Available online at www.sciencedirect.com
ScienceDirect
Metabolism Clinical and Experimental 57 (2008) 140 – 148
Metabolism
Clinical and Experimental
www.elsevier.com/locate/metabol

Nateglinide prevents fatty liver through up-regulation of lipid oxidation pathway in Goto-Kakizaki rats on a high-fat diet

Tomoyuki Mine, Kyoko Miura, Toshifumi Kajioka, Yoshiro Kitahara*

Pharmaceutical Research Laboratories, Ajinomoto Co, Inc, Kawasaki-ku, Kawasaki 210-8681, Japan

Received 22 February 2007; accepted 28 August 2007

Abstract

Dyslipidemia and fatty liver are important components of the metabolic syndrome and are the factors most commonly associated with the development of nonalcoholic fatty liver disease. Delayed and excessive insulin secretion in response to food intake is a key element in the onset of these risk factors. Nateglinide (NAT) is known to restore early-phase insulin secretion. We assessed the effect of NAT on postprandial hypertriglyceridemia and fatty liver in type 2 diabetic Goto-Kakizaki (GK) rats. The GK rats fed a high-fat diet containing 30% beef tallow twice a day were administered either the vehicle alone or NAT (50 mg/kg) before each meal for 12 weeks. Delayed insulin secretion and an increase of total insulin release were caused by feeding 30% beef tallow to the rats. This diet also induced postprandial hypertriglyceridemia and increased the hepatic triglyceride content. Treatment with NAT restored early-phase insulin secretion without any increase of total insulin release and also reduced postprandial hypertriglyceridemia and the hepatic triglyceride content. There was up-regulation of the hepatic expression of peroxisome proliferators–activated receptor α and its downstream enzymes after 12 weeks of NAT treatment, as well as normalization of the plasma total ketone body level. Furthermore, NAT also up-regulated hepatic expression of the adiponectin receptor AdipoR2, although there was no effect on the plasma adiponectin level. These findings indicate that long-term treatment with NAT prevented the development of fatty liver through the up-regulation of hepatic lipid oxidation pathways. Restoration of early-phase insulin secretion and suppression of recurrent postprandial hypertriglyceridemia might be involved in these effects of NAT. The present results may support the use of NAT to prevent the onset and progression of the metabolic syndrome and chronic liver disease.

1. Introduction

Dyslipidemia and fatty liver are important components of the metabolic syndrome. Delayed and excessive insulin secretion combined with loss of early-phase insulin secretion after food intake is a characteristic pathophysiological abnormality of patients with type 2 diabetes mellitus or impaired glucose tolerance, and it plays a key role in the development of these risk factors along with insulin resistance [1-3]. In addition, hyperinsulinemia is the commonest factor associated with the development of nonalcoholic fatty liver disease [4,5], which is one of the commonest causes of chronic liver disease. Excessive dietary fat intake is also involved in the occurrence of these abnormalities [6]. Therefore, intervention to prevent dyslipidemia and fatty liver by restoring early-phase insulin secretion may be a potential therapeutic approach for preventing progression of the metabolic syndrome and chronic liver disease.

Nateglinide (NAT) is known to restore early-phase insulin secretion after food intake and also suppresses postprandial hyperglycemia [7-10]. Uchino et al [8] reported that NAT enhanced early-phase insulin secretion without increasing total postprandial insulin release in obese patients with type 2 diabetes mellitus and also suppressed postprandial hyperglycemia. They pointed out that restoration of early-phase insulin secretion is necessary for the normalization of postprandial hepatic glucose metabolism. In addition, recent preclinical and clinical studies have shown that NAT suppresses the postprandial increase of plasma triglycerides (TG), very low-density lipoprotein (VLDL) TG, remnant-like particles TG, and remnant-like particles cholesterol [11-14], whereas such effects were not observed with glibenclamide or an α-glucosidase inhibitor [11,12]. These findings suggest that restoration of early-phase insulin secretion is also necessary to improve postprandial

* Corresponding author. Tel.: +81 44 210 5824; fax: +81 44 210 5875.
E-mail address: yoshiro_kitahara@ajinomoto.com (Y. Kitahara).

0026-0495/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2007.08.017

Table 1
Composition of the experimental diets

	5BT	30BT
Casein	20	20
L-Cys_2	0.3	0.3
Corn starch	51.75	26.75
α-Starch	13.2	13.2
Oil	5	30
Cellulose	5	5
Mineral mix (AIN93)	3.5	3.5
Vitamin mix (AIN93)	1.0	1.0
Choline bitartrate	0.25	0.25

Weight percentage.

dyslipidemia. However, it is still unclear whether recurrent postprandial hypertriglyceridemia is a risk factor for the development of fatty liver and related diseases, and restoration of early-phase insulin secretion is efficacious for prevention of hepatic lipid dysmetabolism.

In the present study, we investigated the effect of long-term NAT therapy on postprandial hypertriglyceridemia and the hepatic TG content in Goto-Kakizaki (GK) rats fed a high-fat diet. The GK rats are known to show impaired early-phase insulin secretion in response to food intake and also display hepatic insulin resistance [15,16]. We previously reported that GK rats showed recurrent postprandial hyperglycemia in the meal-feeding regimen [16,17]. In this study, the same feeding protocol was adopted, and GK rats received a high-fat diet for 12 weeks as a model of recurrent postprandial hypertriglyceridemia. Afterward, the hepatic TG content and hepatic expression of genes related to lipid metabolism were assessed after long-term NAT therapy.

2. Materials and methods

2.1. Animals

Male GK rats and Wistar rats were purchased from Clea Japan (Tokyo, Japan) at 6 weeks of age, and each rat was housed in a polycarbonate cage with wood chip bedding. Water was provided ad libitum, and the experimental diet was provided according to the meal-feeding regimen described below. The animal room was kept on a 12-hour light/dark cycle (7:00 AM to 7:00 PM, dark; 7:00 PM to 7:00 AM, light), with a temperature range of 22°C ± 1°C and a relative humidity of 55% ± 5% throughout the experimental period.

2.2. Measurements of blood glucose, plasma insulin, plasma TG, plasma nonesterified fatty acids, plasma total ketone bodies, and plasma adiponectin

Blood glucose and plasma TG were measured with an autoanalyzer (Fuji Dri-Chem 5500; Fuji, Tokyo, Japan). Plasma nonesterified fatty acids (NEFA) were measured by using a commercial kit (NEFA C; Wako, Tokyo, Japan). Plasma insulin was measured by enzyme-linked immunosorbent assay (ELISA) (Morinaga insulin ELISA kit; Morinaga, Tokyo, Japan). Plasma total ketone bodies were measured with a commercial kit (Ketone Test B; Sanwa, Nagoya, Japan). The plasma adiponectin level was determined by an ELISA kit (Otsuka adiponectin kit; Otsuka, Tokushima, Japan). Fractionation of plasma lipids was performed by agarose gel electrophoresis, and the TG level in each lipoprotein fraction was determined by TG-specific staining using the Chol/Trig Combo system (Helena Laboratories, Saitama, Japan).

