hepatocytes protected mice against high-fat diet-induced hepatic steatosis.¹²⁾ These findings suggest a strong relationship between hepatic steatosis and elevated PPAR γ levels. However, it has been reported that administration of rosiglitazone, a ligand of PPAR γ , improves hepatic steatosis in patients with NAFLD.^{13,14)} In contrast, prolonged troglitazone treatment of KKAY mice, which express high levels of PPAR γ in the liver, resulted in severe hepatic steatosis.¹⁰⁾ Consequently, whether PPAR γ is either a causal factor or a consequence of hepatic steatosis is still controversial.

We previously showed that sustained hyperglycemia could be induced in ddY mice by re-feeding them with standard chow pellets after fasting for 48 h.¹⁵⁾ We have attempted to

select those mice with induced serious hyperglycemia by selective breeding based on serum glucose levels after re-feeding. Finally, two strains, namely spontaneous insulin resistant mice (ddY-H) and non-insulin resistant mice (ddY-L), have been isolated from ddY mice by inbreeding.¹⁶⁾ In livers of ddY mice, marked accumulation of triglycerides (TG) and an elevation of PPAR γ expression, even if mice were fed with the standard chow pellets, without high-fat diet feeding or specific gene knockout. In this study, we investigated the characteristics of hepatic triglycerides accumulation in these mice.

MATERIALS AND METHODS

Animal Care Four-week-old male ddY mice were purchased from SLC Inc. (Hamamatsu, Japan), and male ddY-H mice and male ddY-L mice from our own colony were used. The mice were maintained on 12 h light/dark cycles (light from 08:00 to 20:00) with free access to standard chow pellets (MF diet, Oriental Yeast Co., Ltd., Japan) and water *ad libitum* until the experiments were carried out. In the experiment for an effect of pioglitazone, mice were fed the standard powder diet (MF diet, Oriental Yeast Co., Ltd.) added with or without pioglitazone (Wako Pure Chemical Industries, Ltd., Osaka, Japan) to take 9 mg/kg body weight (BW)/d from nine-week old for 6 weeks. Blood was collected and livers were excised at 13:00 after a 4 h-fasting.

Animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the University of Shizuoka, Japan.

Measurement of TG Contents in Serum and Liver Serum and livers were obtained from mice at 13:00 after a 4 h-fasting. Serum TG was measured using Triglyceride E-Test Wako (Wako Pure Chemical Industries, Ltd.). For hepatic

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TG determination, TG in 0.2 g liver was extracted by 4 mL of 2-propanol and aliquot of the filtrate was measured by using the above kit.

Histological Examination of the Liver To evaluate histological changes, liver specimens were fixed and subjected to hematoxylin–eosin staining. For Sudan III staining, liver tissue was frozen in an O.C.T. compound (Daiichi Kigyo, Tokyo, Japan) on dry ice. Fixing and staining were carried out.

Measurement of TG Absorption from Intestine To evaluate TG absorption from the intestine as described by Abe *et al.*,¹⁷⁾ olive oil (10 mL/kg BW) was orally administered to overnight-fasted mice at 12 weeks of age. After 0.5, 1, 2, 4, and 6 h, blood was collected from the caudal vein, and serum TG concentrations were measured. The area of increased TG concentration after olive oil loading in serum TG concentration–time curve was calculated as TG absorption.

Measurement of Triglyceride Secretion from the Liver into Blood Triton WR1339 (tyloxapol; Sigma-Aldrich, St. Louis, U.S.A.) was dissolved in saline (20%, w/v) and injected (2 mL/kg) intravenously into caudal veins of overnight-fasted mice at 12 weeks of age, as described by Sasase *et al.*¹⁸⁾ At 0 h, 1 h and 2 h, blood was collected and serum TG concentrations were measured.

Glucose Tolerance Test Mice were orally administered glucose (3 g/kg BW) and blood was collected from the caudal vein after 0 min, 30 min, 60 min, and 120 min. Serum glucose was measured by Glucose CII-Test Wako (Wako Pure Chemical Industries, Ltd.).

Hepatic Gene Expression Analysis Mice were killed, and livers were excised at 13:00 after a 4-h fasting to avoid acute effects of food intake. Total RNA was isolated by RNeasy Lipid Tissue Mini Kit (QIAGEN, U.S.A.). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using TaKaRa RNA PCR Kit (TaKaRa, Tokyo, Japan). Primers listed in Table 1 were purchased from Invitrogen (Carlsberg, CA, U.S.A.) and were used for RT-PCR. RT-PCR was carried out as described in references listed in Table 1.

