



*Original Article*

## Increased hepatic triglyceride level induced by a glucokinase activator in mice

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**ABSTRACT** — Glucokinase (GK) is an enzyme that catalyzes the phosphorylation of glucose to glucose-6-phosphate, and plays an important role in maintaining glucose homeostasis by regulating secretion of insulin from pancreatic  $\beta$ -cells and glucose metabolism in the liver. Recently, GK activators are expected as a novel therapeutic agent for Type 2 diabetes mellitus (T2DM). However, the increase in plasma triglyceride (TG) levels is one of the major issues for the development of GK activators. In this study, we evaluated the effects of the GK activator GKA50 on the plasma and hepatic TG in mice. Male CD-1 mice received a single oral dose of vehicle or GKA50 (15, 30, or 60 mg/kg), and plasma glucose and insulin levels were measured. Next, CD-1 mice received oral doses of vehicle or GKA50 (20 or 60 mg/kg) once daily for 4 days, and clinical signs, body weight, food consumption, blood chemistry, and hepatic TG were evaluated. In the single oral dose study, dose-dependent decrease in plasma glucose levels and increase in plasma insulin levels were observed. In the 4-day repeated dose study, there were no treatment-related changes in clinical signs, body weight, food consumption, or plasma TG levels. In the 60 mg/kg group, a significant increase in the hepatic TG level was observed. Additionally, the detailed analysis of TG species composition revealed marked increases in TGs mainly composed of 18:1 fatty acids. This study revealed that GKA50 enhanced insulin secretion and hepatic glucose utilization, and increased hepatic TGs, which were mainly composed of 18:1 fatty acids.

**Key words:** Glucokinase activator, GKA50, Triglyceride, Liver, Mice

### INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a metabolic disease characterized by hyperglycemia mainly resulting from impaired insulin secretion, defects in insulin action, or both (American Diabetes Association, 2014). Although many classes of drugs are currently available for glycemic control in T2DM, there remains a great need for drugs with new mechanisms of action that can be added to or replace currently available drugs (Mittermayer *et al.*, 2015).

Glucokinase (GK) is an enzyme that catalyzes the phosphorylation of glucose to glucose-6-phosphate, and is predominantly expressed in the pancreas, liver, brain, and gastrointestinal tract (Massa *et al.*, 2011; Iynedjian *et al.*, 1986; Matschinsky *et al.*, 2006). GK plays an important role in maintaining glucose homeostasis by regulating secretion of insulin from pancreatic  $\beta$ -cells and glucose metabolism in the liver (Matschinsky, 1990). Therefore, GK activators are expected to be novel therapeutic agents for T2DM (Matschinsky, 2009). Several GK activators have been reported to demonstrate clinically significant

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reduction in hemoglobin A1c (HbA1c) in T2DM patients (Meininger *et al.*, 2011; Wilding *et al.*, 2013; Kiyosue *et al.*, 2013; Kazierad *et al.*, 2013). However, clinical trials of several GK activators also revealed a loss of efficacy over time with sustained treatment and an increase in plasma triglyceride (TG) levels. The increase in plasma TG levels is one of the major issues for the development of GK activators. Accordingly, it is important to characterize the effects of GK activators on plasma and hepatic TG levels.

In this study, we evaluated the effects of the GK activator GKA50 on the plasma and hepatic TG levels in mice. In addition, we also evaluated TG species composition in the livers of mice treated with GKA50 in order to clarify the contribution of individual TG species to the changes in hepatic TG level, which will help us to better understand the mechanism underlying the changes.

## MATERIALS AND METHODS

### Reagents

All reagents were of analytical grade. GKA50 (6-({3-[(1S)-2-methoxy-1-methylethoxy]-5-[(1S)-1-methyl-2-phenylethoxy]benzoyl}amino)nicotinic acid) was purchased from Sigma-Aldrich (St. Louis, MO, USA). GKA50 was weighed and suspended in distilled water at 1 w/v% hydroxyethyl cellulose (HEC) purchased from Wako-Pure Chemical Industry Co., Ltd. (Osaka, Japan).