2.3. Study protocol

This study was approved by the Animal Care and Use Committee of Ajinomoto. The rats were trained to consume their chow within 1 hour, and it was provided twice a day during the dark period (9:00 AM to 10:00 AM and 3:00 PM to 4:00 PM), as described previously [16,17]. The animals were acclimatized to the laboratory condition for 3 weeks. After this dietary conditioning, the GK rats were divided into 3 groups. One group was fed a basal diet containing 5% beef tallow (5BT), and the vehicle (0.5% methylcellulose [MC]) was administered by oral gavage twice daily just before each meal for 12 weeks (5BT/MC group). The other 2 groups were fed a high-fat diet containing 30% beef tallow (30BT), and either the vehicle (30BT/MC group) or 50 mg/kg of NAT (30BT/NAT group) was administered by oral gavage twice daily just before each meal for 12 weeks. Wistar rats (Wis group) were fed the 5BT diet, and the vehicle was administered by oral gavage twice daily just before each meal for 12 weeks. The composition of each diet is shown in Table 1. At the end of the study (week 12), blood was collected from the inferior vena cava during laparotomy under pentobarbital anesthesia, and plasma samples were prepared. Afterward, the liver was rapidly removed, frozen in liquid nitrogen, and stored at −80°C until analysis.

Table 2
Primer used for real-time quantitative RT-PCR

Genes		Primer sequences
PPARα	Forward	5′-CTCGTGCAGGTCATCAAGAA-3′
	Reverse	5′-CAGCCCTCTTCATCTCCAAG-3′
ACS	Forward	5′-GTGAAAGGGGCAAATGTGTT-3′
	Reverse	5′-TCGCTCCGCAGGTAGATATT-3′
CPT-1	Forward	5′-AGCCATGGAGGTTGTCTACG-3′
	Reverse	5′-GGCTTGTCTCAAGTGCTTCC-3′
SREBP1c	Forward	5′-GCCATGGATTGCACATTTG-3′
	Reverse	5′-TGTGTCTCCTGTCTCACCCC-3′
SCD-1	Forward	5′-CTGTTAGCCCAGCCTCACTC-3′
	Reverse	5′-TATTAGCAGCCCAGGGAGAA-3′
ME	Forward	5′-ACCACGGCTGAGGTCATATC-3′
	Reverse	5′-TCTTTGTTTTGGGGTTCAGG-3′
AdipoR2	Forward	5′-ACCCACAACCTTGCTTCATC-3′
	Reverse	5′-AGAGGGCAGCTCCTGTGATA-3′
β-Actin	Forward	5′-CTCCAAGTATCCACGGCATAG-3′
	Reverse	5′-AAGCAATGCTGTCACCTTCC-3′

2.4. Measurement of the hepatic TG content

Triglycerides were extracted from the harvested liver tissue by the chloroform-methanol (2:1, vol/vol) method of Folch et al [18] and measured enzymatically using a commercial kit (TG test, Wako).

2.5. Real-time quantitative reverse transcriptase polymerase chain reaction

Total RNA was extracted from liver samples using an RNA extraction kit (ISOGEN; Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Afterward, complementary DNAs were synthesized by using Superscript II Rnase H Reverse Transcriptase and an oligo-dT primer (Invitrogen, Tokyo, Japan). The resulting complementary DNAs were amplified by using a SYBR Green polymerase chain reaction (PCR) kit (Applied Biosystems Japan, Tokyo, Japan). Quantitative PCR was performed using an ABI PRISM 7700 sequence detection system (Applied Biosystems Japan). The primer sequences for detecting the target genes are listed in Table 2.

2.6. Statistical analysis

Statistical analysis was performed with StatView version 5.0 software (SAS Institute, Cary, NC). Results are expressed as the means ± SEM. Data between 2 groups were analyzed by Student *t* test and that among all groups by 1-way analysis of variance, followed by Tukey-Kramer test. $P < .05$ was considered to indicate significance.

3. Results

3.1. Effect of NAT treatment on blood parameters

There were no significant differences of daily food intake between the groups. The total calorie intake of rats