Data and Statistical Analysis All data are expressed as mean $\pm$ S.E. One-way analysis of variance (ANOVA) was used to compare the means among different groups, and Tukey's test was used in the *post hoc* multiple comparison. Statistical significance was set at $p < 0.05$.

RESULTS

Triglyceride Content in Liver and Serum Figure 1 depicts alterations of hepatic TG (A) and serum TG (B) in ddY-H mice, ddY-L mice and ddY mice which were fed the standard chow pellets. There were no differences between TG contents in livers and serum of ddY-L mice and ddY mice through the experimental periods. However, in ddY-H mice, hepatic TG and serum TG significantly increased from seven-weeks of age and from five weeks of age, respectively. Marked increases were observed both in liver and serum at 20-weeks of age.

Histological Examination Liver specimens of ddY-H mice and ddY-L mice were stained with hematoxylin–eosin and Sudan III. Examination of liver histology revealed that any changes were not observed in livers of ddY-L mice at 15-weeks of age (Figs. 2A,D). On the other hand, small fat droplets appeared in pericentral parenchymal cells of liver of ddY-H mice at 9-weeks of age (Fig. 2F). Sizes and numbers of fat droplets increased at 12- and 15-weeks of ages (Figs. 2G,H). But histological changes such as infiltrations of any inflammatory cells and degenerations were not observed (Figs. 2B,C). These findings, together with increased TG contents in liver, revealed that hepatic steatosis was spontaneously induced in ddY-H mice without loading high fat diet.

Triglyceride Absorption from Intestine Since TG contents in liver and serum of ddY-H mice were elevated, fat absorption was examined in mice administered olive oil. As shown Fig. 3, serum TG concentration in ddY-H mice and ddY-L mice reached a maximum level at 1 h and then decreased gradually. However, TG concentrations in ddY-H mice were significantly high compared to those in ddY-L mice and TG content absorbed for 6 h after olive oil administration in ddY-L mice was 1.5 fold, suggesting higher absorption of TG from intestine in ddY-H mice.

Triglyceride Secretion from Liver Triton WR1339 is known well as an inhibitor of lipoprotein lipase. An increase in serum TG concentration after its injection is known to reveal TG secretion from the liver in fasted mice.^{17,18)} TG concentration in serum of ddY-H mice and ddY-L mice after triton WR1339 injection elevated at 1 and 2 h (Fig. 4). However, there were no differences between two groups at any time, suggesting that TG secretion from liver was not changed in ddY-H mice.

Table 1. Primers Used for RT-PCR

Gene	Forward primers (5'→3')	Reverse primers (5'→3')	Reference
PPAR γ	CACTCGCATTCCTTTGACATC	GGCATACTCTGTGATCTCTTG	1)
CD35	TGTACCTGGGAGTTGGCGAG	CTGCTGTCTTTGCCACGTC	2)
aP2	TGGAAGACAGCTCCTCTCG	CCGCCATCTAGGGTTATGATG	2)
CPT-1a	TCCACCTGAGGCATCTATT	ATGACCTCCTGGCATTCTCC	2)
ACC	CTTCCTCCTGATGAGCAACTCT	CGTGAGTTTCCCAAATAAGC	3)
FAS	TCAACTCACTGGCAGAAGAGAA	CCAGAGCGTGGTTGTTAGAAAG	3)
SREBP-1	ACAGTCCAGCCTTTGAGGATAG	GACACAGAAAGGCCAGTACACA	3)
MTP	TGGTGAAAGGGCTTATTCTGTT	TTGCAGCTGAATATCCTGAGAA	3)
apoB	AAACATGCAGAGCTACTTTGGAG	TTTAGGATCACTTCCTGGTCAAA	3)
PPAR α	AGGCAGATGACCTGGAAAGTC	ATGCGTGAACCTCCGTAGTGG	4)
fsP27	AGTAGCCCTTTCCCAAG	CCTGTAGCAGTGCAGGTCA	5)
β -Actin	TGGAATCCTGTGGCATCCATGAAAC	TAAACGCAGCTCAGTAACAGTCCG	6)

1): *Biochem. Biophys. Res. Commun.*, **30**, 22–27 (2003). 2): *Life Sci.*, **82**, 983–990 (2008). 3): *Hepatology*, **48**, 1799–1809 (2008). 4): *Brit. J. Nutr.*, **97**, 58–66 (2007). 5): *Toxicol. Lett.*, **211**, 29–38 (2012). 6): *Biochem. Biophys. Res. Commun.*, **295**, 630–635 (2002).