### Animals

Male CD-1 mice [CrI:CD1(ICR)] (SPF) were obtained from Charles River Japan, Inc. (Yokohama, Japan). The mice were quarantined and acclimatized for 1 week, and assigned to each treatment group by stratified randomization (StatLight group allocation, Yukums Co., Ltd, Kanagawa, Japan) according to body weight. All animals were housed under a 12-hr/12-hr light cycle (light on 6:00 AM-6:00 PM) under controlled conditions (room temperature, 22-26°C; humidity, 40-70%), and fed a laboratory chow diet (CE-2pellets; CLEA Japan, Inc.) and water *ad libitum*. All animal experiments were performed in accordance with the “Animal Experiment Regulations” and “Regulations on animal Euthanasia” of the Institute for Bio-medical Research, Teijin Pharma Limited.

### Experimental design

#### Study 1

CD-1 mice at 8 weeks of age were divided into 4 groups (n = 4/ each group), and given a single oral dose of vehicle (1w/v% HEC) or GKA50 at 15, 30 or 60 mg/kg (10 mL/kg). Blood samples were obtained for

measurement of plasma glucose and insulin levels at several time points (0, 1, 2, 4, 8 or 24 hr) after administration. Blood samples were collected from the outside saphenous vein into heparin coated glass capillary tubes and plasma was obtained by centrifugation. The obtained plasma samples were kept frozen at -20°C before use. Plasma levels of glucose and insulin were measured using a glucose kit (Wako-Pure Chemical Industry Co., Ltd., Osaka, Japan) and an enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Inc., Kanagawa, Japan) following the manufacturer’s protocols.

#### Study 2

CD-1 mice at 8 weeks of age were divided into three groups (n = 6/ each group), and vehicle (1 w/v% HEC) or GKA50 at 20 or 60 mg/kg (10 mL/kg) was orally administered once daily for 4 days. Observations of clinical signs, and measurements of body weight and food consumption were performed daily during the treatment period. The last compound administration was given one day before sacrificing. The day of sacrifice, animals were fasted for approximately 2 hr and anaesthetized using isoflurane. Blood was taken from the postcava for blood chemistry examination, and then livers were removed, snap-frozen in liquid nitrogen and stored at -80°C for subsequent hepatic TG assays.

### Blood chemistry

The plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, TGs, total cholesterol, phospholipid, non-esterified fatty acid (NEFA) and lactic acid were measured using a 7180 Clinical Analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan).

### Measurement of hepatic TG level

TGs were extracted from the liver with 2-propanol. The TG concentration was assayed using a commercial TG kit (Wako L-Type TG M kit, Wako-Pure Chemical Industry Co., Ltd., Osaka, Japan) following the manufacturer’s instructions.

### Detailed analysis of TG species composition

The detailed analysis of TG species composition was conducted at the Chemicals Evaluation and Research Institute, Japan. The analysis was performed using livers of 3 animals in the vehicle control group and 3 animals in the 60 mg/kg group. A necessary amount (20 mg) of the frozen sample of the liver was weighed and 500 µL of methanol was added. It was sonicated for

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10 min, and then shaken at 2500 rpm for 5 min. Additionally, 500  $\mu$ L of chloroform was added and shaken at 2500 rpm for 5 min. It was centrifuged (4°C, 9000 g, 5 min), and the supernatant was collected. Analysis samples were prepared by diluting 10 times with acetone containing 1  $\mu$ g/mL of internal standard (TG tri13:0). The QC sample was prepared by mixing 20  $\mu$ L of each analysis sample. For high-performance liquid chromatography (HPLC), UFLC XR (Shimadzu Corp., Kyoto, Japan) and reversed-phase column Sunrise C28 (150  $\times$  2 mm i.d., 3- $\mu$ m particle size; Chromanik Technologies Inc., Osaka, Japan) were used. Mobile phase A was acetonitrile, and mobile phase B was acetone. The flow rate was 0.4 mL/min, the column temperature was 15°C and the sampler temperature was 4°C. Ammonium acetate (10 mmol/L) was added post-column at a flow rate of 0.1 mL/min. The injection volume for the analysis was 3  $\mu$ L. For mass spectrometry, an ion trap-orbitrap mass spectrometer (LTQ Orbitrap XL; Thermo Fisher Scientific K.K., Kanagawa, Japan) equipped with heated electrospray ion source was used. Data dependent MS/MS (TopN3 experiment) was operated at 60,000 resolution from  $m/z$  245 to 1,300 in positive ESI mode. For identification of TG, Lipid Search™ (Mitsui Knowledge Industry, Tokyo, Japan) was employed and the product search mode was used. Identification by the product search mode was performed based on the accurate mass of precursor ions and MS/MS spectral pattern (Stranska-Zachariasova *et al.*, 2016). The mass tolerance was set to 5 ppm mass window. The relative intensity threshold of product ions was set to 1%. The m-score (the score calculated based on the number of matches with product ion peaks in the spectrum) threshold was set to 5. As adduct ions,  $[M + H]^+$ ,  $[M + NH_4]^+$ ,  $[M + Na]^+$  and  $[M + K]^+$  were searched for. As the quality data filter, identification results that met all five conditions described below were adopted: 1) the peak was detected in all the QC samples and coefficient of variance was less than 15%, 2) mean intensity of the QC samples was more than 10,000, 3) mean signal to noise ratio in the QC samples was more than 3, 4) retention time was reasonable and 5) specific product ions were detected. The detected peak area in each sample was corrected by the peak area of the internal standard.