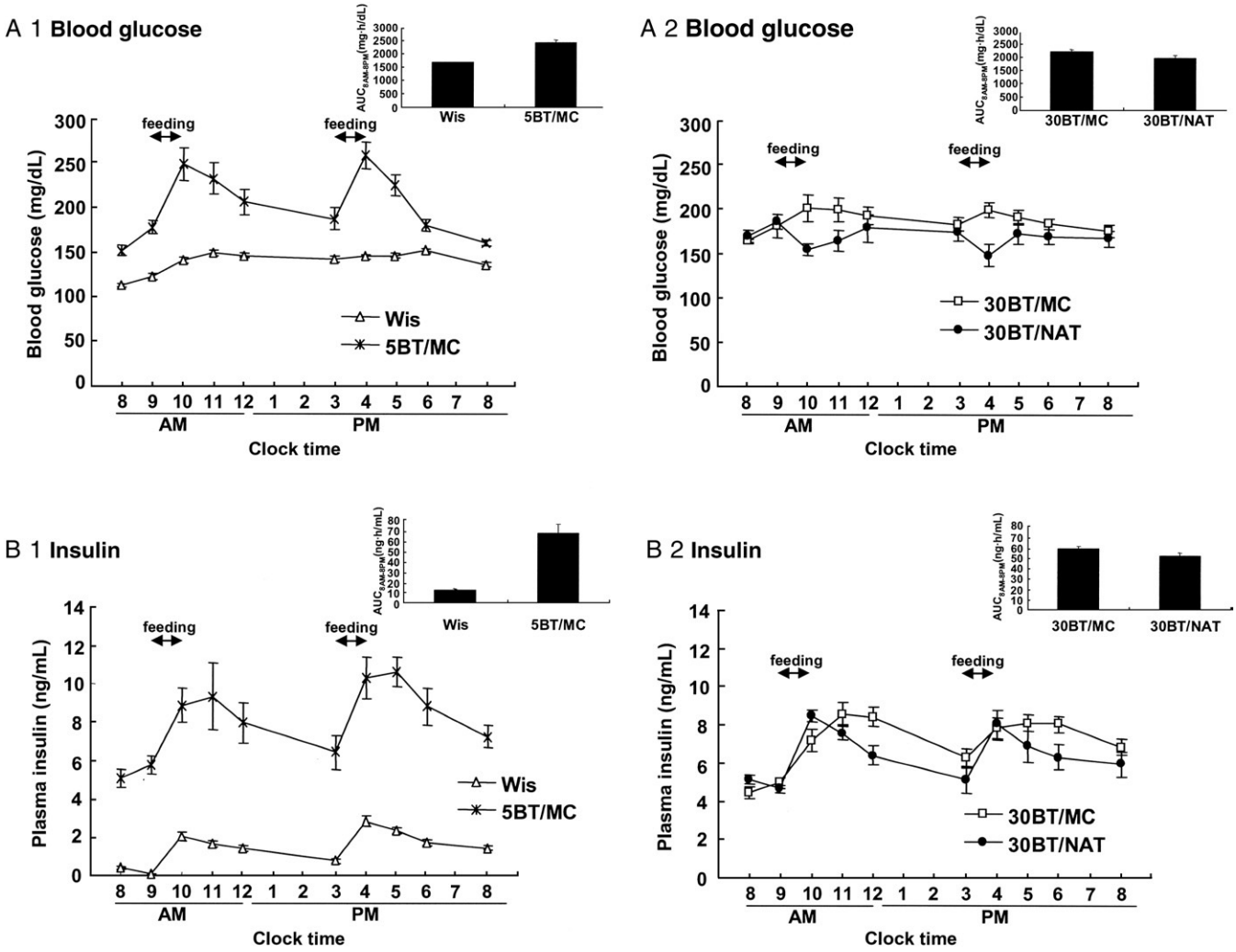

Fig. 1. Diurnal profiles of blood glucose (A-1, A-2), plasma insulin (B-1, B-2), plasma TG (C-1, C-2), and plasma NEFA (D-1, D-2) in each group. The experimental diet was given as described in Materials and methods. Either MC or NAT was administered just before each meal. Blood samples were taken from the tail vein at each time point. The AUC from 8:00 AM to 8:00 PM was calculated and superimposed on the right side of each graph. Data are expressed as the mean ± SEM. n = 8.

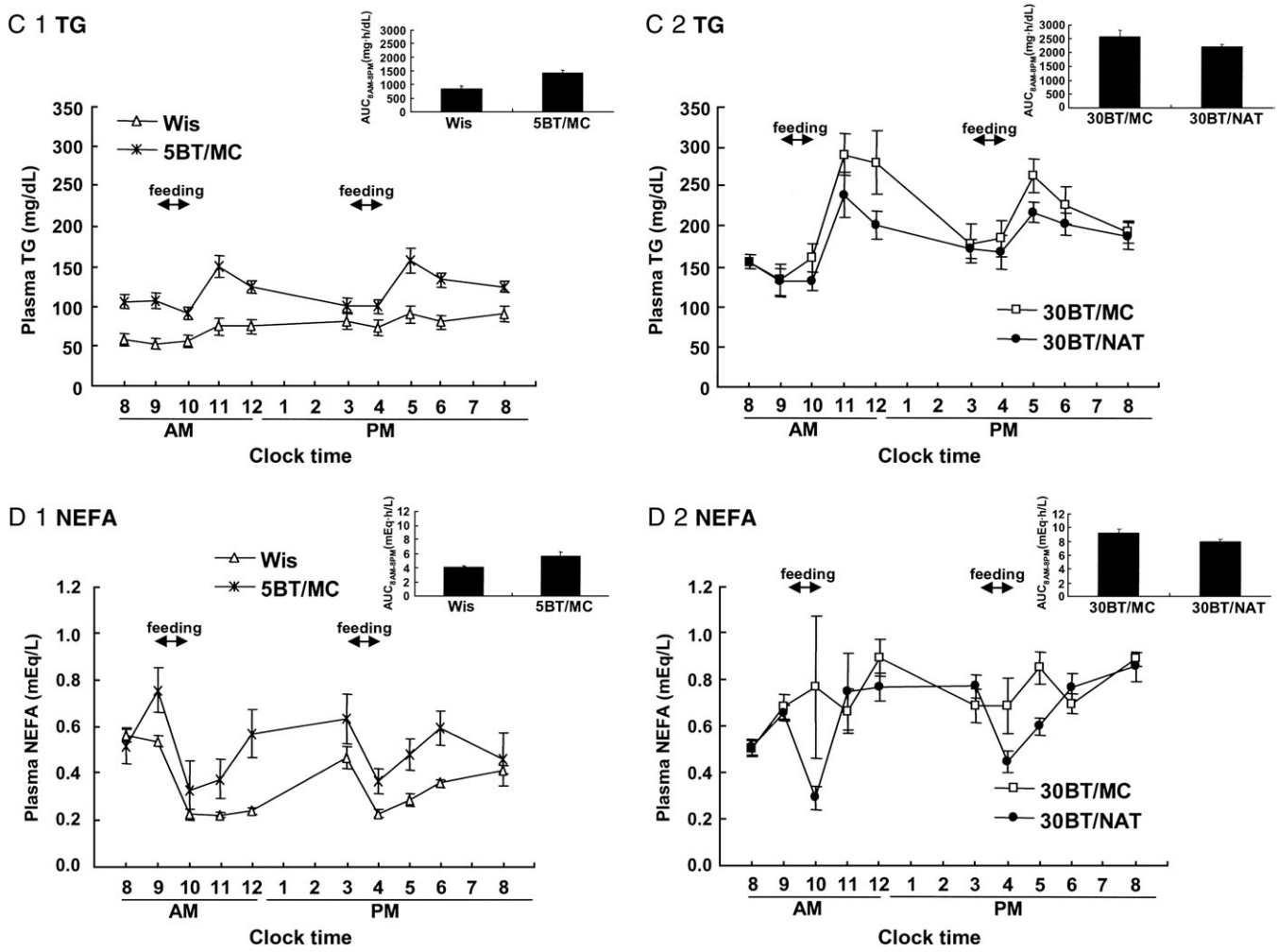

Fig. 1 (*continued*).