Fig. 1. Alteration of Hepatic (A) and Serum (B) Triglycerides in ddY Mice, ddY-H Mice and ddY-L Mice

ddY mice: open square, ddY-H mice: closed circle, ddY-L mice: closed triangle. Each point represents mean $\pm$ S.E. for eight mice. ** $p < 0.01$ vs. ddY-L.

Fig. 2. Histological Appearance of Livers in ddY-H Mice and ddY-L Mice

Liver sections were stained with hematoxylin-eosin (A-C) and with Sudan III (D-H) as described in Materials and Methods. (A) and (D); 15-week-old ddY-L mice, (E); five-week-old ddY-H mice, (F); nine-week-old ddY-H mice, (B) and (G); 12-week-old ddY-H mice, 15-week-old ddY-H mice; (C) and (H).

Fig. 3. Increase in Serum TG Concentration after Olive Oil Administration

Olive oil (0.5 mL/50 BW) was orally administered to 12-week-old ddY-H mice and ddY-L mice, and blood was collected from the caudal vein after 0.5, 1, 2, 4 and 6 h and serum TG was determined. (A) Area of increased TG concentration after olive oil administration in (A) was calculated for TG absorption from intestine as described in Materials and Methods. (B) Each column represents mean $\pm$ S.E. for six mice. ***p* < 0.01 vs. ddY-L.

Fig. 4. Increase in Serum TG Concentration after Triton WR1339 Injection in ddY-H Mice and ddY-L Mice

Mice were intravenously injected Triton WR1339 (0.4 g/kg BW) and blood was collected from the caudal vein after 60 min and 120 min. Serum TG concentrations in ddY-H mice (closed column) and ddY-L mice (open column) were determined as described in Materials and Methods. Each column and bar represents mean $\pm$ S.E. for eight mice.

Gene Expression of Factors Involved in Lipid Metabolism in the Liver Since PPAR γ is known to be a promoting factor in the generation of fatty liver, mRNA expression of PPAR γ was analyzed in livers of ddY-H mice at 5-, 9-, 12- and 15-weeks of age. The expression in liver of five-week-old ddY-H mice was similar to those in livers of ddY-L mice and ddY mice. (Fig. 5B) However it increased from 9-weeks of age, and marked increase was observed at 12- and 15-weeks of age (Fig. 5A). The time course of its increase was similar to that of TG accumulation in liver. Its increased expression was observed in the liver, but not in adipose tissue (data not shown). Fatty acid translocase (CD36) and adipocyte fatty acid-binding protein (aP2) are known as the target genes of PPAR γ and involved in fatty acid transportation into the liver. Gene expression of CD36 in liver of ddY-H mice at 5-weeks of age was not changed, but that at 12-weeks of age were

increased compare with those of ddY-L mice and ddY mice (Fig. 5C). Marked increase in aP2 gene expression was not observed (Fig. 5C). These results may be indicating that incorporation of fatty acids from blood into the liver could be accelerated. On the other hand, no changes in the livers of ddY-H mice were observed in gene expressions of factors of lipogenesis (acetyl-CoA carboxylase (ACC)), fatty acid synthase (FAS) and sterol regulatory element binding protein-1c (SREBP-1c), factors of fatty acid oxidation (carnitine palmitoyl transferase 1a (CPT-1a)) and peroxisome proliferator-activated receptor α (PPAR α), factors of lipoprotein secretion (microsomal triglyceride transfer protein (MTP)) and apolipoprotein B (apoB) (Fig. 6).

Hepatic Triglyceride Content, Serum Triglyceride Concentration and Glucose Tolerance in Pioglitazone Treated Mice Pioglitazone, a ligand of PPAR γ , was administered to ddY-H mice and ddY-L mice for six weeks. No influence of pioglitazone was observed in hepatic TG contents and serum TG concentration in ddY-L mice (Figs. 7A,B). However, TG content in the livers of ddY-H mice significantly increased by pioglitazone treatment, indicating deterioration of hepatic steatosis (Fig. 8A). Pioglitazone improved serum TG concentration in ddY-H mice. On the other hand, pioglitazone administrations did not influence decreased glucose tolerance activity in ddY-H mice (Fig. 8).