### Statistical analysis

Data were presented as means  $\pm$  standard deviation (S.D.) for each group. Statistical analysis was performed using SAS Systems version 9.2 (SAS Institute, Cary, NC). Significant differences between the mean values were tested by Bartlett's test followed by Dunnett's test

or Steel's test or by F-test followed by Student's t-test or Aspin-Welch's t-test. P-values less than 0.05 were considered to indicate significance.

## RESULTS

### Effects of GKA50 on plasma glucose and insulin levels

CD-1 mice received a single oral dose of GKA50 at 15, 30 or 60 mg/kg, and plasma glucose and insulin levels were measured. Dose-dependent decrease in plasma glucose levels (Fig. 1A) and increase in plasma insulin levels were observed (Fig. 1B). Plasma glucose levels decreased in 15, 30 and 60 mg/kg groups from a mean ( $\pm$  S.D.) of  $159.3 \pm 24.0$ ,  $161.3 \pm 10.9$  and  $177.8 \pm 21.1$  mg/dL before dosing to  $97.3 \pm 13.0$ ,  $94.4 \pm 5.7$  and  $78.0 \pm 7.6$  mg/dL 4 hr after dosing, respectively. Plasma insulin levels increased in 30 and 60 mg/kg groups from a mean ( $\pm$  S.D.) of  $0.683 \pm 0.137$  and  $0.652 \pm 0.328$  ng/dL before dosing to  $0.899 \pm 0.264$  and  $2.364 \pm 1.193$  ng/dL 4 hr after dosing, respectively.