fed the 30BT diet was about 1.3-fold greater than that of rats fed the 5BT diet, whereas the total carbohydrate intake of rats on the 30BT diet was about one half of that in the 5BT/MC group (because the carbohydrate content of 30BT was about one half of that in 5BT). Fig. 1 shows the characteristic diurnal changes of blood glucose, plasma insulin, TG, and NEFA under the meal-feeding regimen. Although the postprandial blood glucose level of the 30BT/MC group was lower than that of the 5BT/MC group (Fig. 1A-1, A-2) because the carbohydrate content of the 30BT diet was lower than that of the 5BT diet, NAT significantly suppressed postprandial hyperglycemia (Fig. 1A-2 and Fig. 2A, B). The GK rats showed hyperinsulinemia throughout a day compared with Wistar rats (Fig. 1B-1), and the peak of insulin secretion after food intake in the 30BT/MC group was slightly delayed (2 to 3 hours after feeding) compared with that in the 5BT/MC group (1 to 2 hours after feeding) (Fig. 1B-1, B-2). Furthermore, total insulin secretion from 8:00 AM to 8:00 PM (area under the curve $[AUC]_{\text{insulin 8:00 AM-8:00 PM}}$) was significantly increased in the 30BT/MC group after 12 weeks of treatment (37.9 ± 5.0 ng·h/mL in week 0 vs 58.7 ± 2.4 ng·h/m in week 12, $P < .05$). Nateglinide enhanced and/or restored the increment of plasma insulin from baseline during the 1-hour feeding period (Fig. 2C, D), but did not cause a marked increase of $AUC_{\text{insulin 8:00 AM-8:00 PM}}$ after 12 weeks of treatment (44.9 ± 5.0 ng·h/mL in week 0 vs 51.8 ± 2.6 ng·h/mL in week 12, not significant). In addition, the 30BT/MC group showed a recurrent postprandial increase of plasma TG at 2 hours after meals (11:00 AM and 5:00 PM) (Fig. 1C-2 and Fig. 2E, F) as well as an increase of $AUC_{8:00\ \text{AM-8:00 PM}}$, whereas this significant increase of TG was not observed in the 5BT/MC group (Fig. 1C-1). Nateglinide also suppressed such postprandial increase of plasma TG (Fig. 1C-2 and Fig. 2E, F). The decrease of TG with NAT therapy was mainly at the origin area and pre-β area on agarose gel electrophoresis, which correspond to the chylomicron and VLDL subfractions, respectively (Fig. 3). Postprandial decrease of NEFA as seen in the Wis group and the 5BT/MC group

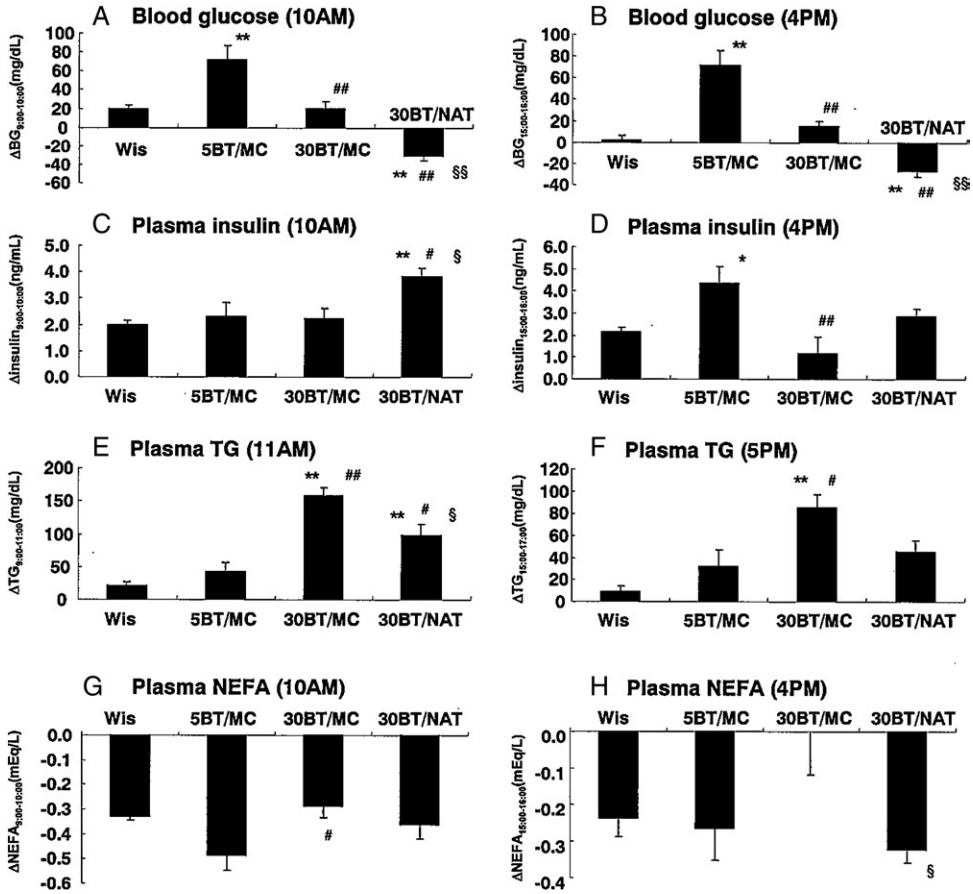

Fig. 2. Increment or decrement of blood glucose (A, B), plasma insulin (C, D), plasma TG (E, F), and plasma NEFA (G, H) from baseline (9:00 AM and 3:00 PM), at 1 hour after food intake (blood glucose, plasma insulin, and plasma NEFA [A, B, C, D, G, H]), and at 2 hours after food intake (plasma TG [E, F]). The experimental diet was given as described in Materials and methods. Either MC or NAT was administered just before each meal. Blood samples were taken from the tail vein just before and at 1 or 2 hours after each meal. Data are expressed as the mean ± SEM. n = 8, $^{*}P < .05$ vs Wis, $^{**}P < .01$ vs Wis, $^{\#}P < .05$ vs 5BT/MC, $^{\#\#}P < .01$ vs 5BT/MC, $^{\S}P < .05$ vs 30BT/MC, $^{\S\S}P < .01$ vs 30BT/MC, Tukey-Kramer test.

(Fig. 1D-1) was diminished in the 30BT/MC group (Fig. 1D-2 and Fig. 2G, H). Nateglinide treatment also restored such postprandial response of NEFA.

3.2. Organ weights and laboratory data

The body weight and organ weights of the GK rats after 12 weeks of treatment are summarized in Table 3. Although the GK rats showed lowered body weight and epididymal fat pad weight compared with the Wistar rats, the high-fat diet caused an increase of body weight and epididymal fat pad weight. Nateglinide therapy did not cause a significant increase of body weight, whereas the increase of epididymal fat pad weight was unaffected. Laboratory data are summarized in Table 4. Fasting blood glucose and fasting plasma insulin in the 5BT/MC group were higher than those in the Wis group, and the 30BT diet induced further elevation of fasting blood glucose. Fasting plasma TG in the 30BT/MC group tended to show an increase in week 12 compared with the 5BT/MC group, but the difference was not significant. Treatment with NAT had no effect on these parameters.

3.3. Effect of NAT treatment on hepatic TG content and plasma total ketone bodies

Intake of the 30BT diet for 12 weeks resulted in greater hepatic accumulation of TG compared with the 5BT/MC group (Fig. 4). In addition, there was a slight decrease of plasma total ketone bodies in the 30BT/MC group (Fig. 5). Nateglinide treatment ameliorated the accumulation of TG in the liver, and total ketone bodies were also normalized in the 30BT/NAT group.