Gene Expression of Factors Involved in Hepatic Steatosis in Pioglitazone Treated Mice Gene expression of PPAR γ , a promoting factor in generation of fatty liver, elevated in the liver of ddY-H mice as described above. However, an elevation of the gene expression was not influenced by pioglitazone treatment for 6 weeks in ddY-H mice (Fig. 9). Gene expressions of CD36, aP2 and fat specific protein27 (fsp27), which were target factors of PPAR γ and were involved in fatty acid transportation into the liver,¹⁹⁾ were increased by pioglitazone treatment. In particular, fsp27 was markedly increased by pioglitazone. In contrast to the liver, pioglitazone did not increase the gene expression of CD36, aP2 and fsp27 in adipose tissue (data not shown).

A ddY-H

B 5 weeks

C 12 weeks

Fig. 5. Gene Expression of PPAR γ and Its Target Factors in Livers of ddY-H Mice, ddY-L Mice and ddY Mice

(A) Gene expressions of PPAR γ , its target factors and β -actin in livers of 5-, 9-, 12- and 15-week-old ddY-H mice. (B) Gene expressions in livers of 5-week-old ddY-H mice, ddY-L mice and ddY mice. (C) Gene expressions in livers of 12-week-old ddY-H mice, ddY-L mice and ddY mice.

Fig. 6. Gene Expression of Factors Involved in Hepatic Lipid Metabolism of 12-Week-Old ddY-H Mice and ddY-L Mice

Gene expressions of factors in five mice examined and typical expression of those were shown.

DISCUSSION

Despite the frequent association of NAFLD with obesity, insulin resistance and diabetes mellitus, which are common

characteristics of the metabolic syndrome, the genetic basis and functional mechanisms of NAFLD are largely unknown. Since type 2 diabetes in humans is a heterogeneous disorder, investigations using genetically and phenotypically homogeneous animal models of diabetes are necessary to elucidate the mechanisms of this disease.²⁰⁾ We have shown that, in ddY-H mice, insulin resistance was spontaneously induced and diabetic symptoms appeared by aging even if mice were fed with the standard chow pellets *ad lib.*¹⁶⁾ In this study, it was certified that hepatic TG contents elevated from low age in concomitant with a high levels of serum TG concentration. Appearance of fat droplets without any degeneration was histologically observed, indicating that hepatic steatosis spontaneously appeared in ddY-H mice. The clinical characteristics of ddY-H mice resemble to a common form of type 2 diabetes in humans, with an age-dependent onset, mild obesity, insulin resistance and hypertriglyceridemia.¹⁶⁾ ddY-H mice may be a hepatic steatosis mouse model.

TG accumulation in the liver could occur by an increase in fat absorption from intestine and incorporation of fatty acid from blood circulation into liver, an increase in hepatic fatty acid synthesis, a decrease in β -oxidation of fatty acid or/and a decrease in secretion of lipoprotein into blood. In ddY-H mice, serum TG concentration was high after 4h-fasting in light time (Fig. 1) but not after over-night fasting in dark time. (Figs. 3, 4) In addition, serum TG concentration after an administration of olive oil was higher than that in ddY-L mice. On the other hand, secretion of TG from the liver was not changed. These findings are indicating that fat absorption

Fig. 7. Effect of Pioglitazone on Serum and Hepatic TG in ddY-H Mice and ddY-L Mice

Mice were fed the standard powder diet (MF) with or without pioglitazone (9mg/kg BW) from nine weeks of age for 6 weeks. Serum and hepatic TG were determined as described in Materials and Methods. LC: control of ddY-L mice, LP: pioglitazone group of ddY-L mice, HC: control of ddY-H mice, HP: pioglitazone group of ddY-H mice. Each point represents mean $\pm$ S.E. for eight mice.

Fig. 8. Effect of Pioglitazone on Glucose Tolerance in ddY-H Mice and ddY-L Mice

(A) After glucose administration (3g/kg), blood was collected at 0, 30, 60, 120min and serum glucose was measured as described in Materials and Methods. (B) Areas under the glucose concentration–time curve were calculated. LC: control of ddY-L mice, LP: pioglitazone group of ddY-L mice, HC: control of ddY-H mice, HP: pioglitazone group of ddY-H mice. Each point and column represents mean $\pm$ S.E. for eight mice.

from intestine increased in ddY-H mice.