### Effects of GKA50 on clinical signs, body weight, food consumption and blood chemistry parameters

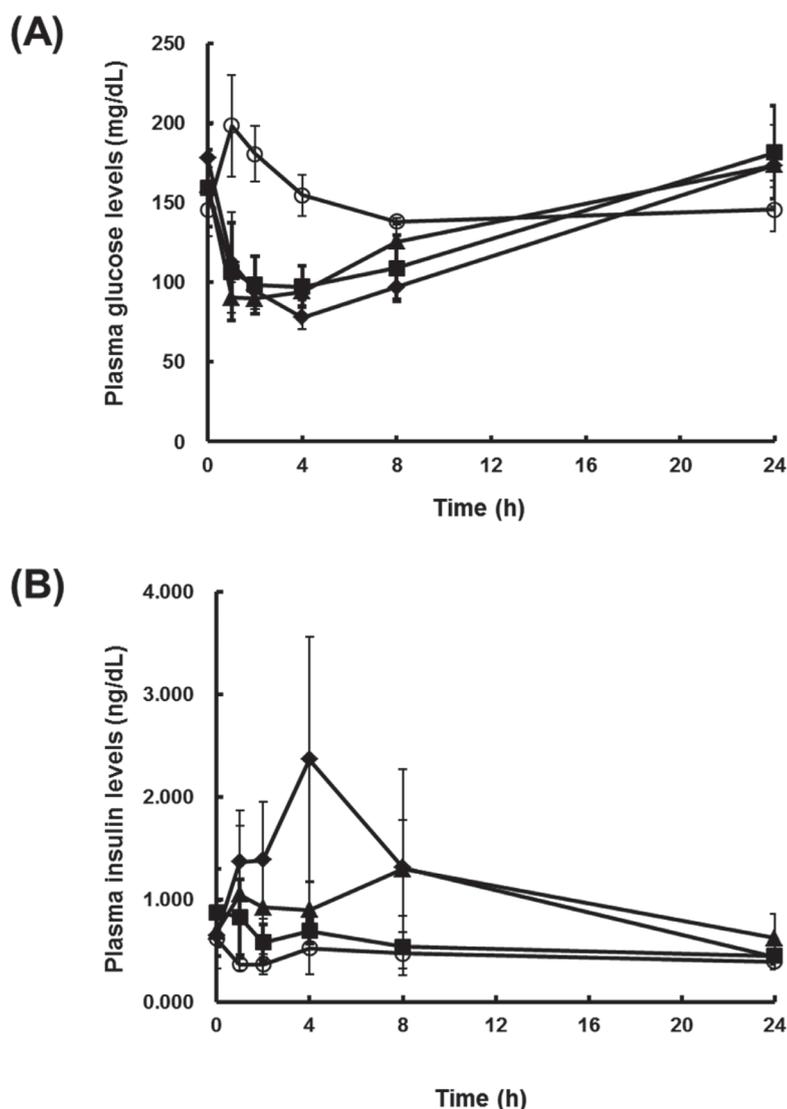
CD-1 mice received oral doses of GKA50 at 20 or 60 mg/kg once daily for 4 days, and clinical signs, body weight, food consumption and blood chemistry parameters were evaluated. There were no treatment-related changes in clinical signs, body weight or food consumption during the dosing period. In the blood chemistry examination at the end of the dosing period, a significant increase in AST ( $P < 0.05$ ) and decrease in total bilirubin ( $P < 0.01$ ) in 20 and 60 mg/kg groups, and a significant increase in total cholesterol ( $P < 0.05$ ) in the 60 mg/kg group were observed (Table 1). There were no treatment-related changes in ALT, TG, phospholipid, NEFA or lactic acid.

### Effects of GKA50 on hepatic TG levels

We evaluated hepatic TG levels in CD-1 mice treated orally with GKA50 (20 or 60 mg/kg) for 4 days. In the 60 mg/kg group, a significant increase in hepatic TG levels ( $P < 0.05$ ) was observed (Fig. 2). The mean ( $\pm$  S.D.) of the hepatic TG levels in the vehicle control, and 20 and 60 mg/kg groups were  $4.9975 \pm 0.6966$ ,  $5.3681 \pm 1.7742$  and  $9.1479 \pm 3.9618$  mg/g tissue, respectively.

### Detailed analysis of TG species composition

We evaluated the contribution of individual TG species to the changes in hepatic TG level by detailed analysis of TG species composition in order to understand



**Fig. 1.** Effects of GKA50 on plasma glucose (A) and insulin (B) levels in CD-1 mice. GKA50 was administered at a single oral dose of 15, 30 or 60 mg/kg. Blood samples were obtained at 0, 1, 2, 4, 8 and 24 hr after administration. Data represent mean  $\pm$  S.D. (n = 4). Open circles: Control; Closed squares: 15 mg/kg GKA50; Closed triangles: 30 mg/kg GKA50; Closed diamonds: 60 mg/kg GKA50.

the mechanism underlying the changes at 60 mg/kg. The number of detected TG species in the vehicle control and 60 mg/kg groups was 61 each. The normalized peak area ratio of each TG species in the vehicle control and 60 mg/kg groups was calculated. Marked increases in TGs mainly composed of 18:1 fatty acids were noted (Fig. 3). The fold changes in TGs [(18:0/18:1/18:1), (18:1/18:1/18:2), (18:1/18:1/20:0) and (18:1/18:1/20:2)] were 6.1, 4.5, 10.9 and 4.9, respectively.

## DISCUSSION

GK activators are expected to be novel therapeutic agents for T2DM (Matschinsky, 2009). However, the effects on plasma and hepatic TG levels are one of the major issues for the development of GK activators (Meininger *et al.*, 2011). In this study, we examined the effects of the GK activator GKA50 on the plasma and hepatic TG levels in CD-1 mice.