3.4. Hepatic gene expression related to lipid oxidation pathway

Hepatic expression of peroxisome proliferators–activated receptor α (PPARα) was slightly decreased in the 30BT/MC group compared with the Wis group, but NAT treatment up-regulated its expression by about 2-fold above the level in the 30BT/MC group (Fig. 6A). In addition, the enzymes acting downstream of PPARα, such as acyl–coenzyme A oxidase (ACO) and carnitine palmitoyltransferase 1 (CPT-1), were up-regulated by NAT treatment (Fig. 6B, C). The expression

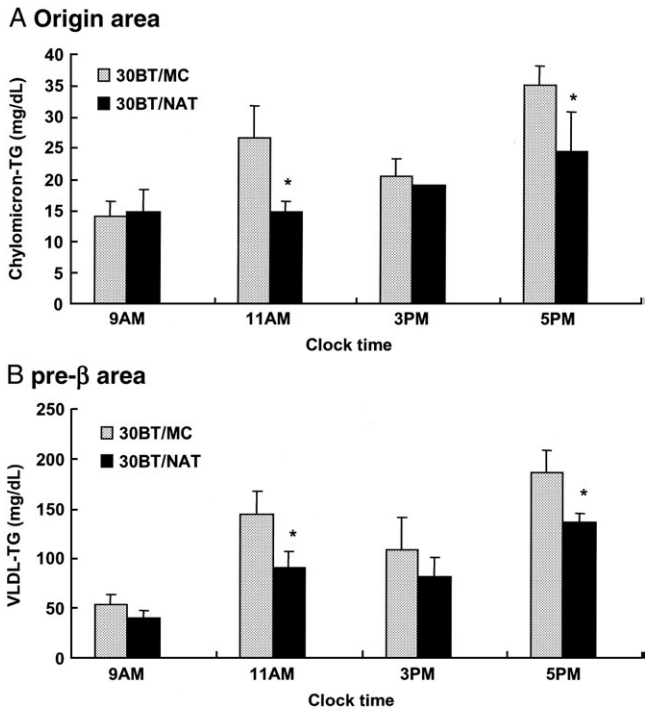

Fig. 3. Changes of plasma TG subfractions (origin area [A] and pre-β area [B]) after food intake. Blood samples were taken from the tail vein just before and 2 hours after feeding, and plasma samples were prepared. Afterward, plasma TG were fractionated and quantified as described in Materials and methods. Data are expressed as the mean ± SEM. n = 8, *$P < .05$ vs 30BT/MC, Student t test.

of ACO and CPT-1 was positively correlated with the expression of PPARα ($r = 0.61$ with ACO, $r = 0.72$ with CPT-1) (Fig. 6B, C). Furthermore, 12 weeks of treatment with NAT resulted in up-regulation of the hepatic expression of adiponectin receptor AdipoR2 by about 2-fold above the level in the 30BT/MC group (Fig. 7A). The plasma adiponectin level after 12 weeks of treatment was reduced by the 30BT diet, and NAT had no effect on it (Fig. 7B).

3.5. Hepatic gene expression related to lipogenesis

Regarding the lipogenic pathway, expression of sterol regulatory element–binding protein 1c (SREBP1c) showed a marked increase in the GK rats, but there was no difference between the groups of GK rats (Fig. 8A). However, NAT treatment suppressed the expression of stearoyl–coenzyme A desaturase 1 (SCD-1) and malic enzyme (ME) (Fig. 8B, C).

Table 3
Body weight and organ weights after 12 weeks of treatment

	Body weight (g)	Epididymal fat pad (g)	Liver (g)
Wis	402.5 ± 7.5	3.4 ± 0.2	9.9 ± 0.5
5BT/MC	321.4 ± 4.6 *	2.3 ± 0.1 *	9.0 ± 0.3
30BT/MC	352.1 ± 5.7 *,†	3.3 ± 0.1 †	9.4 ± 0.3
30BT/NAT	335.1 ± 6.3 *	3.1 ± 0.2 †	8.9 ± 0.3

Mean ± SEM, n = 8.
* $P < .01$ vs Wis, Tukey-Kramer test.
† $P < .01$ vs 5BT/MC, Tukey-Kramer test.

4. Discussion

In this study, meal feeding of the 30BT diet did not have a major impact on the fasting plasma TG level. However, GK rats fed the 30BT diet twice a day showed marked postprandial hypertriglyceridemia accompanied by delayed insulin secretion. Occurrence of fatty liver was also observed after 12 weeks in the 30BT/MC group. The elevated plasma TG level after consumption of the 30BT diet was mainly due to an increase of the chylomicron and VLDL subfractions. These data suggested that although elevation of fasting plasma TG levels were modest, recurrent postprandial hypertriglyceridemia combined with delayed insulin secretion causes hepatic lipid dysmetabolism and increases the risk of fatty liver.

Table 4
Fasting blood glucose, TG, insulin, and NEFA after 12 weeks of treatment

	FBG (mg/dL)	TG (mg/dL)	Insulin (ng/mL)	NEFA (mEq/L)
Wis	116.5 ± 1.7	157.5 ± 16.0	1.7 ± 0.2	0.81 ± 0.04
5BT/MC	151.6 ± 6.0 *	107.3 ± 10.0	5.0 ± 0.5 *	0.76 ± 0.10
30BT/MC	164.0 ± 2.6 *,†	133.3 ± 19.7	4.5 ± 0.3 *	0.68 ± 0.05
30BT/NAT	169.5 ± 7.1 *,†	132.0 ± 16.6	5.1 ± 0.3 *	0.65 ± 0.04

After 17 hours of fasting (18 hours after the last dosing), blood samples were taken from the tail vein. Mean ± SEM, n = 8. FBG indicates fasting blood glucose.
* $P < .01$ vs Wis, Tukey-Kramer test.
† $P < .01$ vs 5BT/MC, Tukey-Kramer test.