PPAR γ is known to be a promoting factor in the generation of fatty liver. In ddY-H mice, gene expression of key factors involved in lipid metabolism, PPAR γ and CD36, were elevated. CD36, aP2, and fsp27 were known as the target genes of PPAR γ and involved in fatty acid transportation into the liver²¹⁾ and fat droplet deposit in the liver.¹⁹⁾ By a treatment with pioglitazone, a ligand of PPAR γ , gene expressions of CD36, aP2 and fsp27 were increased and hepatic steatosis was deteriorated. These results suggest that elevated expressions of these genes could increase incorporation of fatty acids from blood into liver and fat deposit could be accelerated in ddY-H mice. In particularly, fsp27 expression was tremendously enhanced by pioglitazone, which may mean an important role of fsp27 in hepatic TG accumulation of ddY-H mice. Gene expression of PPAR γ was increased at 9-, 12-, and 15-weeks of age in ddY-H mice compared to in ddY-L mice. Its increased

expression was observed in the liver, but not in adipose tissue. (data not shown) It is not clear why PPAR γ gene are differently expressed in liver and adipose tissue.

On the other hand, no changes in the livers of ddY-H mice were observed in gene expressions of factors of lipogenesis (ACC, FAS and SREBP-1c), factors of fatty acid oxidation (CPT-1 α and PPAR α), and factors of lipoprotein secretion (MTP and apoB). These results suggest lipogenesis, fatty acid oxidation and lipoprotein secretion may not be a major cause of TG accumulation in liver.

Hypertriglyceridemia is one of the major abnormality found in diabetes with insulin deficiency.²²⁾ Elevated levels of serum TG were observed in ddY-H mice from a low age. Decreased signals of insulin cause diabetic hyperphagia,²³⁾ and are an explanation for hypertriglyceridemia. In a diabetic state, hyperphagia leads to intestinal hypertrophy,²⁴⁾ which contributes to increased lipid absorption. An increase of 15–20% in intake

Fig. 9. Effect of Pioglitazone on mRNA Expression of PPAR- γ and Its Target Factors in Livers of 12-week-old ddY-H Mice and ddY-L Mice

Gene expressions of factors in five mice examined and typical expression of those were shown.

of the diet was observed in ddY-H mice compared to ddY-L mice (data not shown). Defect in plasma TG clearance is another cause of diabetic hypertriglyceridemia.²⁵⁾ TG absorbed from intestine is transported into circulation, and is cleared from circulation by lipoprotein lipase. Therefore, the impaired TG absorption from the small intestine contributes to hypertriglyceridemia in diabetes.²⁵⁾ Earlier studies indicate that increased TG absorption from intestine contributes to hypertriglyceridemia in diabetes.²⁶⁾ Serum TG concentration was high and was decreased by the treatment of pioglitazone in ddY-H mice, but not in ddY-L mice. This finding suggest, in ddY-H mice, clearance of TG from circulation may function but may decrease to some extent, since lipoprotein lipase is activated by pioglitazone, an clearance of serum lipid was increased.²⁷⁾ Therefore, elevation of serum TG in ddY-H mice might be mainly caused by the increased TG absorption. Although, activities of lipogenesis, fatty acid degradation and lipoprotein secretion in the livers of ddY-H mice have yet to be clarified, findings in gene expression analysis suggest fatty acid origin of accumulated TG in the liver comes from the elevations of fat absorption from intestine and fatty acid incorporation from circulation into liver could be a main cause.

PPAR γ activates genes involved in lipid storage and metabolism.^{28,29)} PPAR γ is expressed at the highest level in adipose tissue.³⁰⁾ and, in contrast, is typically expressed in murine liver at only 10–30% of the levels found in adipose tissue.³¹⁾ However, it is expressed at markedly elevated levels in the severe NAFLD of various murine models of diabetes or obesity.^{6–9)} In ddY-H mice, markedly elevated PPAR γ was observed in the liver but not in adipose tissue (data not shown). Also, enhanced expressions of CD36, aP2, fsp27, the target genes of PPAR γ , were not induced in adipose tissue by pioglitazone (data not shown). This is consistent with the finding that pioglitazone did not improve decreased glucose tolerance in ddY-H mice. It is not clear why elevated expression of PPAR γ was observed in the liver but not in adipose tissue.

In conclusion, hepatic steatosis is spontaneously induced by the increase in incorporation of fatty acid into the liver via increased PPAR γ expression in the liver of ddY-H mice.

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