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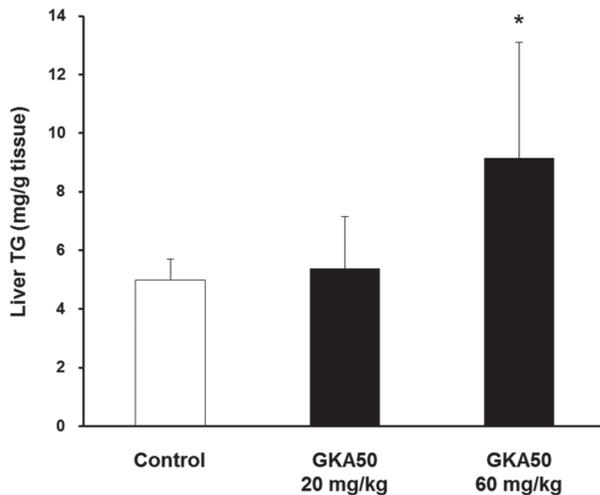
**Table 1.** Effects of GKA50 on blood chemistry parameters in CD-1 mice.

	Vehicle control	GKA50 20 mg/kg	GKA50 60 mg/kg
AST (U/L)	36 ± 3	43 ± 4*	42 ± 6*
ALT (U/L)	19 ± 3	21 ± 5	22 ± 2
T. bilirubin (mg/dL)	0.15 ± 0.03	0.10 ± 0.01**	0.11 ± 0.01 **
Triglyceride (mg/dL)	119 ± 47	107 ± 35	115 ± 25
T. cholesterol (mg/dL)	101 ± 11	104 ± 16	125 ± 10*
PL (mg/dL)	204 ± 19	198 ± 23	219 ± 10
NEFA (μEq/L)	533 ± 69	536 ± 146	536 ± 42
LA (mg/dL)	38.8 ± 5.1	35.8 ± 5.6	32.0 ± 5.5

Data represents mean ± S.D. (n = 6).

\*, \*\*: There are significant differences compared with the vehicle control group

(\*: P < 0.05, \*\*: P < 0.01, Dunnett's test or Steel's test)