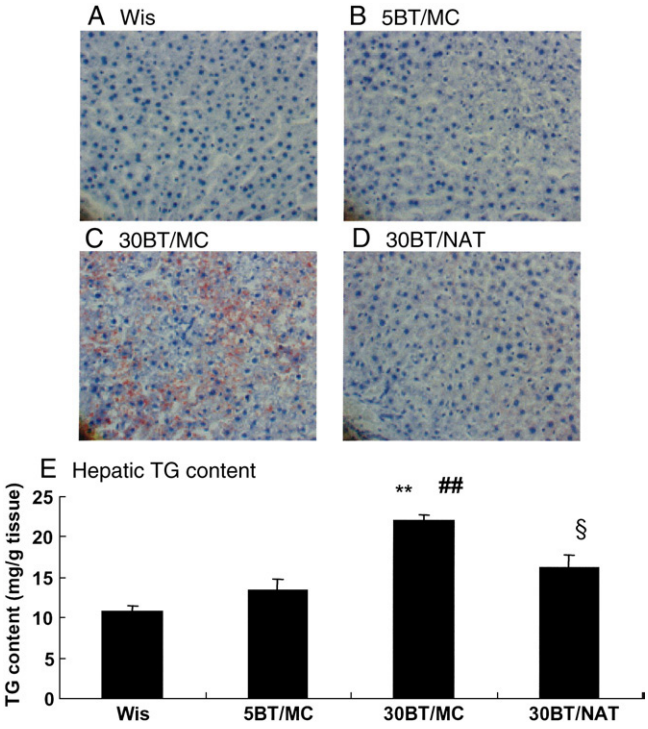

Fig. 4. Histology and TG content in the liver after 12 weeks of treatment. Representative hepatic histology of the Wis group (A), the 5BT/MC group (B), the 30BT/MC group (C), and the 30BT/NAT group (D). Liver sections were stained with oil red O. Magnification, 40×. Hepatic TG content (E) was assessed as described in Materials and methods. Data are expressed as the mean ± SEM. n = 8, $^{**}P < .01$ vs Wis, $^{\#\#}P < .01$ vs 5BT/MC, $^{\S}P < .05$ vs 30BT/MC, Tukey-Kramer test.

It is known that polyunsaturated free fatty acids suppress lipogenesis by down-regulating SREBP1c expression in the liver [19]. However, the fat added to the diet used in this study was beef tallow, and its polyunsaturated free fatty acid content is known to be very low, whereas the content of saturated free fatty acids is high. Moreover, hyperinsulinemia has been reported to activate lipogenesis through up-regulation of SREBP1c [20], and the 30BT/MC group showed a significant increase of diurnal total insulin secretion by the end of the present study. Therefore, up-regulation of SREBP1c seemed likely to contribute to the

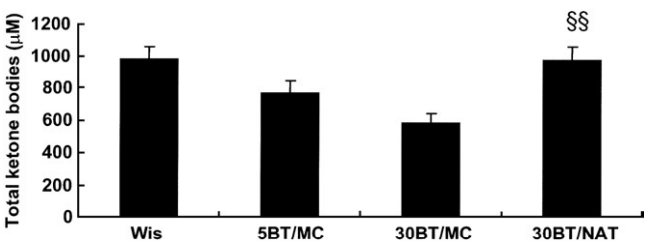

Fig. 5. Plasma total ketone body level after 12 weeks of treatment. A blood sample was obtained after an overnight fast, and plasma total ketone bodies were measured as described in Materials and methods. Data are expressed as the mean ± SEM. n = 8, $^{\S\S}P < .01$ vs 30BT/MC, Tukey-Kramer test.

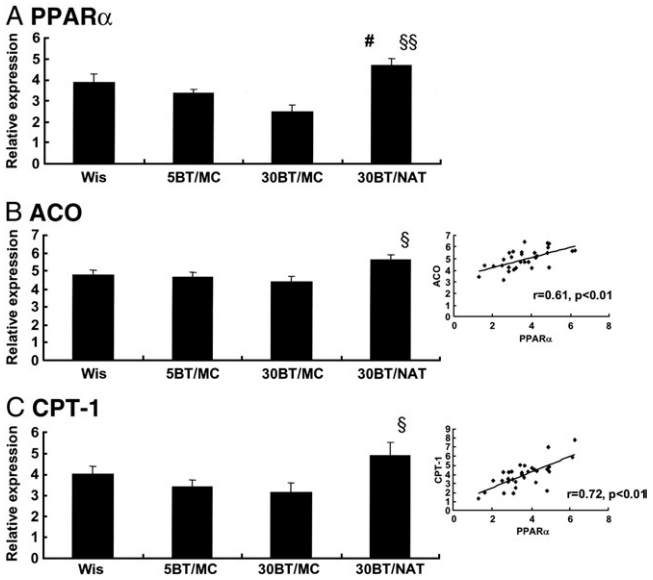

Fig. 6. Hepatic expression of PPARα (A), ACO (B), and CPT-1 (C) after 12 weeks of treatment. The liver was excised from each rat, and total RNA was extracted for quantitative reverse transcriptase (RT) PCR analysis described in Materials and methods. β-Actin was used as the internal control. Correlation between the PPARα level and the ACO or the CPT-1 level was superimposed on the right side of each graph. Data are expressed as the mean ± SEM. n = 8, $^{\#}P < .05$ vs 5BT/MC, $^{\S}P < .05$ vs 30BT/MC, $^{\S\S}P < .01$ vs 30BT/MC, Tukey-Kramer test.

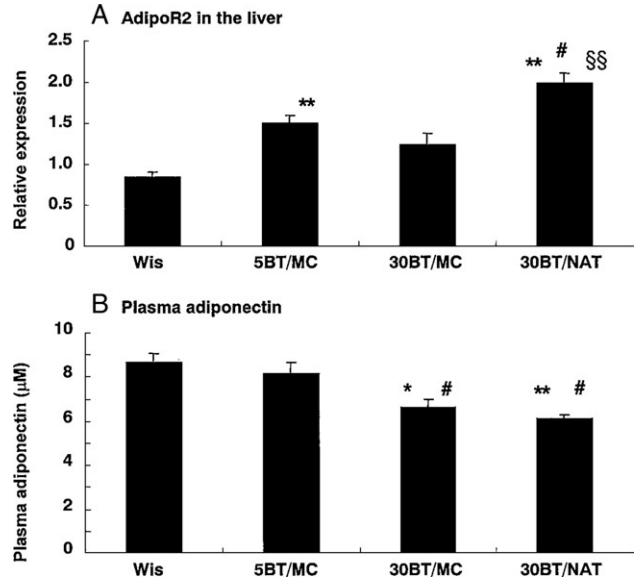

Fig. 7. Hepatic expression of AdipoR2 (A) and plasma adiponectin level (B) after 12 weeks of treatment. The liver was excised from each rat, total RNA was extracted, and relative expression of AdipoR2 was measured by quantitative RT-PCR analysis as described in Materials and methods. β-Actin was used as the internal control. Blood samples were obtained after an overnight fast, and plasma adiponectin was measured by ELISA. Data are expressed as the mean ± SEM. n = 8, $^{*}P < .05$ vs Wis, $^{**}P < .01$ vs Wis, $^{\#}P < .05$ vs 5BT/MC, $^{\S\S}P < .01$ vs 30BT/MC, Tukey-Kramer test.

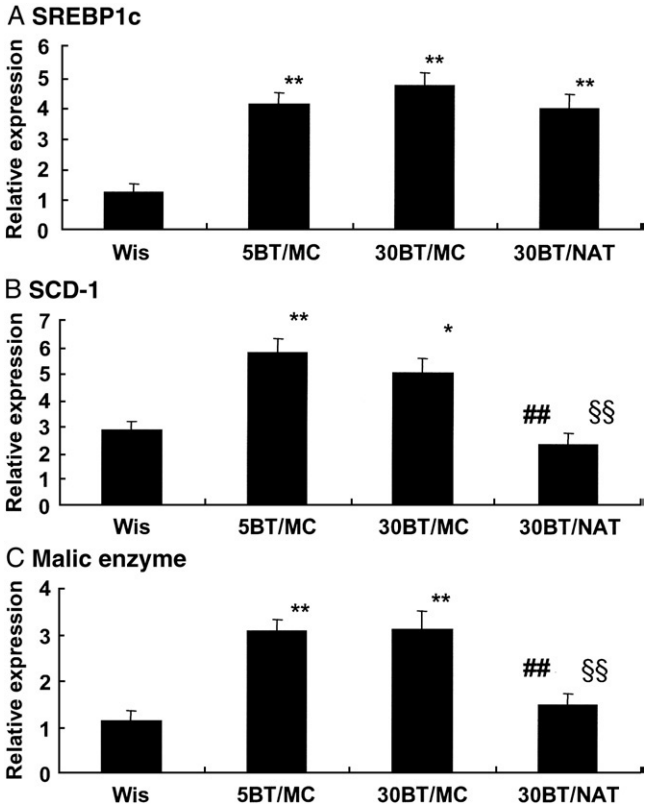

Fig. 8. Hepatic expression of SREBP1c (A), SCD-1 (B), and ME (C) after 12 weeks of treatment. The liver was excised from each rat, and total RNA was extracted for quantitative RT-PCR analysis described in Materials and methods. β-Actin was used as the internal control. Data are expressed as the mean ± SEM. n = 8, $^{*}P < .05$ vs Wis, $^{**}P < .01$ vs Wis, $^{\#\#}P < .01$ vs 5BT/MC, $^{\S\S}P < .01$ vs 30BT/MC, Tukey-Kramer test.

development of fatty liver in the 30BT/MC group. However, from the current results, although a significant elevation of SREBP1c expression was seen in GK rats compared with normal Wistar rats, there was no difference between the 5BT/MC group and the 30BT/MC group, despite showing a significant increase of hepatic TG content in the 30BT/MC group. In addition, NAT treatment had no effect on hepatic expression of SREBP1c. Nevertheless, the expression of SCD-1 and ME, which are the enzymes acting downstream of SREBP1c, was down-regulated by NAT treatment, and hepatic TG content was decreased. Therefore, suppression of lipogenesis by NAT treatment might contribute to suppression of the development of fatty liver in this model. In the present study, SREBP1c expression was measured in the fasting state just before the first meal on the final day of the experiment, whereas up-regulation or activation of SREBP1c is reported to be induced by food intake [21]. Therefore, it may be necessary to measure SREBP1c expression in the postprandial or postabsorptive state to clarify the involvement of lipogenic pathway in the development of fatty liver in this model.

On the other hand, there was decreased hepatic expression of PPARα and a lower plasma total ketone body level in the 30BT/MC group. Peroxisome proliferators–activated receptor α regulates the mitochondrial and peroxisomal fatty acid β-oxidation systems as well as the microsomal ω-oxidation system. Its defect has been reported to lead to the defect of hepatic fat utilization as an energy source and lead to hypoketonemia that has a prominent impact on the pathogenesis of fatty liver disease [22-25]. In addition, impairment of hepatic insulin sensitivity has been also known to be involved in impairment of ketogenesis in the liver and hepatic TG accumulation [26]. In this study, we used type 2 diabetic GK rats that show hepatic insulin resistance [15]. Feeding the high-fat diet to the GK rats exacerbated hyperinsulinemia and caused a decrease of hepatic PPARα. Therefore, the decrease of total ketone body level observed in the 30BT/MC group might be due to the defect of fat utilization in the liver. In this model, hepatic TG accumulation was suppressed by NAT treatment along with up-regulation of PPARα and its downstream enzymes and with normalization of the plasma total ketone body level. These data indicated that NAT suppressed hepatic accumulation of TG through up-regulation of lipid oxidation pathway. Because measurements of gene expression are not the same as measurements of biologic function, further investigation might be necessary for confirming the effect of NAT treatment on lipid oxidation pathway. However, because positive relationship between the expression of PPARα and CPT-1 or ACO was observed in the present study, transcriptional regulation of lipid oxidation pathway by PPARα might play a critical role for the preventive effect of NAT on fatty liver. Morita et al [27] reported that 16 weeks of treatment with NAT led to improvement of histologic changes in the livers of nonalcoholic fatty liver disease (nonalcoholic steatohepatitis) patients. Therefore, although the mechanism by which NAT regulates hepatic expression of PPARα remains unclear, it might be a potential agent for use in the prevention of fatty liver disease, such as nonalcoholic steatohepatitis. There was no acute effect of NAT on hepatic PPARα expression (data not shown). Therefore, long-term treatment with NAT may be necessary for such changes to occur. Suppression of the increase of total insulin release by restoration of early-phase insulin secretion might be an important factor for this effect of NAT. However, we cannot exclude the possibility that NAT directly regulates hepatic lipid metabolism.

We also found that hepatic expression of AdipoR2 was unexpectedly up-regulated by NAT treatment. Because this study was not designed to evaluate the role of adiponectin receptors in mediating the effects of NAT, it is difficult to clarify whether the up-regulation of AdipoR2 is involved in the prevention of fatty liver by NAT treatment. However, it has been demonstrated that the impairment of adiponectin signaling pathway is involved in the pathogenesis of fatty liver disease [28-30]. In the present study, long-term intake of the 30BT diet lowered the plasma adiponectin level.

Although NAT treatment had no effect on the plasma adiponectin level, it might be possible that adiponectin activity in the liver was enhanced by up-regulation of AdipoR2 expression. Therefore, activation of the adiponectin signaling pathway might also be involved in the effects of NAT detected in the present study.

In conclusion, this was the first study to show that long-term treatment with NAT can prevent the development of fatty liver through enhancement of lipid oxidation pathway in the liver in rats with type 2 diabetes mellitus fed a high-fat diet. Although further investigation is necessary to clarify whether the current results are also applicable to the pathophysiological basis of dyslipidemia and fatty liver in human subjects with type 2 diabetes mellitus, our findings support the efficacy of NAT for preventing the onset and progression of fatty liver and related diseases.

Acknowledgment

The authors thank Yoshiharu Tsuchiya and Kyoko Yuasa for their excellent technical assistance.