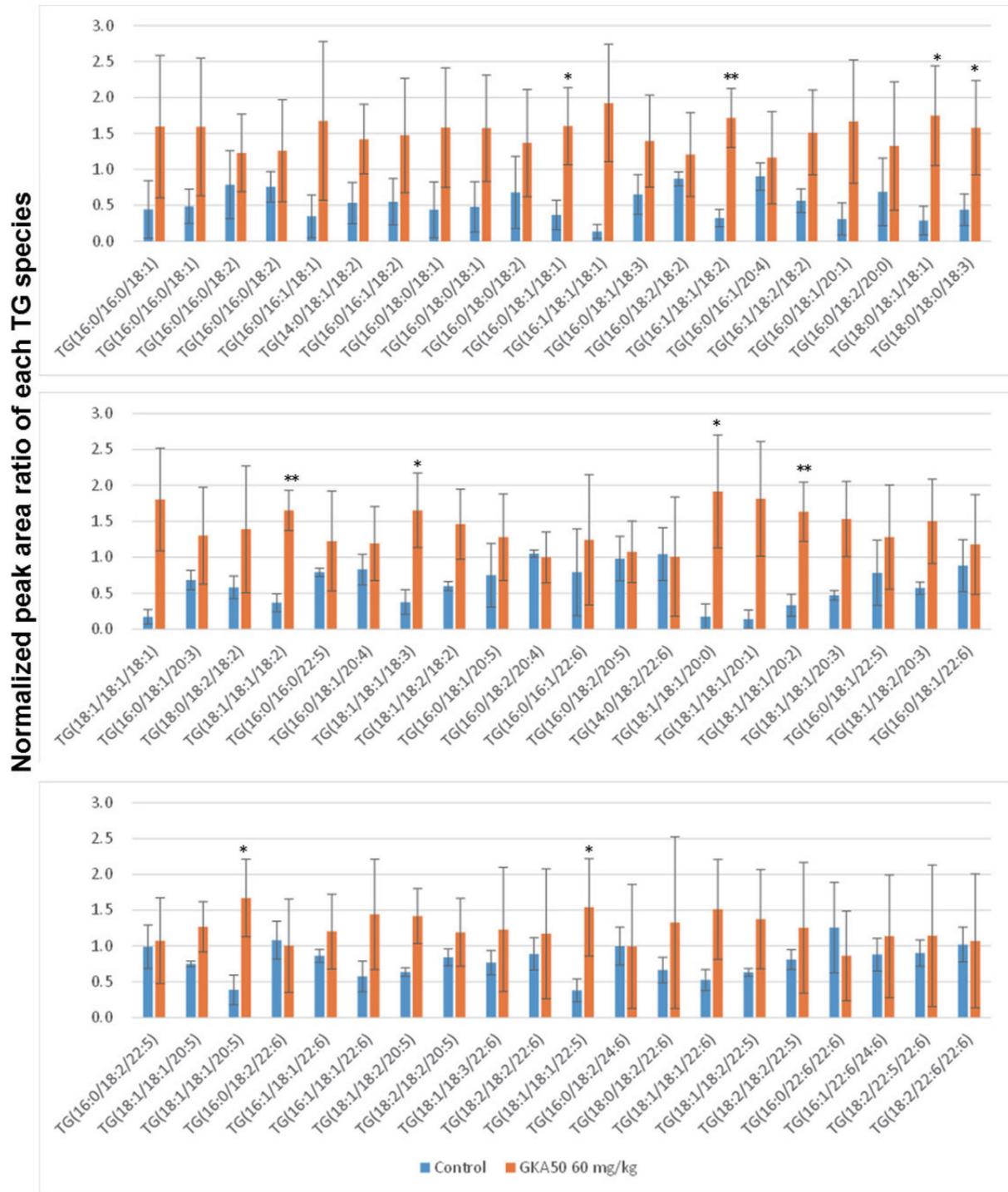
**Fig. 2.** Effects of GKA50 on hepatic TG level in CD-1 mice. CD-1 mice were administered vehicle (1 w/v% HEC) or GKA50 (20 or 60 mg/kg) orally once daily for 4 days. Animals were sacrificed after 4 days of treatment. The hepatic TG level was measured as described in the Materials and Methods section. Data represent mean ± S.D. (n = 6). (\*P < 0.05; Dunnett's test).

The primary findings in this research are as follows: 1) Dose-dependent decrease in plasma glucose levels and increase in plasma insulin levels were observed in CD-1 mice treated with GKA50 in study 1. 2) In the high dose (60 mg/kg) group of study 2, a significant increase in hepatic TG levels (P < 0.05) was observed. 3) The detailed analysis of TG species composition in the animals with increased hepatic TG levels revealed marked increases in TG mainly composed of 18:1 fatty acids.

In study 1, a dose-dependent decrease in plasma glu-

cose levels and increase in plasma insulin levels were observed in CD-1 mice treated with GKA50. The findings in this study are consistent with other studies that have reported that GKA50 stimulated glycogen synthesis in rat hepatocytes and insulin secretion from rodent and human pancreatic  $\beta$  cells, and demonstrated strong blood-glucose-lowering effects in experimental animals, including *db/db* mice, ZDF rats and Wistar rats (De Ceuninck *et al.*, 2013; Johnson *et al.*, 2007; McKerrecher *et al.*, 2006). Additionally, other GK activators have been reported to have strong blood-glucose-lowering effects in humans as well as in experimental animals (Meininger *et al.*, 2011; Wilding *et al.*, 2013; Kiyosue *et al.*, 2013; Kazierad *et al.*, 2013). In the high dose group of this study, a marked decrease in plasma glucose levels and increase in plasma insulin levels were observed, suggesting that insulin secretion and hepatic glucose utilization were enhanced by excessive activation of GK.