References

[1] Kelley DE, McKolanis TM, Hegazi AF, et al. Fatty liver in type 2 diabetes mellitus: relation to regional adiposity, fatty acids, and insulin resistance. Am J Physiol Endcrinol Metab 2003;285:E906-16.

[2] Dimitriadis G, Boutati E, Lambadiari V, et al. Restoration of early insulin secretion after a meal in type 2 diabetes: effects on lipid and glucose metabolism. Eur J Clin Invest 2004;34:490-7.

[3] Ravikumar B, Carey PE, Snaar JEM, et al. Real-time assessment of postprandial fat storage in liver and skeletal muscle in health and type 2 diabetes. Am J Physiol Endocrinol Metab 2005;288:E789-97.

[4] Utzschneider KM, Kahn SE. The role of insulin resistance in non-alcoholic fatty liver disease. J Clin Endocrinol Metab 2006;91: 4753-61.

[5] Clark JM. The epidemiology of nonalcoholic fatty liver disease in adults. J Clin Gastroenterol 2006;40(3 Suppl 1):S5-10.

[6] Westerbacka J, Lammi K, Hakkinen A-M, et al. Dietary fat content modifies liver fat in overweight nondiabetic subjects. J Clin Endocrinol Metab 2005;90:2804-9.

[7] Ikenoue T, Akiyoshi M, Fujitani S, et al. Hypoglycemic and insulinotropic effects of a novel oral antidiabetic agent, [(−)-*N*-(*trans*-4-isopropylcyclohexanecarbonyl)-D-phenylalanine], (A-4166). Br J Pharmacol 1997;120:137-45.

[8] Uchino H, Niwa M, Shimizu T, et al. Impairment of early insulin response after glucose load, rather than insulin resistance, is responsible for postprandial hyperglycemia seen in obese type 2 diabetes: assessment using nateglinide, a new insulin secretagogue. Endcr J 2000;47:639-41.

[9] Horton ES, Clinkingbeard C, Gatrin M, et al. Nateglinide alone and in combination with metformin improves glycemic control by reducing mealtime glucose levels in type 2 diabetes. Diabetes Care 2000;23: 1660-5.

[10] Barnett AH, Anderson DM, Shelley S, et al. A placebo-controlled crossover study comparing the effects of nateglinide and glibenclamide on postprandial hyperglycaemia and hyperinsulinaemia in patients with type 2 diabetes. Diabetes Obes Metab 2004;6:104-13.

[11] Mori Y, Kitahara Y, Miura K, et al. Role of early insulin secretion in postglucose-loading hyperglycemia and postfat-loading hyperlipidaemia: comparing nateglinide and glibenclamide for acute effects on insulin secretion in OLETF rats. Diabetes Obes Metab 2004;6:422-31.

[12] Mine T, Miura K, Kitahara Y, et al. Nateglinide suppresses postprandial hypertriglyceridemia in Zucker fatty rats and Goto-Kakizaki rats: comparison with voglibose and glibenclamide. Biol Pharm Bull 2002; 25:1412-6.

[13] Mori Y, Kuriyama G, Tajima N. Effects of nateglinide on elevation of postprandial remnant-like particle levels in Japanese patients with type 2 diabetes assessment by meal tolerance test. Endocrine 2004;25: 203-6.

[14] Ai M, Tanaka A, Ogita K, et al. Favorable effects of early insulin secretion by nateglinide on postprandial hyperlipidemia in patients with type 2 diabetes. Diabetes Care 2006;29:1180.

[15] Goto Y, Suzuki K, Ono T, et al. Development of diabetes in the non-obese NIDDM rat (GK rat). Adv Exp Med Biol 1988;256:29-31.

[16] Kitahara Y, Miura K, Takesue K, et al. Decreased blood glucose excursion by nateglinide ameliorated neuropathic changes in Goto-Kakizaki rats, an animal model of non-obese type 2 diabetes. Metabolism 2002;51:1452-7.

[17] Azuma K, Kawamori R, Toyofuku Y, et al. Repetitive fluctuations in blood glucose enhance monocyte adhesion to the endothelium of rat thoracic aorta. Arterioscler Thromb Vasc Biol 2006;26:2275-80.

[18] Folch J, Lees M, Sloane-Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957; 226:497-509.

[19] Sekiya M, Yahagi N, Najima Y, et al. Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression. Hepatology 2003;38:1529-39.

[20] Shimomura I, Bashmakov Y, Ikemoto S, et al. Insulin selectively increases SREBP-1c mRNA in the livers of rats with streptozotocin-induced diabetes. Proc Natl Acad Sci U S A 1999;96:13656-61.

[21] Matsumoto M, Ogawa W, Akimoto K, et al. PKCλ in liver mediates insulin-induced SREBP-1c expression and determines both hepatic lipid content and overall insulin sensitivity. J Clin Invest 2003;112: 935-44.

[22] Yeon JE, Choi KM, Baik SH, et al. Reduced expression of peroxisome proliferators–activated receptor–α may have an important role in the development of non-alcoholic fatty liver disease. J Gastroenterol Hepatrol 2004;19:799-804.

[23] Svegliati-Baroni G, Candelaresi C, Saccomanno S, et al. A model of insulin resistance and nonalcoholic steatohepatitis in rats. Am J Pathol 2006;169:846-60.

[24] Reddy JK, Rao MS. Lipid metabolism and liver inflammation. II. Fatty liver disease and fatty acid oxidation. Am J Physiol Gastrointest Liver Physiol 2006;290:G852-8.

[25] Hashimoto T, Cook WS, Qi C, et al. Defect in peroxisome proliferators–activated receptor α–inducible fatty acid oxidation determines the severity of hepatic steatosis in response to fasting. J Biol Chem 2000;37:28918-28.

[26] Wolfrum C, Asilmaz E, Luca E, et al. Foxa2 regulates lipid metabolism and ketogenesis in the liver during fasting and in diabetes. Nature 2004;432:1027-32.

[27] Morita Y, Ueno T, Sasaki N, et al. Nateglinide is useful for nonalcoholic steatohepatitis (NASH) patients with type 2 diabetes. Hepatogastroenterology 2005;52:1338-43.

[28] Vuppalanchi R, Marri S, Kolwankar D, et al. Is adiponectin involved in the pathogenesis of nonalcoholic steatohepatitis? A preliminary human study. J Clin Gastroenterol 2005;39:237-42.

[29] Bugianesi E, Pagotto U, Manini R, et al. Plasma adiponectin in nonalcoholic fatty liver is related to hepatic insulin resistance and hepatic fat content, not to liver disease severity. J Clin Endocrinol Metab 2005;90:3498-504.

[30] Mendez-Sanchez N, Chavez-Tapia N, Villa AR, et al. Adiponectin as a protective factor in hepatic steatosis. World J Gastroenterol 2005;11: 1737-41.