In study 2, GKA50 was administered orally once daily for 4 days at doses of 20 or 60 mg/kg/day to CD-1 male mice to evaluate the effects on the plasma and hepatic TG levels. The high dose level was set at 60 mg/kg/day because a marked decrease in plasma glucose levels and increase in plasma insulin levels were observed at 60 mg/kg in study 1. There were no treatment-related changes in clinical signs, body weight, food consumption or plasma TG levels during or at the end of the 4-day dosing period. On the other hand, a significant increase in hepatic TG levels (P < 0.05) was observed in the high-dose group. The findings from this study are consistent with other studies in which small molecule GK activators increased hepatic TG levels in *db/db* mice, ZDF rats and Wistar rats (De Ceuninck *et al.*, 2013). In order to elucidate the mechanism underlying the changes in the high-dose group, we examined the contribution of individual TG species to the



**Fig. 3.** Detailed composition of hepatic TGs in CD-1 mice treated with GKA50. The normalized peak area ratio of each TG species in the vehicle control and 60 mg/kg groups was calculated. The compositions of three fatty acids on the glycerol backbone were identified and listed without regard to their binding position. The detailed analysis of TG species composition was performed as described in the Materials and Methods section. Data represent mean  $\pm$  S.D. (n = 3). (\*: P < 0.05, \*\*: P < 0.01, Student's t-test or Aspin-Welch's t-test).

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changes in hepatic TG level by detailed analysis of TG species composition. We found marked increases in TGs composed mainly of 18:1 fatty acids by detailed analysis of TG species composition. Dietary derived fatty acids were less likely to contribute to the increased hepatic TG levels because food consumption was not affected in this study and the main component of fatty acids included in the diet was 18:2 fatty acids (Erion *et al.*, 2014). Given that a marked decrease in plasma glucose levels and increase in plasma insulin levels were observed in animals with increased hepatic TG levels, increased *de novo* lipogenesis by enhanced hepatic glucose utilization is a possible reason for the increased hepatic TG levels. Additionally, the increase in plasma insulin levels may enhance hepatic *de novo* lipogenesis through activation of the sterol regulatory element binding protein (SREBP1c) (Kim *et al.*, 1998; Shimomura *et al.*, 1999) and suppression of hepatic gluconeogenesis through FOXO1-PGC-1- $\alpha$  interaction (Puigserver *et al.*, 2003). Glucose and insulin are reported to increase the gene expression of stearoyl-CoA desaturase-1 (SCD1), an enzyme that catalyzes the conversion of stearate to oleate, through activation of SREBP1c (Miyazaki *et al.*, 2006; Ntambi and Miyazaki, 2003). Although the precise mechanism for GKA50-induced increases in TGs composed of 18:1 fatty acids is not clear, they may be associated with increased hepatic SCD1 activity due to the enhanced insulin secretion and hepatic glucose utilization.

GK activators are divided into two types: those that reduce blood glucose levels by increased insulin secretion from pancreatic  $\beta$  cells and hepatic glucose uptake resulting from activation of GK systemically, and those that reduce blood glucose levels by selective activation of hepatic GK (Grewal *et al.*, 2014; Pfefferkorn *et al.*, 2012). As GKA50 dose-dependently increased plasma insulin levels, GKA50 is a GK activator that activates GK systemically. As this research suggests that excessive GK activation both in the pancreas and liver is likely to increase hepatic TG levels, a hepatoselective GK activator, which acts primarily on the liver without increasing plasma insulin levels resulting from pancreatic GK activation, may reduce the effects on plasma and hepatic TG levels. Additionally, given that diabetic patients and diabetic animal models have lower hepatic GK activity (Caro *et al.*, 1995; Torres *et al.*, 2009), restoring the activity to normal levels or increasing the activity more mildly may be a feasible strategy to control blood glucose levels without affecting plasma and hepatic TG levels. It has been reported that transgenic mice with high hepatic GK activity (five-fold higher than control) developed hypertriglyceridemia concomitant with the increased hepatic

TG with aging and showed mild hyperinsulinaemia and hyperglycaemia (Ferre *et al.*, 2003). On the other hand, it has been reported that transgenic mice with a more mild increase in hepatic GK activity (lower than two-fold) did not exhibit increased plasma or hepatic TG levels (Hariharan *et al.*, 1997; Shiota *et al.*, 2001). Taken together, the present study suggests that excessive GK activation both in the pancreas and liver may be associated with increased hepatic TG levels.

In summary, we examined the effects of GKA50, a systemic GK activator, on plasma and hepatic TG levels in mice, and speculated that the systemic GK activator GKA50 increased *de novo* lipogenesis by enhanced insulin secretion and hepatic glucose utilization.

## ACKNOWLEDGMENT

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**Conflict of interest----** The authors declare that there is no conflict of interest.

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